When emulsions meet saliva

A physical-chemical, biochemical and sensory study

Promotor:

Prof.dr.ir. W. Norde
Hoogleraar Bionanotechnologie

Co-promotoren:

Dr. G.A.van Aken, Senior wetenschappelijk onderzoeker, Nizo food research, Ede
Dr. M.H. Vingerhoeds, Wetenschappelijk onderzoeker, AFSG, Wageningen Universiteit en Researchcentrum

Promotiecommissie:

Prof. dr. ir. H. Gruppen (Wageningen Universiteit)
Prof. dr. L. Lindh (Universiteit Malmo, Zweden)
Prof. dr. E.C.I. Veerman (Vrije Universiteit Amsterdam)
Dr. R.J. de Vries (Wageningen Universiteit)

Dit onderzoek is uitgevoerd binnen de onderzoeksschool VLAG

When emulsions meet saliva

A physical-chemical, biochemical and sensory study

Erika M.G. Silletti

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof.dr. M. J. Kropff,
in het openbaar te verdedigen
op vrijdag 1 februari 2008
des namiddags te 13.30 uur in de Aula.

ISBN: 978-90-8504-821-3

To Arno,
ad Arno.

Abstract

Upon consumption food emulsions undergo various structural and compositional changes in the mouth. One of these changes is that mixing of an emulsion with saliva induces droplet flocculation

In the study described in this thesis we investigated the influence of saliva on emulsions properties, the mechanism of flocculation and the role in sensory perception. Firstly, we started with evaluating the effect of parameters related to emulsions on flocculation (i.e. differently charged surfactants and proteins such as β-lactoglobulin and lysozyme used as emulsifiers and oil-volume fraction). Among the obtained results, we observed that the sign and the density of the charge on the surface of the droplets determine the (ir-)reversibility of flocculation upon dilution with water and shearing. Secondly, the effect of saliva-related parameters was analyzed. Among other aspects, it appeared that an increase in salivary protein concentration increased emulsion flocculation, and that extensive flocculation is typically found for unstimulated saliva. This approach shows that both emulsion and saliva properties affect the flocculation behavior of emulsions/saliva mixtures.

To investigate the nature of the flocculation, we characterized the salivary protein composition in both the continuous phase of the emulsion/saliva mixture and on the emulsion droplets. Different physical-chemical and biochemical techniques were used. For this approach, we focused on β-lactoglobulin and lysozyme stabilized emulsions, which flocculated reversibly and irreversibly, respectively, upon mixing with saliva. A large number of salivary proteins and peptides in the molecular mass (M_r) range between 0.8 kDa and 100 kDa and the salivary mucins MUC5B and MUC7 (M_r > 200 kDa) associated with emulsion droplets of the emulsions. The results also indicate that the emulsifying protein at the oil-water interface determines which salivary components associate with the droplets in the flocs. A hypothesis is formulated that emulsion flocculation is mainly driven by a complex formation involving specific interactions and electrostatic attraction between salivary peptides/proteins and the emulsifying proteins at the droplets surface.

The importance of the saliva-induced droplet flocculation was demonstrated with a sensory paneling study. Emulsions stabilized by whey protein isolate, (predominantly

composed of β-lactoglobulin) showed reversible flocculation and were perceived as creamy. In contrast, emulsions stabilised by lysozyme showed irreversible flocculation and were perceived as dry, rough and astringent.

To conclude, this thesis shows that saliva-induced emulsion flocculation is driven mainly by association of salivary peptides and proteins to the droplets surface. Because of this, flocculation is determined by the composition of the droplet interface as well as the composition of the saliva, and can be controlled by variation of emulsion parameters (charge, pH, ionic strength). This interaction between emulsions and saliva may help to improve our understanding an control the sensory perception of emulsions.

Keywords: Emulsion, flocculation, bridging, saliva, salivary protein, salivary peptides, lysozyme, β-lactoglobulin, complex formation, LC-MS, SELDI-TOF-MS, proteomics.

Table of contents

Chapter 1

Introduction

Part of this chapter is published in the review

R.G. Schipper, E. Silletti and M.H. Vingerhoeds

Archives of Oral Biology 52 (2007)1114-1135

The research presented in this thesis is part of a multi-disciplinary project which aims at understanding the relationship between the structure of food emulsions under oral conditions and their sensory properties. The human perception of food products, e.g. emulsions, is becoming increasingly important for the food industry in relation to product design and evaluation. Nevertheless, a gap in the knowledge between emulsion properties under oral conditions and perceived texture is present.

Saliva plays an important role in digestion of food, formation of the bolus (consisting in masticated food, shaped in a round mass by chewing and mixing with saliva), sensory perception and flavor release [1-5]. However, most of the studies addressing sensory-physical chemical relations, do not consider the changes taking place during oral processing, which in our view, is fundamental to understand the sensory behavior of a product. This thesis focuses on gaining knowledge on the effect of saliva on emulsion properties. Physical-chemical and biochemical approaches were used to study the influence of saliva on the changes in the structure of food emulsions upon mixing and to investigate which salivary components, i.e. proteins and peptides, are likely to play a role. Furthermore, sensory analysis was performed as a tool to relate the behavior of the emulsion observed *in vitro*, with perception *in vivo*.

This chapter provides an overview of several aspects of emulsions, including stability and rheological characteristics. Moreover, an introduction of saliva composition, structure, functions and rheology is presented. Lastly, the aim and the outline of this thesis are described.

Emulsions

Emulsions are mixtures of at least two of immiscible liquids one of which is dispersed as droplets in the other, which forms the continuous phase. In everyday life, emulsions have numerous applications such as in inks, paints, coatings, latex preparations and food. Examples of food emulsions are milk products, mayonnaises and margarine which can be prepared as oil in water (o/w) or water in oil (w/o) [6]. In this study, o/w emulsions were stabilized mainly by proteins and in some cases, by surfactants (Chapter 2). Chicken egg-white lysozyme and bovine β-lactoglobulin (β-lg) have been largely used to stabilize emulsions throughout the whole study. Lysozyme is a 129 amino acid residues enzyme with a molecular mass (M_r) of approximately 14.3 kDa constituting about 3% of the total

chicken egg-white proteins content. It is a small ellipsoid molecule, with a high isoelectric point (pI ~10.5) [7, 8] and has a slight tendency to form dimers when in solution at neutral pH [9]. Bovine β-lactoglobulin is a major whey protein abundant in cow's milk [10, 11]. It consists of 162 amino acid residues with a M_r of about 18.2 and a pI of about 4.9. At neutral pH, bovine β-lg forms a dimer, while a monomeric native state is stable at pH values below 3 [12].

Preparation and stability of emulsions

Emulsion preparation necessitates oil, water and emulsifiers. Because of the existence of a surface tension between oil and water, the formation of the droplets requires energy [6]. Droplets resist deformation and break up because of their Laplace pressure, which becomes larger as droplet size decreases. The required energy, which is generally provided by intense agitation using a homogenizer, can be reduced if the interfacial tension, hence the Laplace pressure, is reduced by addition of emulsifiers. Because the system contains an excess of free energy, emulsions are unstable. This means that, with time, emulsions will tend to separate into the two phases, e.g. an oil(y) and an aqueous phase. In practice, complete separation of all oil from the liquid phase, also called continuous phase, can be prevented by using different ingredients which increase emulsion stability. Stability of emulsion is achieved if creaming, coalescence and flocculation, as illustrated schematically in Figure 1, are prevented to occur [13].

Creaming refers to the movement of emulsion droplets to the top of a container. Apart from the difference in density between the dispersed and the aqueous phases, one of the important factors affecting the creaming rate is the size of the droplets since large droplets, as well as aggregates, will cream faster than smaller droplets. Generally, emulsions with droplets smaller than 1 μm may be considered stable with respect to creaming [14]. Creaming can also be prevented by increasing the viscosity of the continuous phase, through addition of so-called thickeners. Thickeners are polymers added to create a network around the droplets which physically prevents the droplets to move.

Coalescence indicates the process that occurs when emulsion droplets encounter each other and merge to form a larger one. Therefore coalescence is primarily impeded by providing electrostatic or steric repulsion between droplets in order to avoid close contact of the droplets.

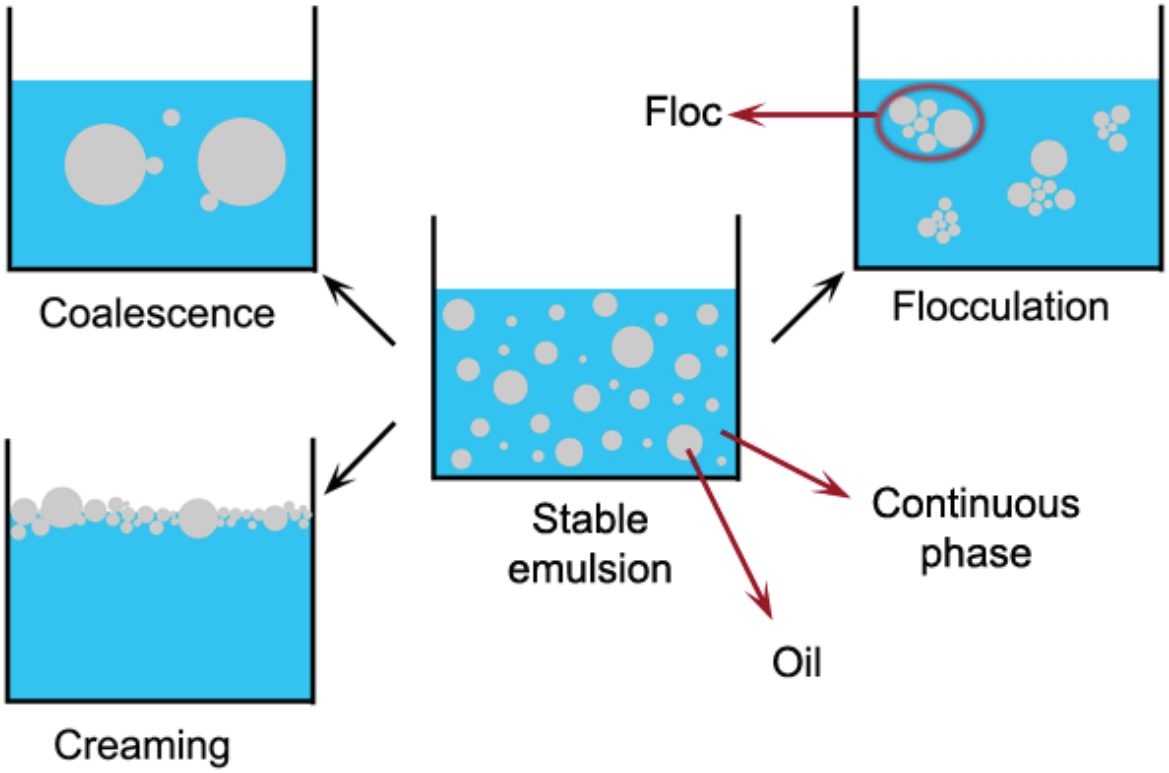

Figure 1 – Stable emulsion compared to the main types of emulsion instability, i.e. coalescence, creaming, and flocculation.

A third type of instability, which, for example, is observed upon mixing emulsion with saliva, is flocculation. In flocculated emulsions the droplets remain at close distance for a prolonged period of time, without rupture of the thin film that separates them. Several terms, i.e. flocculation, coagulation, aggregation, are used as synonyms in literature. Aggregation is mostly used as a very general term, while flocculation and coagulation often indicate various types of aggregation according to the degree of reversibility. Flocculation refers to reversible aggregation, as for example by depletion, whereas coagulation implies irreversibility [15]. However, bridging flocculation is also used to indicate irreversible aggregation caused by adsorption of (bio)polymers to different emulsion droplets. In this thesis, flocculation is used as a general term to indicate the phenomenon of aggregated droplets irrespectively of the reversibility of the process.

Emulsion flocculation by depletion and bridging

Addition of polysaccharides or polymers to a colloidal system has been observed to induce flocculation as schematically represented in Figure 2. Depletion flocculation of emulsions is caused by a solution of non-adsorbing polymer molecules. The center of mass of the molecule cannot approach the droplet surface closer than a distance of about its radius of gyration. If the separation distance between the droplets is smaller than the diameter of the polymer, a region of pure solvent, called depletion zone, occurs. The resulting difference in polymer concentration between the depletion zone and the bulk solution leads to an osmotic potential. A movement of solvent from the depletion zone into the bulk, to reduce the concentration gradient, induces an attractive force between the droplets [16]. The depletion interaction free energy (G_{dep}) for two particles of radius a is given by

$$G_{dep} = - \pi R^2_g (2a + 4 R_g/3) \Pi f(h) \qquad \text{Eq.1}$$

where R_g is the radius of gyration of the polymer, Π is the osmotic pressure of the polymer solution and $f(h)$ is a function that decreases from unity at zero separation of the particle (h=0), to zero for large particle separations (h>2 R_g) [17]. The thickness of the depletion zone, which depends on the radius of gyration, is a measure of the range of the depletion interaction. Therefore, the larger the non-adsorbed species are, the more long-ranged is the force and the lower the critical polymer concentration necessary to induce flocculation is. In addition to R_g, the shape of the non-adsorbing macromolecules was found to play a role in depletion flocculation [18, 19]. Other important parameters are the concentration and M_r of the polymer, which affects Π, and the emulsion droplets radius a, as indicated in equation 1. Increasing the polymer concentration adds to the osmotic pressure leading to an increase of the depletion effect [16]. An increase in the M_r of the polymer causes at first an increase in depletion, and then a decrease. Furthermore, for a given polymer, depletion was observed to increase with the particle size.

Several examples of emulsion flocculation caused by depletion are reported in literature. In particular, the influence of polysaccharides on the stability of food-related emulsions at pH values close to 7 has been extensively studied [20-24]. Polysaccharides as guar gum, methylcellulose, dextran, xantan gum are very often used in studies regarding instability of emulsions through depletion.

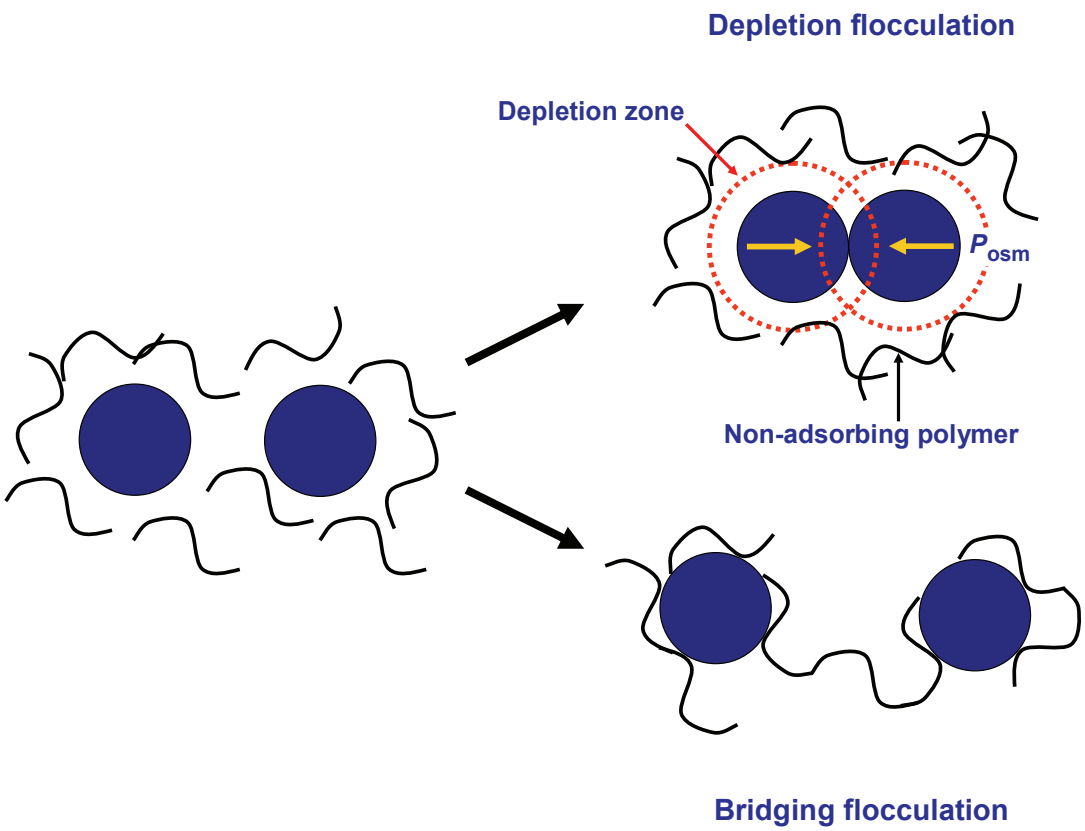

Figure 2 – Schematic representation of depletion and bridging flocculation.

Moreover, depletion may as well be caused by fibrillar protein assemblies [25] and micelles, for example casein micelles [26-28], where sub-micelles of 10-20 nm in diameter could generate appreciable depletion forces.

In contrast to depletion, the basic principle of bridging flocculation consists, as above-mentioned, in a polymer chain bridging two or more particles via adsorption to the particles [29]. Evidence of the structures formed in this process has been obtained using transmission electron microscopy (TEM). The results indicate a structure consisting of a large filamentous polymer matrix holding small particles together in aggregates [30, 31]. It is known that attractive interaction between adsorbed proteins at an interface, e.g. in o/w emulsions, and the hydrocolloids present in the bulk phase can induce bridging [32]. Parameters such as chemical composition and charge of the polymer, as well as particle surface charge and ionic strength of the medium, are important for polymer adsorption onto the oil droplets. The result is usually the formation of a firm and irreversible drop-drop bond which can grow to a range of relatively large sized aggregates (diameter > 100 μm)

[33]. In food-related systems, bridging flocculation has been reported for example in a mixture of a β-lg emulsion and carboxymethylcellulose at pH 3 [34] and for bovine serum albumin emulsions with ι-carrageenan [35].

Rheology of emulsions

The rheological characteristics of emulsions are of considerable importance from both fundamental and applied points of view because they relate to the interaction forces occurring in the system. Several factors are affecting emulsion rheology. The volume fraction of the dispersed phase (ϕ), which in most food emulsions varies between 0.01 and 0.4, and the viscosity of the continuous phase (η_0), are the most important ones. Low oil-volume fraction o/w emulsions, as in this study, behave Newtonian, i.e. the viscosity (η) is independent of the shear rate [36], since the influence of colloidal interparticle interactions is negligible. The viscosity of emulsions for non-interacting oil droplets and ϕ up to 0.2 can be calculated, for example, using Batchelor equation [37]

$$\eta = \eta_0 (1 + 2.5\phi + 6.2\ \phi^2) \qquad \text{Eq.2}$$

where η_0 is the viscosity of the continuous phase.

In case of flocculated emulsions, e.g. by saliva, the rheological behavior depends on the type and strength of the interaction between droplets as well. As known from literature, flocculation of emulsion droplets affects the viscosity by increasing the effective volume fraction, as result of liquid entrapment in the floc structure. Flocculated emulsions exhibit, initially, a higher viscosity than non-flocculated and, with the increased shear rate, a shear-thinning behavior [27]. In flow-curve measurements, the viscosity decreases, until it reaches a Newtonian plateau. This behavior can be explained considering the structure of the system. When a shear force is applied to a flocculated emulsion, the aggregate structures are disrupted leading to a reduced effective volume fraction and consequently a lower viscosity. Since flocculated emulsions are viscoelastic, oscillatory measurements are commonly used to assess the storage modulus (*G'*) and loss modulus (*G"*) [27, 34, 38-40]. *G'* and *G"* are the elastic and viscous components of the complex modulus, corresponding to the energy stored and dissipated in a cycle of oscillation, respectively. When emulsions flocculate, a rapid increase in the storage modulus occurs [39] since *G'* is determined by the

attraction between the droplets. Therefore changes in the linear viscoelastic region where *G'* and *G"* are constant with the strain amplitude, may be used as indication of flocculation. In particular, a decrease in the critical strain above which the structure breaks down as well as an increase of *G'*, may be seen in case of strong flocculation. Furthermore, as reported by Blijdenstein [34], both depletion and bridging can be distinguished by comparing the *G'* behavior over the applied deformation range. Depletion flocculation shows a gradual decrease in *G'* with the applied strain, while bridging flocculated emulsions show higher *G'* and a sudden decrease at larger strain [34].

More extensive information about fundamental principles and application of rheology to study emulsion flocculation can be found, for example, in two useful papers by Tadros [39, 40].

Saliva

Saliva has multifunctional roles in speech, lubrication, digestion of food and maintaining oral and general health [41, 42]. Interest in saliva increased even more with the finding that saliva contains components that may serve to detect systemic disease or evidence of exposure to various harmful substances, as well as provide biomarkers of health and disease status [43-45].

Human saliva is mainly composed of water, proteins and inorganic and trace substances [42, 46, 47]. It is produced by the contra-lateral major glands, i.e. parotid glands (PAR), submandibular glands (SM), and sublingual glands (SL), and minor salivary glands present in the mucosa of the tongue (Von Ebner glands), cheeks, lips and palate (Pal) [48, 49]. The glands differ in the type of secretion they produce, which is caused by the ratio of serous to mucous glandular cells. Serous cells in PAR, SM, Pal and lingual glands secrete a watery fluid, essentially devoid of mucus; this secretion is strongly activated by stimuli. Mucous cells, present in SM, SL, labial (Lab), Pal and lingual glands, produce a mucus-rich secretion. The amount and composition of secreted human saliva depends on many factors, such as flow rate, circadian rhythm [50-52], type and size of the salivary gland [53, 54], duration and type of the stimulus [55-57], diet, drugs, age, gender and blood type [58, 59] and physiological status [60]. Secretion of saliva ranges from 0.3 to 7 mL saliva per minute [61] with about 0.5-1.5 L of saliva secreted per day [42]. Saliva pH can range from 6.2 to 7.4, with the higher pH exhibited upon increased secretion. Several methods have been

suggested to collect resting (unstimulated) and stimulated saliva [54, 62]. Most commonly, saliva is collected by draining or spitting into a tube, or chewing on an absorbent material. Collection of pure glandular secretions is possible with the use of special collection devices, for example the Lashley-cup for collection of parotid saliva.

Biochemical characterization, protein composition and function of salivary proteins

Human saliva contains a large array of proteins and peptides with a total concentration varying in the range 1-2 mg mL^{-1}. Although the major families of salivary proteins have been studied by conventional biochemical strategies (Table 1), the exact number of proteins and peptides is not precisely known.

Nowadays, analysis of salivary proteins/peptides is conducted using proteomic techniques. Several investigators have used two-dimensional (2D) gel electrophoresis to separate the protein components followed by mass spectrometry (MS) to subsequently identify the peptides produced from in-gel digests of the proteins of interest (2D-MS) [63-67]. With this approach more than 300 proteins were identified. Although 2D-MS is a very powerful approach to protein separation, it has limitations when dealing with small M_r proteins, highly acidic or basic proteins, very hydrophobic proteins or proteins present in low quantity. In addition, the technique requires a relatively large amount of sample, is labor-intensive, and high gel-to-gel reproducibility is hard to achieve [62]. An alternative approach is the combination of liquid chromatography (LC) as the separation step, with the mass spectrometer (LC-MS) [67-71]. Using both 2D-MS and LC-MS, more than 1050 proteins in whole saliva have been identified [72]. Lists of different salivary proteins, with information about database accession number, pI, M_r can be found in several proteomics pubblications [63-65, 67] or at the websites of the Human Salivary Proteome Project (HSPP) of the UCLA at the address www.hspp.ucla.edu/cgi-bin/search.cgi. A summary of the proteomic techniques used to investigate saliva and different salivary secretions is provided in Table 2.

Table 1 – Methods for fractionation and isolation of salivary proteins from whole saliva and glandular secretions.

Protein isolation/ fractionation	**Saliva**	**Chromatography**				**Other techniques**	**[a]Ref**
		Anion exchange	**Cation-exchange**	**Gel filtration**	**Affinity**		
Fractionation							
To study histadine rich peptide	PAR	DEAE- Sephadex A25		Bio-Gel P10			[73]
To study PRP				Bio-Gel P60			[74]
To study PRP				Sephacryl S-200			[75]
For adsorption study at solid/liquid and air/liquid interfaces	WS			Superdex 200 Hiload		Phenil-superose for hydrophobic interaction chromatography	[76]
To study vine astringency correlation	WS					RP-HPLC with octadecylsilane packing AMQ-303, S-5	[1]
Isolation							
Amylase	WS	Mono-Q	Mono-P	Toyo Soda-G3000-SW			[77]
Amylase	PAR	Mono-Q		Bio-Gel P60			[78]
Cystatin	SMSL			Sephadex G-50		RP-HPLC with LKBUltropac TSK-ODS 120T 5	[79]
Cystatin		DE32	CM-Sepharose CL6B	Sephacryl S-200			[80]
Cystatin	SMSL	D52 cellulose; Mono-Q		Sephadex G-200; Bio-Gel P60			[81]

Cystatin	SM; SL; Pal; Lab	Mono-Q	SP-Sephadex C-25			[82]
Cystatin	SMSL	DEAE-Sepharose CL-6B; DEAE-cellulose D52; Mono-Q	Mono SHR5/5	Sephadex G-200; Sepharose CL-2B; Sephacryl S-300; Sephadex G-25		[83]
Deglycosilated glycoproteins	PAR		SP-Sephadex C-25	Sephadex G-200		[84]
Histadine rich peptide	PAR		Bio-Rex 70	Sephadex G-25		[85]
Histatins	PAR; SMSL				RP-HPLC C 18 column TSK-GEL 5 mm ODS-120T	[53, 86]
LMW peptides	PAR			Bio-Gel P2	RP-HPLC Vydac C 18	[87]
Minor components of PRP family	PAR	DEAE-Sephadex A25		Sephadex G-75; Sephadex G-10		[88]
PRP	PAR	DEAE- Sephadex	SP-Sephadex C-25	Sephadex G-150; Sephadex G-50		[89]
PRP	PAR; SMSL	GenPak FAX				[90]
PRP	PAR	Mono-Q; GenPakFAX	DEAE-Agarose			[91]
PRP		DEAE-Sepharose CL-6B		Sephadex G-100	Suprose-12	[92]
PRP	SMSL	DEAE-Sepharose CL-6B; DEAE-cellulose D52; Mono-Q	Mono-S	Sephadex G-200; Sepharose CL-2B; Sephacryl S-300; Sephadex G-25		[83]

Table 1 Continued

PSP	WS				IgG anti-PSP coupled to CNBr-Sepharose		[93]
SAP-1		DE32					[94]
Statherin	SMSL	DEAE-Sepharose CL-6B; DEAE-cellulose D52; Mono-Q	Mono-S	Sephadex G-200; Sepharose CL-2B; Sephacryl S-300; Sephadex G-25			[83]
Statherin	SMSL		Sephadex G-50			RP-HPLC LKBUltropac TSK-ODS 120T5	[95]
TPST	WS; PAR				IgG anti-TSPT coupledto CNBr-Sepharose 4B		[96]
Vitamin B_{12} binding protein	WS	DEAE-cellulose	CM-52-cellulose	Sephadex G-150			[97]
			Isolation of mucins and micelles				
Mucins	SMSL	DEAE-Sepharose CL-6B; DEAE-cellulose D52; Mono-Q	Mono-S	Sephadex G-200; Sepharose CL-2B; Sephacryl S-300; Sephadex G-25			[83]
Different MUC5B species	WS			Sephacryl HR-500		CsCl density-gradient centrifugation	[98]
MUC5B	SMSL			Sephadex G-200; Sepharose CL-2B;			[78]
MUC5B	WS			TSK 5000 PW			[44]

MUC5B	WS; SMSL; other secretion		Sepharose CL-2B; Sepharose CL-4B	CsCl density-gradient centrifugation	[99-102]
MUC5B	WS; SMSL			CsCl density-gradient centrifugation	[103, 104]
MUC7	SMSL		Sephadex G-200; Sepharose CL-4B; Sephacryl S-300		[78]
MUC7	SMSL		Sephadex G-200; Superose 6		[105]
MUC7	WS	Mono-Q	Sepharose CL-4B		[106]
MUC7	SMSL		Sepharose CL-2B; Superose 6		[107]
Salivary micelles	PAR		Sephacryl S-1000		[108]

WS: whole saliva; SMSL: submandibular(SM) and sublingual (SL) saliva; PAR: parotid saliva; Pal: palatal saliva; Lab: labial saliva; PRP: proline-rich protein; LMW: low-molecular-weight; PSP: parotid secretory protein; TPST: tyrosylprotein sulfotransferase; SAP-1: salivary acidic protein-1; [a] *Ref: reference.*

Table 2 – Global analysis of salivary protein/peptides using proteomic techniques.

Method	Number of peaks/spots detected	Proteins/peptides Assigned	[a] Ref
Whole saliva			
HPLC-MS	Not indicated	Basic PRPs	[109]
HPLC-MS	33	Defensins, histatins, PRPs and statherin	[65]
2-DE coupled to MALDI-TOF-MS	101	Albumin, α-amylase, calgranuline A, calgranuline B, cystatins, fatty acid binding protein, Igs, lipocalin-1, lysozyme, prolactin, Zn-α-2-glycoprotein and others	[63]
2-DLC-MS/MS	102	Albumin, α-Amylase, calgranulin A, calgranulin B, carbonic anhydrase VI, cystatins, fatty acid binding protein, Igs, kallikrein, lacto-peroxidase, lactotransferrin, lipocalin-1, lysozyme, prolactin, PRPs, statherin , Zn-α-2-glycoprotein and others	[110]
2-DE coupled to MALDI-TOF-MS	110	Albumin, α-Amylase, calgranulin B, cystatins, lipocalin-1, myosin heavy chains, Zn-α-2-glycoprotein and others	[111]
2-DE coupled to MALDI-TOF-MS, ESI-QoTOF-MS	138	Albumin, α-amylase, cystatins, statherin, calgranulin A	[66]
2-DE coupled to MALDI-TOF-MS	192	Actin, Albumin, α-Amylase, calgranulin A, calgranulin B, carbonic anhydrase VI, cystatins, fatty acid binding protein, Igs, kallikrein, lacto-peroxidase, lactotransferrin, lipocalin-1, lysozyme, prolactin, PRPs, statherin, Zn-α-2-glycoprotein and others	[112]
2-DE coupled to MALDI-TOF-MS, oMALDI-QqTOF- MS/MS	200	Albumin, α-amylase, calgranulin A, cystatins, Igs, lactotransferrin, prolactin, PRP, Zn-α-2-glycoprotein and others	[113]
2-DE coupled to MALDI-TOF-MS	> 200	α-Amylase, calgranulin A, calgranulin B, carbonic anhydrase VI, cystatins, fatty acid binding protein, histatins, Igs, lysozyme, prolactin, Zn-α-2-glycoprotein and others	[65]
Capillary LC coupled to ESI-Q-TOF-MS	266	Albumin, α-amylase, calgranulin A, calgranulin B, cystatins, cytokeratins, defensins, histatins, Igs, kallikrein 1, lysozyme, MUC5B, MUC7, phospholipase C-beta-3, prolactin, PRPs, PRGP, salivary peroxidase, statherin and others	[67]
2-DE coupled to MALDI-MS and LC ESI-Q-TOF-MS	> 300	Albumin, α-amylase, cystatins, cytokeratins, fatty acid binding protein, Igs, Zn-α-2-glycoprotein and others	[67]
Capillary Isoelectric Focusing/NanoRPLC coupled to ESI-Q-TOF-MS	1381	Albumin, α-amylase, calgranulin A, calgranulin B, calgranulin C, cystatins, cytokeratins, defensins, histatins, Igs, kallikrein 1, lipocalin, lysozyme, phospholipase C-beta-3, prolactin, PRPs, superoxide dismutase, thymosin β-4 and others	[114]

SELDI-TOF-MS	80 (CM10), 108 (Q10), 99 (H4), 118 (IMAC-Cu)	Protein candidates: Albumin, calgranulin A, calgranulin B, calgranulin C, cystatins, defensins, fatty acid binding protein, histatins, kallikrein, lysozyme, PRPs, superoxide dismutase and thymosin β-4	[115]
Stimulated whole saliva			
SELDI-TOF-MS	Not indicated	Protein candidates: Albumin, CA mucins, *c-erb*B-2,cystatins, EGFR, Igs, lactoferrin, lactoperoxidase, Mm23, nNOS/NOS-1, P21 and Rb protein	[45]
PAR saliva			
2-DE coupled to MALDI-TOF-MS, MALDI-QoTOF-MS or ESI-QoTOF-MS	55	Albumin, calgranulin B, cytokeratins, lysozyme, histatins, phosphodiesterase and statherin	[66]
2-DE coupled to MALDI-TOF-MS, oMALDI-QqTOF- MS/MS and MALDI-TOF/TOF- MS/MS	92	Albumin, carbonic anhydrase VI, cystatins,histatins, Igs, lysozyme, PRPs, PRG, Zn-α-2-glycoprotein and others	[64]
Stimulated PAR saliva			
2-DE coupled to MALDI-TOF-MS	58	Albumin, α-amylase, carbonic anhydrase VI, Igs, lactoperoxidase, lactotransferrin, lipocalin-1, PRPs, Zn-α-2-glycoprotein and others	[112]
SELDI-TOF- MS and/or 2D-DIGE	81	α-Amylase, carbonic anhydrase VI, lactoferrin, B2-microglobulin, Igs, lysozyme, cystatin C Protein candidates: α-amylase, calgranulin B, cystatin S, PRB2 and PRP	[43]
Stimulated SM/SL saliva			
2-DE coupled to MALDI-TOF-MS	63	Albumin, α-amylase, carbonic anhydrase VI, cystatins, histatins, MUC7, PRPs, Zn-α-2-glycoprotein and others	[112]
Gengival crevicular fluid			
HPLC coupled to ESI-MS	16	Albumin, cystatin A, defensins, basic PB salivary peptide and statherin	[69]
In vivo pellicle			
2-DE coupled to MALDI-TOF-MS, MALDI-QoTOF-MS, ESI-QoTOF-MS	138	Albumin, α-amylase, calgranulin B, cystatins, cytokeratins, histatins, phosphodiesterase and statherin	[66]
In vitro PAR gland			
RP-HPLC coupled to ESI-MS	9	PRPs	[70]

Table 2 Continued

In vitro SM gland			
RP-HPLC coupled to ESI-MS	13	Cystatins, histatins, lysozyme and PRPs	[70]

RP-HPLC: reverse-phase high performance liquid chromatography; NanoRPLC; nanoflow reverse-phase liquid chromatography 2DLC: two-dimensional liquid chromatography; MS: mass spectrometry; MS/MS: tandem MS; MALDI; matrix-assisted laser desorption ionization; SELDI: surface-enhanced laser desorption ionization; TOF: time-of-flight; QoTOF: quadrupole/quadrupole time-of-flight; ESI: electrospray ionization; 2DE: two-dimensional electrophoresis; 2D DIGE: two-dimensional difference gel electrophoresis. [a] *Ref: reference.*

Saliva is mainly constituted of glycoproteins (e.g mucins, proline-rich glycoproteins), enzymes (e.g., α-amylase, carbonic anhydrase), immunoglobulins and a wide range of peptides such as cystatins, statherin, histatins and proline-rich proteins (PRPs) [42, 47, 116, 117]. Posttranslational modifications (PTMs), such as glycosilation, phosphorilation and amino acids substitutions originate different salivary proteins isoforms [64, 71, 109, 117-121], as for example observed in α-amylase or PRPs [71, 77, 109]. Increasing evidence suggests that proteolysis, which occurs prior to or during secretion, can be considered a common PTM of salivary proteins [87, 118]. Examples of proteolytic activity as PTM involved histatin family members, as demonstrated by the identification of 60 small histatin fragments with anti-fungal activity [64], statherins [95], PRPs [122] and cystatins [68].

In spite of the large efforts in saliva proteome studies, functions of most salivary components are still poorly understood. A brief overview of the primary functions of the main proteins in saliva is provided in Figure 3. Lubrication properties have been ascribed to several salivary proteins, including mucins [78, 123, 124], statherin [125-128], amylase [78], proline-rich glycoproteins [129] and acidic PPRs [125]. The role of carbohydrate moieties in lubrication is unclear, and seems to be protein-dependent [78, 129]. Anti-bacterial and anti-fungal properties are performed by small proteins as cystatins, histatins, which together with mucins and immunoglobulin and the antimicrobial enzymes, i.e. lysozyme, lactoperoxidase and chitinase, constitute part of the defense system present in the oral cavity.

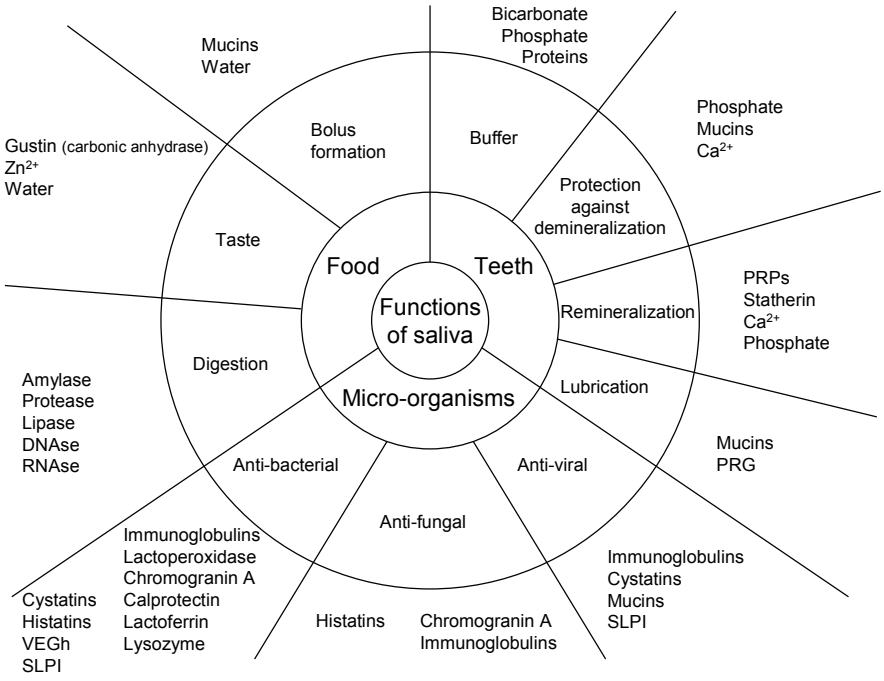

Figure 3 – Schematic presentation of the main functions of saliva in the relation to its constituents (from reference [130]).

Macromolecular organization of saliva

Already in the early decades of the twentieth century, it was acknowledged that saliva could not be regarded as a simple solution, due to the presence of elastic threadlike components [131-133]. In 1987, Schwartz hypothesized that saliva contains simultaneously different phases, i.e. a liquid phase, a gaseous phase (bubbles) and gel phase [134] and a couple of years later, Glantz suggested that saliva should be probably better classified as a fluid tissue than as a solution [135]. At the end of the nineties, based on microscopy studies, Glantz proposed a refined model for the structure of saliva [136]. According to this model, saliva contains four levels of organization, (a) a continuous phase composed of electrolytes in water, (b) lipoid material, bacterial and epithelial cells and two main structural features: (c) a scaffold-like continuous network structure and (d) salivary micelles and/or other salivary globular structures observed inside the saliva network filaments.

The network structure of saliva, which is mainly constituted of the high M_r mucin MUC5B, was found in the bulk fluid, as well as on oral surfaces. The gel properties of the network are attributed to entanglements of these mucin molecules [99, 137-139] most likely due to hydrophobic and carbohydrate-carbohydrate interactions and calcium-mediated crosslinks

[102, 140]. Besides MUC5B, other proteins, such as the secretory IgA (sIgA), lactoferrin, lysozyme, MUC7 and the parotid agglutinin have been identified [103, 141]. Furthermore, it seems that MUC7 and lactoferrin are present as small heterodimeric complexes isolated in submandibular/sublingual secretion [101, 142].

The presence of globular structures lying within the network was first described using microscopy techniques by Glantz and co-workers [135, 143], followed by publications from Rykke and co-workers [144-146]. These globular structures, nowadays called salivary micelles, have been identified in both PAR and stimulated saliva [145, 146] and have been reported to have a negative surface potential at physiological pH [146, 147]. The micelles consist of individual particles, or clusters of particles with different sizes and shapes [146, 148]. The size, as estimated by photon correlation spectroscopy and electron microscopy, ranges from 40 to 400 nm [146, 148] and increases with time due to aggregation [145, 149]. The amino acid composition of salivary micelles differs from whole saliva [148, 150] but largely resembles that of the acquired pellicle [144, 150] and PAR saliva, with the exception of the high proline content [108]. According to Soares, who conducted the experiments at acidic pH, the micelles are composed of MUC7, sIgA, lactoferrin, amylase, glycosylated PRPs and lysozyme [148]

As above-mentioned, analysis of saliva structure has been mostly performed with scanning- and transmission electron microscopy (SEM and TEM, respectively) [135, 145, 146, 151]. TEM is especially useful for determination of particles at the nano scale, as for example the globular structures (100-200 nm) inside the saliva network. In this thesis, instead, another microscopic technique, Confocal Laser Scanning Microscopy (CLSM), was used. Flocculation of emulsions after mixing with saliva and the determination of complex formation between saliva and positively charged emulsifiers was performed with this technique as described in Chapter 3. Compared to electron microscopy, CLSM offers the advantage that the sample can be studied without drying or freezing, which could induce structural artifacts. However, some limitations of the technique may restrict the field of application since for example, high magnifications, such as those obtained by TEM, cannot be reached. As an example, the structure of saliva examined with both electron microscopy, e.g. SEM, and CLSM is shown in Figure 4.

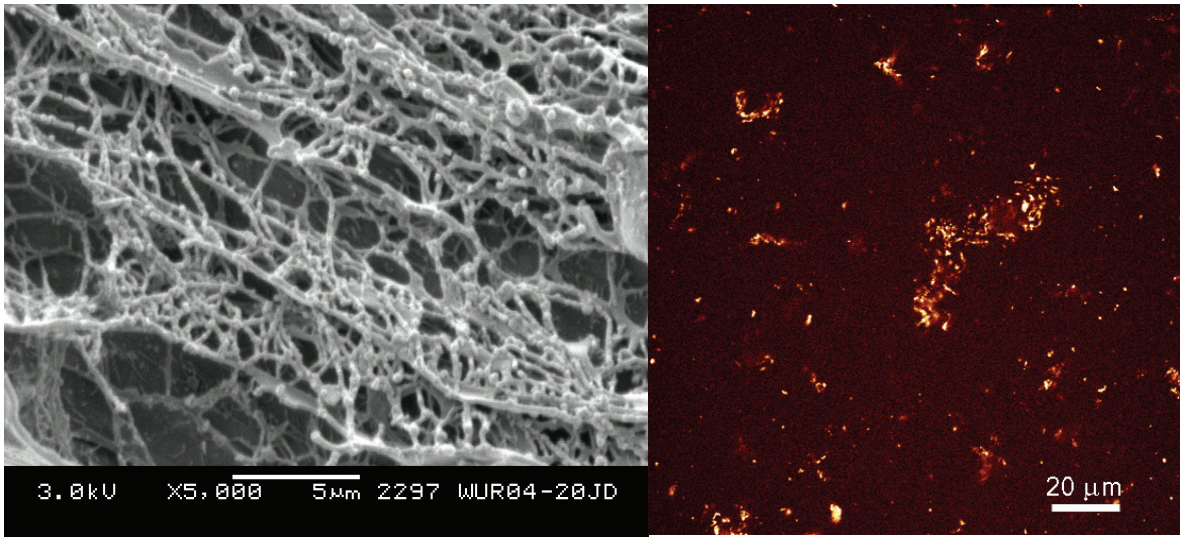

Figure 4 – (Left) Fresh saliva determined with Cryo SEM. A drop of centrifuged fresh saliva was rapidly frozen in liquid nitrogen, cut, ice crystal water was sublimated and two gold layers were applied. The structure looks filamentous and very porous. (Right) CLSM image of centrifuged fresh saliva of which the proteins have been stained with Rhodamine B. The large structures are clearly visible.

Rheological properties of saliva

Whole saliva can be classified as a non-Newtonian fluid [134, 152-154]. The main reason for the shear-thinning character of whole saliva is the presence of large glycoproteins, like mucins, causing the above described weak gel character of saliva [155]. This is supported by different studies showing that the viscosity of PAR saliva, which does not contain high M_r mucins, is shear rate independent with a viscosity slightly higher than that of water [153, 155]. In addition, treatment of saliva by homogenization destroys the weak gel and resulted in a 3-4 fold reduction in viscosity [155]. The origin and type of mucin appear to play a dominant role, i.e. the viscosity of SM and Pal saliva were shown to be hardly dependent on the shear rate opposite to SL saliva showing a clear shear-thinning behavior [153]. Table 3 lists an overview of the reported viscosities of whole saliva as well as viscosities of saliva from different glands.

Another parameter affecting saliva and mucin properties is the pH. The viscosity of salivary mucins was greatest at pH 4 [155], while pig gastric mucin showed a optimum value at pH 2 [156], where the formation of large aggregates is occurring.

Table 3 – Rheological parameters of whole saliva and different salivary secretions as reported in literature.

Saliva origin and Stimulation	Viscosity * (mPa.s)	Elasticity ** (mPa.s)	Shear rate (s^{-1}) Frequency (Hz) Temperature (°C)	Method	[a] Ref.
Whole saliva					
None	2.5, 100	Nd	95 s^{-1}; 35°C 0.02 s^{-1}; 35°C	Low shear rheometer (Contraves)	[152]
None	4.2	Nd	20 s^{-1}; 37°C	Low shear rheometer (Contraves)	[155]
None	1.5-1.6	0.6	1-300 s^{-1}; 37°C	Oscillating capillary rheometer (Vilastic)	[153]
None	1.6-1.8	0.5-0.8	1-300 s^{-1}; 23°C	Oscillating capillary rheometer (Vilastic)	[157]
None	1.1	Nd	70 Hz; 25°C	Couette-type (Contraves)	[158]
None	3.8-8.8 (mean 5.7)	Nd	90 s^{-1}; 37°C	Cone-and-plate viscometer (Brookfield)	[159]
None	1.3	Nd	26°C	Capillary rheometer (Cannon)	[160]
Chewing gum	10	Nd [#]	100 s^{-1}; 25°C	Weissenberg rheogoniometer (cone-and-plate); dynamic viscosity	[134]
Parafilm	1.9	Nd	Ca. 50 s^{-1}; 37°C	Dynamic rheometer (ReoCue; Bohlin)	[161]
Parafilm	2.4 1.6	Nd	450 s^{-1}; 20°C 450 s^{-1}; 37°C	Cone-and-plate viscometer (Brookfield)	[162]
Paraffin	1.1-2.0	0.1-4	1-66 Hz; 25°C	Coaxial cylindrical viscometer	[163]
Paraffin	3	Nd	90 s^{-1}; 37°C	Cone-and-plate viscometer (Brookfield)	[159]
PAR saliva					
None	0.8	<0.05	1-300 s^{-1}; 37°C	Oscillating capillary rheometer (Vilastic)	[153]
None	0.8	Nd	20 s^{-1}; 37°C	Low shear rheometer (Contraves)	[155]
None	1.3	Nd	230 s^{-1}; 25°C	Cone-cup viscometer (Brookfield)	[164]
Citric acid	2.0	Nd	230 s^{-1}; 25°C	Cone-cup viscometer (Brookfield)	[164]
Citric acid	1.0	Nd	450 s^{-1}; 37°C	Cone-and-plate viscometer (Brookfield)	[165]
Citric acid	0.78	Nd	450 s^{-1}; 37°C	Cone-and-plate viscometer (Brookfield)	[78]
Citric acid	0.88	Nd	450 s^{-1}; 37°C	Cone-and-plate viscometer (Brookfield)	[154]
Not indicated	1.0	0.06	66 Hz; 25°C	Coaxial cylindrical viscometer	[163]
SM saliva					
None	1.4-1.5	0.4-0.6	1-300 s^{-1}; 37°C	Oscillating capillary rheometer (Vilastic)	[153]
Citric acid	0.9-2.0	0.1-3	1-66 Hz ; 25°C	Coaxial cylindrical viscometer	[163]

SL saliva					
None	2.9-4.6	1.8-4.9	1-300 s^{-1}; 37°C	Oscillating capillary rheometer (Vilastic)	[153]
SMSL saliva					
None	1.3	Nd	20 s^{-1}; 37°C	Low shear rheometer (Contraves)	[155]
None	3.0	Nd	230 s^{-1}; 25°C	Cone-cup viscometer (Brookfield)	[164]
Citric acid	2.8	Nd	230 s^{-1}; 25°C	Cone-cup viscometer (Brookfield)	[164]
Citric acid	1.2	Nd	450 s^{-1}; 37°C	Cone-and-plate viscometer (Brookfield)	[78]
Citric acid	2.1	Nd	450 s^{-1}; 37°C	Cone-and-plate viscometer (Brookfield)	[165]
Citric acid	1.8	Nd	450 s^{-1}; 37°C	Cone-and-plate viscometer (Brookfield)	[154]
Pal saliva					
None	1.6-1.8	0.4-0.7	1-300 s^{-1}; 37°C	Oscillating capillary rheometer (Vilastic)	[153]

*In some cases clarified saliva samples were used [153, 154, 157]; it was noted that centrifugation did not affect the rheological properties [153, 154], whereas others found a viscosity decrease [160]. * apparent viscosity (η_a) or viscous component of viscoelasticity (η'); ** elastic component (η') of viscoelasticity; #G' was reported, i.e. 0.1 Pa at 1.0 Hz; Nd: not determined. [a] Ref: reference.*

This optimum vanishes at increased ionic strength, emphasizing the role of electrostatic interactions in the pH dependent behavior. In some cases, lowering the pH resulted in a decrease in viscosity of whole unstimulated saliva and a small viscosity increase of stimulated saliva [166]. Moreover, it was found that the viscosity of centrifuged whole unstimulated saliva was not affected by pH [166]. It should be taken into consideration that a low pH might cause proteolysis, leading to a collapse of the gel structure of isolated mucins [167], or may induce conformational changes in salivary proteins [166].

A point of attention is that the shear rate dependent viscosity, reported by several authors varies with (1) measuring methods, (2) saliva collection and handling, (3) circadian rhythm and (4) individual variation [153, 158, 159, 161, 163]. When no precautions are taken with respect to protease inhibition or centrifugation, the viscosity of whole saliva decreases upon storage within a few hours [162]. To prevent viscosity changes, it is advisable to add protease inhibitors directly after saliva collection [155], to handle saliva in the cold, and/or to minimize the time between collection and measurement. For these reasons, standardized conditions for saliva sampling and handling, as described in Chapter 2, were strictly followed during the whole study.

Aim of the thesis and outline

The focus of this thesis is to study the behavior of low viscosity emulsions upon mixing with saliva. Three different approaches were taken during this study. Physical-chemical experiments (Chapter 2 to 4) were conducted to characterize different emulsion/saliva mixtures and to evaluate the effect of parameters related to emulsions and saliva. Biochemical, proteomics and physical-chemical techniques (Chapters 3, 5 and 6), were used to investigate the mechanism of interaction between saliva and emulsions and to determine which salivary proteins are involved. Finally, sensory analysis (Chapter 7) was performed to relate knowledge obtained from biochemical and physical-chemical techniques to sensory attributes in order to provide a broader picture of how saliva is affecting the emulsions properties.

In more detail, Chapter 2 evaluates the effect of using different emulsifiers, and therefore the droplets charge, on emulsion flocculation. A first mechanistic insight into the flocculation occurring after mixing emulsions with saliva is provided. Chapter 3 focuses on the flocculation of positively charged emulsions stabilized by lysozyme. Using CLSM, lysozyme was observed to form complexes with salivary proteins, both in solution and at the oil-water interfaces. Based on turbidity measurements, upon increasing the ionic strength these complexes have been found to be electrostatically driven. Chapter 4 illustrates how emulsion-related parameters such as oil-volume fraction and saliva-related parameters (type of saliva and salivary protein content) influence flocculation and consequently the rheological properties of emulsion/saliva mixtures for emulsions stabilized by β-lactoglobulin and by lysozyme.

Chapter 5 and Chapter 6 are dedicated to the identification of salivary proteins and peptides which were found to be associated with the droplets of emulsions stabilized by β-lactoglobulin and lysozyme. These chapters illustrate biochemical and proteomics techniques which are commonly used for protein detection and identification, and provide new insights in the role of saliva components in the in-mouth behavior of food. Chapter 7 describes the effect of saliva-induced flocculation on sensory perception of emulsions which flocculate reversibly and irreversibly upon mixing with saliva. Particular attention is given to correlate sensory attributes scored by the sensory panel with physical-chemical parameters, e.g. viscosity, storage and loss moduli and the retention to the tongue surface. Furthermore, this chapter combines different biochemical techniques in an attempt to clarify possible mechanisms responsible for perception, such as astringent after-feel. To

conclude, Chapter 8 provides a general discussion of the findings, hypothesizes a saliva-induced flocculation mechanism and offers suggestions for further studies.

References

1. Kallithraka, S., Bakker, J., Clifford, M.N., and Vallis, L., *Correlations between saliva protein composition and some T-I parameters of astringency.* Food Quality and Preference, 2001. **12**: p. 145.
2. Vingerhoeds, M.H., Silletti, E., de Groot, J., Schipper, R. G., and van Aken, G.A., *Relating the effect of saliva-induced emulsion flocculation on rheological properties and adherence to the tongue surface with sensory perception.* Submitted, 2007.
3. Engelen, L., de Wijk, R.A., Prinz, J.F., and Bosman, F., *The relation between saliva flow after different stimulations and the perception of flavor and texture attributes in custard desserts.* Physiology & Behavior, 2003. **78**: p. 165.
4. Engelen, L., De Wijk, R.A., Prinz, J.F., Janssen, A.M., van der Bilt, A., Weenen, H., and Bosman, F., *A comparison of the effects of added saliva, alpha-amylase and water on texture perception in semisolids.* Physiology & Behavior, 2003. **78**: p. 805.
5. Engelen, L., van der Keybus, P.A.M., de Wijk, R.A., Veerman, E.C.I., van Nieuw Amerongen, A., van der Bilt, A., Prinz, J.F., and Bosman, F., *The effect of saliva composition on texture perception in semi solids.* Archives of Oral Biology, 2007. **52**: p. 518.
6. Walstra, P., *Physical chemistry of foods*. 2003, New York: Marcel Dekker.
7. Jolles, J., Jauregui, A.J., Bernier, I., and Jolles, P., *La structure chimique du lysozyme de blanc dóeuf de poule: Etude detailee.* Biochimica et Biophysica Acta, 1963. **78**: p. 668.
8. Lechevalier, V., Croguennec, T., Pezennec, S., Guerin-Dubiard, C., Pasco, M., and Nau, F., *Ovalbumin, ovotrasferrin, lysozyme: Three model proteins for structural modifications at the air-water interface.* Journal of Agricoltural and Food Chemistry, 2003. **51**: p. 6354.
9. Deonier, R.C. and Williams, J.W., *Self-association of muramidase (lysozyme) in solution at 25°, pH 7.0, and I = 0.20.* Biochemistry, 1970. **9**: p. 4260.
10. Godovac-Zimmermann, J., *The structural motif of beta-lactoglobulin and retinol-binding protein: A basic framework for binding and transport of small hydrophobic molecules?* Trends in Biochemical Sciences, 1988. **13**: p. 64.
11. Hambling, S.G., McAlpine, A.S., and Sawyer, L., *Advance dairy chemistry -1: Proteins*, E.A. Science, Editor. 1992: Barking, UK. p. 141.
12. McKenzie, H.A., *Milk protein chemistry and molecular biology*, ed. M. HA. Vol. 2. 1971, New York: Academic press. 257.
13. Walstra, P. and de Roos, A.L., *Proteins at air-water and oil-water interfaces - static and dynamic aspects.* Food Reviews International, 1993. **9**: p. 503.
14. Walstra, P., *Emulsion stability in encyclopedia of emulsion technology*, ed. P. Becher. 1996, New York: Marcel Dekker
15. Walstra, P., *Dispersed systems: Basic considerations* in *Food chemistry* O.R. Fennema, Editor. 1996, CRC Press: New York. p. 96.
16. Vrij, A., *Polymers at interfaces and the interactions in colloidal dispersions.* Pure & Applied Chemistry, 1976. **48**: p. 471.
17. Walstra, P., *Introduction in aggregation phenomena in food colloids* in *Food colloids and polymers: Stability and mechanical properties*, E. Dickinson and P. Walstra, Editors. 1993, Royal Society of Chemistry: Cambridge. p. 3.
18. Asakura, S. and Oosawa, F., *On interaction between two bodies immersed in a solution of macromolecules.* The Journal of Chemical Physics, 1954. **22**: p. 1255.
19. Asakura, S. and Oosawa, F., *Interaction between particle suspended in solution of macromolecules.* Journal of Polymer Science, 1958. **33**: p. 183.
20. Cao, Y., Dickinson, E., and Wedlock, D.J., *Creaming and flocculation in emulsions containing polysaccharide.* Food Hydrocolloids, 1990. **4**: p. 185.
21. Blijdenstein, T.B.J., Hendriks, W.P.G., van der Linden, E., van Vliet, T., and van Aken, G.A., *Control of strength and stability of emulsion gels by a combination of long- and short-range interactions.* Langmuir, 2003. **19**: p. 6657.
22. Blijdenstein, T.B.J., Zoet, F.D., van Vliet, T., van der Linden, E., and van Aken, G.A., *Dextran-induced depletion flocculation in oil-in-water emulsions in the presence of sucrose.* Food Hydrocolloids, 2004. **18**: p. 857.
23. Reiffers-Magnani, C.K., Cuq, J.L., and Watzkea, H.J., *Depletion flocculation and thermodynamic incompatibility in whey protein stabilised o/w emulsions.* Food Hydrocolloids, 2000. **14**: p. 521.

24. Tuinier, R. and de Kruif, C.G., *Phase separation, creaming, and network formation of oil-in-water emulsions induced by an exocellular polysaccharide.* Journal of Colloid and Interface Science, 1999. **218**: p. 201.
25. Blijdenstein, T.B.J., Veerman, C., and van der Linden, E., *Depletion-flocculation in oil-in-water emulsions using fibrillar protein assemblies.* Langmuir, 2004. **20**: p. 4881.
26. Koczo, K., Nikolov, A.D., Wasan, D.T., Borwankar, R.P., and Gonsalves, A., *Layering of sodium caseinate submicelles in thin liquid films- a new stability mechanism for food dispersions.* Journal of Colloid and Interface Science, 1996. **178**: p. 694.
27. Dickinson, E., Golding, M., and Povey, M.J.W., *Creaming and flocculation of oil-in-water emulsions containing sodium caseinate.* Journal of Colloid and Interface Science, 1997. **185**: p. 515.
28. Dickinson, E. and Golding, M., *Rheology of sodium caseinate stabilized oil-in-water emulsions.* Journal of Colloid and Interface Science, 1997. **191**: p. 166.
29. Ruehrwein, R.A. and Ward, D.W., *Mechanism of clay aggregation by polyelectrolytes.* Soil Science, 1952. **73**: p. 485.
30. Buffle, J. and Leppard, G.G., *Characterization of aquatic colloids and macromolecules .1. Structure and behavior of colloidal material.* Environmental Science & Technology, 1995. **29**: p. 2169.
31. Perret, D., Newman, M., Negre, J.C., Chen, Y.W., and Buffle, J., *Submicron particles in the rhine river, part 1. Physico-chemical characterization.* Water Research, 1994. **28**: p. 91.
32. Healy, T.W. and Lamer, V.K., *Energetics of flocculation and redispersion by polymers.* Journal of Colloid Science, 1964. **19**: p. 323.
33. Biggs, S., Habgood, M., Jameson, G.J., and Yan, Y., *Aggregates structure formed via a bridging flocculation mechanism.* Chemical Engineering Journal, 2000. **80**: p. 13.
34. Blijdenstein, T.B.J., van Winden, A.J.M., van Vliet, T., van der Linden, E., and van Aken, G.A., *Serum separation and structure of depletion- and bridging-flocculated emulsions: A comparison.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2004. **245**: p. 41.
35. Dickinson, E. and Pawlowsky, K., *Influence of iota-carrageenan on flocculation, creaming and rheology of a protein-stabilized emulsion.* Journal of Agriculture and Food Chemistry, 1997. **45**: p. 3799.
36. Dickinson, E., *Milk protein interfacial layers and the relationship to emulsion stability and rheology.* Colloids and Surfaces B: Biointerfaces, 2001. **20**: p. 197.
37. Batchelor, G.K., *Effect of brownian-motion on bulk stress in a suspension of spherical-particles.* Journal of Fluid Mechanics, 1977. **83**: p. 97.
38. Ibanoglu, E., *Rheological behaviour of whey protein stabilized emulsions in the presence of gum arabic.* Journal of Food Engineering, 2002. **52**: p. 273.
39. Tadros, T., *Application of rheology for assessment and prediction of the long-term physical stability of emulsions.* Advances in Colloid and Interface Science, 2004. **108-09**: p. 227.
40. Tadros, T.H., *Fundamental principles of emulsion rheology and their applications.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 1994. **91**: p. 39.
41. Mandel, I.D., *A contemporary view of salivary research.* Critical Reviews in Oral Biology and Medicine 1993. **4**: p. 599.
42. Humphrey, S.P. and Williamson, R.T., *A review of saliva: Normal composition, flow and function.* Journal of Prosthetic Dentistry, 2001. **85**: p. 162.
43. Ryu, O.H., Atkinson, J.C., Hoehn, G.T., Illei, G.G., and Hart, T.C., *Identification of parotid salivary biomarkers in Sjogren's syndrome by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional difference gel electrophoresis.* Rheumatology, 2006. **45**: p. 1077.
44. Saari, H., Halinen, S., Ganlöv, K., Sorsa, T., and Konttinen, Y.T., *Salivary mucous glycoprotein MG1 in Sjögren's syndrome.* Clinica Chimica Acta, 1997. **259**: p. 83.
45. Streckfus, C.F., Bigler, L.R., and Zwick, M., *The use of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry to detect putative breast cancer markers in saliva: A feasibility study.* Journal of Oral Pathology and Medicine, 2006. **35**: p. 292.
46. Zalewska, A., Zwierz, K., Zólkowski, K., and Gindzienski, A., *Structure and biosynthesis of human salivary mucins.* Acta Biochimica Polonica, 2000. **47**: p. 1067.
47. Van Nieuw Amerongen, A., Bolscher, J.G.M., and Veerman, E.C.I., *Salivary proteins: Protective and diagnostic value in cariology?* Caries Research, 2004. **38**: p. 247.
48. Silvers, A.R. and Som, P.M., *Salivary glands.* Radiologic Clinics of North America, 1998. **36** p. 941.
49. Young, J.A. and van Lennep, E.W., *The morphology of the salivary glands.* 1978, London: Academic Press.

50. Ferguson, D.B. and Botchway, C.A., *A comparison of circadian variation in the flow rate and composition of stimulated human parotid, submandibular and whole salivas from the same individuals.* Archives of Oral Biology, 1980. **25**: p. 559.
51. Ferguson, D.B., Fort, A., Elliott, A.L., and Potts, A.J., *Circadian rhythms in human parotid saliva flow rate and composition.* Archives of Oral Biology, 1973. **18**: p. 1155.
52. Dawes, C., *Circadian rhythms in human salivary flow rate and composition.* Journal of Physiology, 1972. **220**: p. 529.
53. Gusman, H., Leone, C., Helmerhorst, E.J., Nunn, M., Flora, B., Troxler, R.F., and Oppenheim, F.G., *Human salivary gland-specific daily variations in histatin concentrations determined by a novel quantitation technique.* Archives of Oral Biology, 2004. **49**: p. 11.
54. Veerman, E.C.I., van der Keybus, P.A.M., Vissink, A., and van Nieuw Amerongen, A., *Human glandular salivas: Their separate collection and analysis.* European Journal of Oral Science, 1996. **104**: p. 346.
55. Christensen, C.M. and Navazesh, M., *Anticipatory salivary flow to the sight of different foods.* Appetite, 1984 **5**: p. 307.
56. Hector, M. and Linden, R., *Reflexes of salivary secretion.* 1999., Basel. : Karger.
57. Lee, V.M. and Linden, R.W., *An olfactory-submandibular salivary reflex in humans.* Experimental physiology, 1992. **77** p. 221.
58. Mandel, I.D., *Relation of saliva and plaque to caries.* Journal of Dental Research, 1974. **53**: p. 246.
59. Shannon, I.L., Suddick, R.P., and Dowd Jr, F.J., *Saliva: Composition and secretion.* Monographs in oral science, 1974. **2**: p. 1.
60. Battino, M., Ferreiro, M.S., Gallardo, I., Newman, H.N., and Bullon, P., *The antioxidant capacity of saliva.* Journal of Clinical Periodontology, 2002. **29**: p. 189.
61. Edgar, W.M., *Saliva and dental health.* British Dental Journal, 1990. **169**: p. 96.
62. Amado, F.M.L., Vitorino, R.M.P., Domingues, P.M.D.N., Lobo, M.J.C., and Duarte, J.A.R., *Analysis of the human saliva proteome.* Expert Review of Proteomics, 2005: p. 521.
63. Ghafouri, B., Tagesson, C., and Lindahl, M., *Mapping of proteins in human saliva using two-dimensional gel electrophoresis and peptide mass fingerprinting.* Proteomics, 2003. **3**: p. 1003.
64. Hardt, M., Thomas, L.R., Newport, G., Agabian, N., Prakobphol, A., Hall, S.C., Witkowska, H.E., Fisher, S.J., and Dixon, S.E., *Toward defining the human parotid gland salivary proteome and peptidome: Identification and characterization using 2S SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry.* Biochemistry, 2005. **44**: p. 2885.
65. Vitorino, R., Lobo, M., J., C., Ferre-Correira, A., J., Dubin, J., R., Tomer, K., B., Domingues, P., M., and Amado, F., M.,L., *Identification of human whole saliva protein components using proteomics.* Proteomics, 2004. **4**: p. 1109.
66. Yao, Y., Berg, E.A., Costello, C.E., Troxler, R.F., and Oppenheim, F.G., *Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches.* The Journal of Biological Chemistry, 2003. **278**: p. 5300.
67. Hu, S., Li, Y., Wong, D.T., Xie, Y., Ramachandran, P., Loo, J.A., and Loo, R.R.O., *Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry.* Proteomics, 2005. **5**: p. 1714.
68. Lupi, A., Messana, I., Denotti, G., Schinina Maria, E., Gambarini, G., Fadda Maria, B., Vitali, A., Cabras, T., Piras, V., Patamia, M., Cordaro, M., Giardina, B., and Castagnola, M., *Identification of the human salivary cystatin complex by the coupling of high-performance liquid chromatography and ion-trap mass spectrometry.* Proteomics, 2003. **3**: p. 461.
69. Pisano, E., Cabras, T., Montaldo, C., Piras, V., Inzitari, R., Olmi, C., Castagnola, M., and Messana, I., *Peptides of human gingival crevicular fluid determined by HPLC-ESI-MS.* European Journal of Oral sciences, 2005. **113**: p. 462.
70. Messana, I., Loffredo, F., Inzitari, R., Cabras, T., Giardina, B., Onnis, G., Piludu, M., and Castagnola, M., *The coupling of RP-HPLC and ESI-MS in the study of small peptides and proteins secreted in vitro by human salivary glands that are soluble in acidic solution.* European Journal of Morphology, 2003. **41**: p. 103.
71. Castagnola, M., Cabras, T., Inzitari, R., Zuppi, C., Rossetti, D.V., Petruzzelli, R., Vitali, A., Loy, F., Conti, G., and Fadda, M.B., *Determination of the post-translational modifications of salivary acidic proline-rich proteins.* European Journal of Morphology, 2003. **41**: p. 93.
72. Hu, S., Loo, J.A., and Wong, D.T., *Human saliva proteome analysis*. Annals of the New York Academy of Sciences, 2007. **1098** : p. 323.
73. Hay, D.I., *Fractionation of human parotid salivary proteins and the isolation of an histidine-rich acidic peptide which shows high affinity for hydroxyapatite surfaces.* Archives of Oral Biology, 1975. **20**: p. 553.

74. Scannapieco. A., Torres G.I., and Levine M.J., *Salivary amylase promote adhesion of oral Streptococci to hydroxyapatite.* Journal oF Dental Research, 1995. **74**: p. 1360.
75. Cannon, R.D., Nand, A.K., and Jenkinson, H.F., *Adherence of Candida Albicans to human salivary components adsorbed to hydroxyapatite.* Microbiology, 1995. **141**: p. 213.
76. Vassilakos, N., Arnebrant, T., and Glantz, P., *Adsorption of whole saliva onto hydrophilic and hydrophobic solid surfaces: Influence of concentration, ionic strength and pH.* Scandinavian Journal of Dental research, 1992. **100**: p. 346.
77. Omichi, K. and Ikenaka, T., *Separation of human salivary [alpha]-amylase isozymes by high-performance liquid chromatography with a continuous monitor system of the activity.* Analytical Biochemistry, 1988. **168**: p. 332.
78. Aguirre, A., Mendoza, B., Reddy, M.S., Scannapieco, F.A., Levine, M.J., and Hatton, M.N., *Lubrication of selected salivary molecules and artificial salivas.* Dysphagia, 1989. **4**: p. 95.
79. Lamkin, M.S., Jensen, J.L., Setayesh, M.R., Troxler, R.F., and Oppenheim, F.G., *Salivary cystatin SA-III, a potential precursor of the acquired enamel pellicle, is phosphorylated at both its amino- and carboxyl-terminal regions.* Archives of Biochemistry and Biophysics, 1991. **288**: p. 664.
80. Isemura, S., Saitoh, E., and Sanada, K., *Characterization of a new cysteine proteinase inhibitor of human saliva, cystatin SN, which is immunologically related to cystatin S.* FEBS Letters, 1986. **198**: p. 145.
81. Al-Hashimi, I., Dickinson, D.P., and Levine, M.J., *Purification, molecular-cloning, and sequencing of salivary cystatin SA-I.* Journal of Biological Chemistry, 1988. **263**: p. 9381.
82. Rathman, W.M., van Zeyl, M.J., van den Keijbus, P.A., Veerman, E.C., and van Nieuw Amerongen, A., *Comparison of salivary 14 kd protein displaying cysteine proteinase inhibitory activity with other salivary cystatins.* Journal de Biologie Buccale, 1990. **18**: p. 9.
83. Ramasubbu, N., Reddy, M.S., Bergey, E.J., Haraszthy, G.G., Soni, S.D., and Levine, M.J., *Large-scale purification and characterization of the major phosphoproteins and mucins of human submandibular-sublingual saliva.* Biochemical Journal, 1991. **280**: p. 341.
84. Kauffman, D.L. and Keller, P.J., *Relationship of the basic glycoprotein to the basic proline-rich proteins in human-parotid saliva.* Archives of Oral Biology, 1983. **28**: p. 61.
85. MacKay, B.B.J., Pollock, J.J.J., Iacono, V.V.J., and Baum, B.B.J., *Isolation of milligram quantities of a group of histidine-rich polypeptides from human parotid saliva.* Infection and immunity, 1984. **44**: p. 688.
86. Flora, B., Gusman, H., Helmerhorst, E.J., Troxler, R.F., and Oppenheim, F.G., *A new method for the isolation of histatins 1, 3, and 5 from parotid secretion using zinc precipitation.* Protein Expression and Purification, 2001. **23**: p. 198.
87. Perinpanayangam, H.E.R., Wuyckhuyze, B.C., Ji, Z.S., and Tabak, L.A., *Characterization of low-molecular-weight peptides in human parotid saliva.* Journal of Dental Research, 1995. **71**: p. 345.
88. Hay, D.I. and Oppenheim, F.G., *The isolation from human parotis saliva of a further group of proline-rich proteins.* Archives of Oral Biology, 1974. **19**: p. 627.
89. Charlton, A.J., Baxter, N.J., Lilley, T.H., Haslam, E., McDonald, C.J., and Williamson, M.P., *Tannin interactions with a full-length human salivary proline-rich protein display a stronger affinity than with single proline-rich repeats.* FEBS Letters, 1996. **382**: p. 289.
90. Hay, D.I., Ahern, J.M., Schluckebier, S.K., and Schlesinger, D.H., *Human salivary acidic proline-rich protein polymorphisms and biosynthesis studied by high-performance liquid-chromatography.* Journal of Dental Research, 1994. **73**: p. 1717.
91. Schlesinger, D.H., Hay, D.I., Schluckebier, S.K., and Ahern, J.M., *Primary structure of a novel human salivary acidic proline-rich protein.* Peptide Research, 1994. **7**: p. 242.
92. Bedi, G.S. and Bedi, S.K., *Purification and characterization of rat parotid glycosylated, basic and acidic proline-rich protein.* Preparative Biochemistry, 1995. **25**: p. 119.
93. Geetha, G., Venkatesh, S.G., Fasciotto Dunn, B.H., and Gorr, S.,-U., *Expression and anti-bacterial activity of human parotid secretory protein (PSP).* Biochemical Society Transactions, 2003. **31**: p. 815.
94. Isemura, S., Saitoh, E., and Sanada, K., *Isolation and amino-acid-sequence of SAP-1, an acidic protein of human whole saliva, and sequence homology with human gamma-trace.* Journal of Biochemistry, 1984. **96**: p. 489.
95. Jensen, J.L., Lamkin, M.S., Troxler, R.F., and Oppenheim, F.G., *Multiple forms of statherin in human salivary secretions.* Archives of Oral Biology, 1991. **36**: p. 529.
96. Kasinathan, C., Ramaprasad, P., and Sundaram, P., *Identification and characterization of tyrosylprotein sulfotransferase from human saliva.* International Journal of Biological Sciences, 2005. **1**: p. 141.

97. Grasbeck, R., Visuri, K., and Stenman, U.H., *Vitamin-B12-binding protein in human saliva isolation, characterization and comparison with unpurified protein.* Biochimica et Biophysica Acta, 1972. **263**: p. 721.
98. Veerman, E.C.I., van der Keybus, P.A.M., Valentijn-Benz, M., and van Nieuw Amerongen, A., *Isolation of different high-Mr mucin species from human whole saliva.* Biochemical Journal, 1992. **283**: p. 807.
99. Raynal, B.D.E., Hardingham, T.E., Thornton, D.J., and Sheehan, J.K., *Concentrated solutions of salivary MUC5B mucin do not replicate the gel-forming properties of saliva.* Biochemical Journal, 2002. **362**: p. 289.
100. Thornton, D.J., Khan, N., Mehrotra, R., Howard, M., Veerman, E.C.I., Packer, N.H., and Sheehan, J.K., *Salivary mucin MG1 is comprised almost entirely of different glycosylated forms of the MUC5B gene product.* Glycobiology, 1999. **9**: p. 293.
101. Iontcheva, I., Oppenheim, F.G., and Troxler, R.F., *Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins.* Journal of Dental Research, 1997. **76**: p. 734.
102. Raynal, B.D.E., Hardingham, T.E., Sheehan, J.K., and Thornton, D.J., *Calcium-dependent protein interactions in MUC5B provide reversible crosslinks in salivary mucus.* Journal of Biological Chemistry, 2003. **278**: p. 28703.
103. Wickström, C., Christersson, C., Davies, J.R., and Carlstedt, I., *Macromolecular organization of saliva: Identification of 'insoluble' MUC5B assemblies and non-mucin proteins in the gel phase.* Biochemical Journal, 2000. **351**: p. 421.
104. Wickström, C., Davies, J.R., Eriksen, G.V., Veerman, E.C.I., and Carlstedt, I., *MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: Identification of glycoforms and c-terminal cleavage.* Biochemical Journal, 1998. **334**: p. 685.
105. Liu, B., Rayment, S.A., Oppenheim, F.G., and Troxler, R.F., *Isolation of human salivary mucin MG2 by a novel method and characterization of its interactions with oral bacteria.* Archives of Biochemistry and Biophysics, 1999. **364**: p. 286.
106. Mehrotra, R., Thornton, D.J., and Sheehan, J.K., *Isolation and physical characterization of the MUC7 (MG2) mucin from saliva: Evidence for self-association.* Biochemical Journal, 1998. **334**: p. 415.
107. Soares, R.V., Liu, B., Oppenheim, F.G., Offner, G.D., and Troxler, R.F., *Structural characterisation of cysteines in a bacterial-binding motif of human salivary mucin MG2.* Archives of Oral Biology, 2002. **47**: p. 591.
108. Rykke, M., Young, A., Devold, T., Smistad, G., and Rølla, G., *Fractionation of salivary micelle-like structures by gel chromatography.* European Journal of Oral sciences, 1997. **105**: p. 495.
109. Messana, I., Cabras, T., Fadda, M.B., Inzitari, R., Zuppi, C., Olmi, C., Giardina, B., Castagnola, M., Lupi, A., and Cordaro, M., *Characterization of the human salivary basic proline-rich protein complex by a proteomic approach.* Journal of Proteome Research, 2004. **3**: p. 792.
110. Wilmarth, P.A., Riviere, M.A., Rustvold, D.L., Lauten, J.D., Madden, T.E., and David, L.L., *Two-dimensional liquid chromatography study of the human whole saliva proteome.* Journal of Proteome Research, 2004. **3**: p. 1017.
111. Hirtz, C., Chevalier, F., Centeno, D., Egea, J.C., Rossignol, M., Sommerer, N., and de Périère, D., *Complexity of the human whole saliva proteome.* Journal of Physiology and Biochemistry, 2005. **61**: p. 469.
112. Walz, A., Stuhler, K., Wattenberg, A., Hawranke, E., Meyer, H.E., Schmalz, G., Bluggel, M., and Ruhl, S., *Proteome analysis of glandular parotid and submandibular-sublingual saliva in comparison to whole human saliva by two-dimensional gel electrophoresis.* Proteomics, 2006. **6**: p. 1631.
113. Huang, C.-M., *Comparative proteomic analysis of human whole saliva.* Archives of Oral Biology, 2004. **49**: p. 951.
114. Guo, T., Rudnick, P.A., Wang, W., Lee, C.S., DeVoe, D.L., and Balgley, B.M., *Characterization of the human salivary proteome by capillary isoelectric focusing/nanoreversed-phase liquid chromatography with esi-tandem ms* Journal of Proteome Research, 2006. **5**: p. 1469.
115. Schipper, R., Loof, A., De Groot, J., Harthoorn, L., Van Heerde, W., and Dransfield, E., *Salivary protein/peptide profiling with SELDI-TOF-MS* in Annals of the New York Academy of Sciences. 2007. **1098**: p. 498.
116. De Smet, K. and Contreras, R., *Human antimicrobial peptides: Defensins, cathelicidins and histatins.* Biotechnology Letters, 2005. **27**: p. 1337.
117. Troxler, R.F., Offner, G.D., Xu, T., Vanderspek, J.C., and Oppenheim, F.G., *Structural relationship between human salivary histatins.* Journal of Dental Research, 1990. **69**: p. 2.

118. Castagnola, M., Inzitari, R., Rossetti, D.V., Olmi, C., Cabras, T., Piras, V., Nicolussi, P., Sanna, M.T., Pellegrini, M., Giardina, B., and Messana, I., *A cascade of 24 histatins (histatin 3 fragments) in human saliva: Suggestions for a pre-secretory sequential cleavage pathway.* Journal of Biological Chemistry, 2004. **279**: p. 41436.
119. Inzitari, R., Cabras, T., Onnis, G., Olmi, C., Mastinu, A., Sanna, M.T., Pellegrini, M.G., Castagnola, M., and Messana, I., *Different isoforms and post-translational modifications of human salivary acidic proline-rich proteins.* Proteomics, 2005. **5**: p. 805.
120. Inzitari, R., Cabras, T., Rossetti, D.V., Fanali, C., Vitali, A., Pellegrini, M., Paludetti, G., Manni, A., Giardina, B., Messana, I., and Castagnola, M., *Detection in human saliva of different statherin and p-b fragments and derivatives.* Proteomics, 2006. **6**: p. 6370.
121. Isemura, S., Saitoh, E., Sanada, K., and Minakata, K., *Identification of full-sized forms of salivary (s-type) cystatins (cystatin SN, cystatin SA, cystatin S, and two phosphorylated forms of cystatin S) in human whole saliva and determination of phosphorylation sites of cystatin S.* Journal of Biochemistry, 1991. **110**: p. 648.
122. Hay, D.I., Bennick, A., Schlesinger, D.H., Minaguchi, K., Madapallimattam, G., and Schluckebier, S.K., *The primary structures of six human salivary acidic proline-rich proteins (PRP-1, PRP-2, PRP-3, PRP-4, Pif-s and Pif-f).* Biochemical Journal, 1988. **255**: p. 15.
123. Hahn Berg, I.C., Rutland, M.W., and Arnebrant, T., *Lubricating properties of the initial salivary pellicle. An AFM study.* Biofouling, 2003. **19**: p. 365.
124. Tabak, L.A., Levine, M.J., Mandel, I.D., and Ellison, S.A., *Role of salivary mucins in the protection of the oral cavity.* Journal of Oral Pathology, 1982. **11**: p. 1.
125. Hahn Berg, I.C., Lindh, L., and Arnebrant, T., *Intraoral lubrication of PRP-1, statherin and mucin as studied by AFM.* Biofouling, 2004. **20**: p. 65.
126. Douglas, W.H., Reeh, E.S., Ramasubbu, N., Raj, P.A., Bhandary, K.K., and Levine, M.J., *Statherin: A major boundary lubricant of human saliva.* Biochemical and Biophysical Research Communications, 1991. **180**: p. 91.
127. Proctor, G.B., Hamdan, S., Carpenter, G.H., and Wilde, P.J., *A statherin and calcium enriched layer at the air interface of human parotid saliva* Biochemical Journal, 2005. **389**: p. 111.
128. Reeh, E.S., Douglas, W.H., and Levine, M.J., *Lubrication of human and bovine enamel compared in an artificial mouth.* Archives of Oral Biology, 1995. **40**: p. 1063.
129. Hatton, M.N., Loomis, R.E., Levine, M.J., and Tabak, L.A., *Masticatory lubrication. The role of carbohydrate in the lubricating property of a salivary glycoprotein-albumin complex.* Biochemical Journal, 1985. **230**: p. 817.
130. van Nieuw Amerongen, A. and Veerman, E.C.I., *Saliva - the defender of the oral cavity.* Oral Diseases, 2002. **8**: p. 12.
131. Aggazzotti, A., *Modificazioni della viscosita' della saliva mista dopo che e'stata secreta in rapporto col potere filante e colla tensione superficiale.* Archivio di Fisiologia, 1922. **20**: p. 3.
132. Columbini, N., *Ricerche sui cosidetti corpi filanti. Sulla diffusione libera di alcune sostanze coloranti.* Bollettino della Societa Italiana di Biologia Sperimentale, 1939. **14**: p. 453.
133. Fano, G., *Contributo allo studio dei corpi filanti.* Archivio di Fisiologia, 1908. **5**: p. 365.
134. Schwartz, W.H., *The rheology of saliva.* Journal of Dental Research, 1987. **66**: p. 660.
135. Glantz, P.O., Friberg, S.E., Wirth, S.M., and Baier, R.E., *Thin-section transmission electron microscopy of human saliva.* Acta Odontologica Scandinavica, 1989. **47**: p. 111.
136. Glantz, P.O., *Interfacial phenomena in the oral cavity.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 1997. **123-124**: p. 657.
137. Waigh, T.A., Papagiannopoulos, A., Voice, A., Bansil, R., Unwin, A.P., Dewhurst, C.D., Turner, B., and Afdhal, N., *Entanglement coupling in porcine stomach mucin.* Langmuir, 2002. **18**: p. 7188.
138. McCullagh, C.M., Soby, L.M., Jamieson, A.M., and Blackwell, J., *Viscoelastic behavior of fractionated ovine submaxillary mucins.* Biopolymers, 1992. **32**: p. 1665.
139. Strous, G.J. and Dekker, J., *Mucin-type glycoproteins.* Critical Reviews in Biochemistry and Molecular Biology, 1992. **27**: p. 57.
140. Bromberg, L. and Barr, D., *Self-association of mucin.* Biomacromolecules, 2000. **1**: p. 325.
141. Biesbrock, A.R., Reddy, M.S., and Levine, M.J., *Interaction of salivary mucin-secretory immunoglobulin a complex with mucosal pathogens.* Infection and Immunity, 1991. **59**: p. 3492.
142. Levine, M.J., *Salivary macromolecules: A structure/function synopsis,* in *Annals of the New York Academy of Sciences*, D. Malamud, Editor. 1993, New York Academy of Sciences: New York. p. 11.
143. Glantz, P.O., Natiella, J.R., Vaughan, C.D., and Baier, R.E., *Structural studies of human saliva.* Acta Odontologica Scandinavica, 1989. **47**: p. 17.
144. Rølla, G. and Rykke, M., *Evidence for presence of micelle-like protein globules in human saliva.* Colloids and Surfaces B: Biointerfaces, 1994: p. 177.

145. Rykke, M., Smistad, G., Rolla, G., and Karlsen, J., *Micelle-like structures in human saliva.* Colloids and Surfaces B: Biointerfaces, 1995. **4**: p. 33.
146. Rykke, M., Young, A., Rølla, G., Devold, T., and Smistad, G., *Transmission electron microscopy of human saliva.* Colloids and Surfaces, B: Biointerfaces, 1997. **9**: p. 257.
147. Rykke, M., Young, A., Smistad, G., Rølla, G., and Karlsen, J., *Zeta potentials of human salivary micelle-like particles.* Colloids and Surfaces, B: Biointerfaces, 1996. **6**: p. 51.
148. Soares, R.V., Lin, T., Siqueira, C.C., Bruno, L.S., Li, X., Oppenheim, F.G., Offner, G.D., and Troxler, R.F., *Salivary micelles: Identification of complexes containing MG2, sIgA, lactoferrin, amylase, glycolsylated proline-rich protein and lysozyme.* Archives of Oral Biology, 2004. **49**: p. 337.
149. Rykke, M., Devold, T.G., Smistad, G., and Vegarud, G.E., *Comparison of bovine casein micelles and human salivary micelle-like structures.* International Dairy Journal, 1999. **9**: p. 365.
150. Young, A., Rykke, M., and Rølla, G., *Quantitative and qualitative analyses of human salivary micelle-like globules.* Acta Odontologica Scandinavica, 1999. **57**: p. 105.
151. Sheehan, J.K. and Thronton, D.J., *Heterogeneity and size distribution of gel-forming mucins*, in *Glycoprotein methods and protocols: The mucins*, C. A.P., Editor. 2000, Humana Press: Totowa NJ. p. 87.
152. Vissink, A., Waterman, H.A., 's-Gravenmade, E.J., Panders, A.K., and Vermey, A., *Rheological properties of saliva substitutes containing mucin, carboxymethylcellulose or polyethylenoxide.* Journal of Oral Pathology, 1984. **13**: p. 22.
153. van der Reijden, W.A., Veerman, E.C.I., and van Nieuw Amerongen, A., *Shear rate dependent viscoelastic behaviour of human glandular salivas.* Biorheology, 1993. **30**: p. 141.
154. Levine, M.J., Aguirre, A., Hatton, M.N., and Tabak, L.A., *Artificial salivas: Present and future.* Journal of Dental Research, 1987. **66**: p. 639.
155. Veerman, E.C.I., Valentijn-Benz, M., and van Nieuw Amerongen, A., *Viscosity of human salivary mucins: Effect of pH and ionic strength and role of sialic acid.* Journal de Biologie Buccale, 1989: p. 297.
156. Bhaskar, K.R., Gong, D., Bansil, R., Pajevic, S., Hamilton, J.A., Turner, B.S., and LaMont, J.T., *Profound increase in viscosity and aggregation of pig gastric mucin at low pH.* The American Journal of Physiology, 199. **261**: p. G827.
157. van der Reijden, W.A., Veerman, E.C.I., and van Nieuw Amerongen, A., *Rheological properties of commercially available polysaccharides with potential use in saliva substitutes.* Biorheology, 1994. **31**: p. 631.
158. Waterman, H.A., Blom, C., Holterman, H.J., 's-Gravenmade, E.J., and Mellema, J., *Rheological properties of human saliva.* Archives of Oral Biology, 1988. **33**: p. 589.
159. Rantonen, P.J.F. and Meurman, J.H., *Viscosity of whole saliva.* Acta Odontologica Scandinavica, 1998. **56**: p. 210.
160. Nordbö, H., Darwish, S., and Bhatnagar, R.S., *Salivary viscosity and lubrication: Influence of pH and calcium.* Scandinavian Journal of Dental Research, 1984. **92**: p. 306.
161. Christersson, C.E., Lindh, L., and Arnebrant, T., *Film-forming properties and viscosities of saliva substitutes and human whole saliva.* European Journal of Oral sciences, 2000. **108**: p. 418.
162. Kusy, R.P., Schafer, D.L., and *Rheology of stimulated whole saliva in typical pre-orthodontic sample population.*Journal of Materials Science - Materials in Medicine, 1995. **6**: p. 385.
163. Mellema, J., Holterman, H.J., Waterman, H.A., Blom, C., and 's-Gravenmade, E.J., *Rheological aspects of mucin-containing solutions and saliva substitutes.* Biorheology, 1992. **29**: p. 231.
164. Gans, R.F., Watson, G.E., and Tabak, L.A., *A new assessment in vitro of human salivary lubrication using a compliant substrate.* Archives of Oral Biology, 1990. **35**: p. 487.
165. Aguirre, A., Mendoza, B., Levine, M.J., Hatton, M.N., and Douglas, W.H., *In vitro characterization of human salivary lubrication.* Archives of Oral Biology, 1989. **34**: p. 675.
166. Nordbö, H., Darwish, S., and Bhatnagar, R.S., *Rate of viscosity changes in five salivary protein fractions following pH alterations.* Scandinavian Journal of Dental Research, 1984. **92**: p. 302.
167. Sellers, L.A., Allen, A., Morris, E.R., and Ross-Murphy, S.B., *Submaxillary mucins: Intermolecular interactions and gel-forming potential of concentrated solutions.* Biochemical Journal, 1988. **256**: p. 599.

Chapter 2

The role of electrostatics in saliva-induced emulsion flocculation

E. Silletti, M.H. Vingerhoeds, W. Norde, and G.A. van Aken

Food Hydrocolloids 21(2007) 596-606

Abstract

Upon consumption food emulsions undergo different processes, including mixing with saliva. It has been shown that whole saliva induces emulsion flocculation [1, 2]. It was hypothesized that depletion flocculation was responsible for the observed flocculation. To further unravel the mechanism, we investigated the role of electrostatics on the behavior of emulsion/saliva mixtures. Emulsions stabilized with differently charged surfactants and proteins were mixed with saliva. Strongly negatively charged emulsions (SDS and Panodan) do not flocculate, likely because the electrostatic repulsion between the droplets overcomes the attractive depletion and van der Waals interactions. Neutral and weakly negatively charged emulsions (Tween 20 and β-lactoglobulin pH 6.7) undergo flocculation, which is reversible upon dilution with water. This is probably due to depletion interactions, induced by large salivary protein like mucins, in combination with the van der Waals interaction and the sufficiently low electrostatic repulsion between the droplets. Positively charged emulsions (CTAB, lysozyme and β-lactoglobulin pH 3.0) show irreversible flocculation leading to rapid phase separation. These findings point to a role of electrostatic attraction between the negatively charged proteins present in saliva and the positively charged surfaces of the emulsion droplets. The results indicate that the sign and the density of the charge on the surface of the droplets contribute significantly to the behavior of an emulsion when mixed with saliva. Depending on the charge, saliva-induced emulsion flocculation is driven by two different main mechanisms: depletion attraction and electrostatic attraction.

Introduction

Human saliva is a complex heterogeneous biological fluid and is generally described as composed of proteins, electrolytes, small organic compounds and water, secreted by different glands [3-5]. Several factors such as stimulus, diet, medication and gender influence the flow rate and the composition. Human saliva is involved in maintaining oral health, in teeth and mucosal surface protection and in oral functions such as eating or swallowing [6]. These functions are carried out by numerous components, predominantly proteins. The protein composition of whole saliva and of the major glandular secretions has largely been investigated [7-12]. Unstimulated saliva, secreted mainly from the submandibular and sublingual glands is composed of several proteins including α-amylase, serum albumin, immunoglobulins (sIgA), cystatins, lysozyme, histatins and mucin. Various isoforms of salivary proteins have been identified [11]. Salivary mucins, which make up for 20-30 % of the protein content of unstimulated saliva [13], belong to a family of negatively charged protein containing heavily glycosylated domains, interspersed with less glycosylated ones.

Two types of secretory mucins are present in human saliva: the oligomeric MUC5B, formerly named MG1, with molecular mass (M_r) >1000 kDa and the monomeric MUC7, formerly called MG2, with M_r 200-300 kDa [3, 14]. Different species of MUC5B varying in the carbohydrate moiety and length of polysaccharides chain have been detected [7, 15-19]. MUC7 differs from MUC5B in the M_r and in the number and type of residues on the carbohydrate chain. Two different isoforms of MUC7 varying in the sialic acid and fucose content have been reported [20].

It is known that MUC5B and MUC7 form distinct types of complexes with other salivary proteins [21, 22]. MUC5B, isolated from human submandibular/sublingual secretion, forms large heterotypic complexes with α-amylase, prolin-rich protein, statherin and histatin [21]. MUC7 forms small heterodimeric complexes with lactoferrin in submandibular/sublingual secretion [23]. In stimulated whole saliva MUC7 also participates in the formation of the so called salivary micelles together with sIgA, lactoferrin, α-amylase, glycosylated prolin-rich protein and lysozyme [22]. Cryo-TEM analysis on stimulated whole saliva and parotid saliva samples confirmed the presence of micelle-like structures as globular negatively charged structures, in the size range about 40-400 nm, appearing in single formation or in clusters [24, 25].

Knowledge of the interaction between saliva and food is important for understanding the oral processing of food. Mixing with saliva can have a diluting effect and plays a role in facilitating food manipulation and bolus formation in the oral cavity [26]. The enzymatic activity of α-amylase favours, for example, initial breakdown of foodstuffs before swallowing. Saliva also influences flavour release [27] and acts as a buffering system affecting the perception of sourness [28]. Research conducted on saliva-food topics has been mostly directed towards the sensory analysis of emulsions in relation to saliva properties such as saliva flow, composition, lubrication properties and towards flavour release [29-32]. Recently, the physical-chemical effect of saliva on protein-stabilized food emulsions was reported. Saliva was shown to induce flocculation in emulsions stabilized by several emulsifiers including sodium caseinate and whey protein isolate [1, 2]. Depletion flocculation has been indicated to be responsible for the droplet flocculation in the saliva/emulsion mixtures. The role of mucins as driving force for flocculation was suggested and pig gastric mucin was used as a model for salivary mucins. However, the authors indicated that other mechanisms such as bridging flocculation could be important as well. Since we are interested in studying emulsion/saliva interaction, in which we ascribed a major role to mucins, we have decided to use unstimulated saliva for our flocculation experiments. Emulsion flocculation has been extensively studied. The influence of biopolymers on the stability of food related emulsions at pH values close to 7, where the emulsions are negatively charged, has been already reported by several authors [33-38]. It is also known that the charge on the droplets surface affects the flocculation mechanism and the demixing behavior of emulsions when mixed with polysaccharides [39].

The aim of this study is to clarify the mechanism of emulsion flocculation induced by saliva. As a first approach, to determine the influence of a number of parameters on the emulsion behavior in a mixture with saliva, we focused on the role of charge on the droplets surface. Low molecular weight surfactants and proteins have been used as emulsifiers for this purpose. To our knowledge, this is the first systematic study that describes the role of charge on the droplet's surface in a mixture with saliva.

Materials and Methods

Materials

Freeze-dried β-lactoglobulin (β-lg) was provided by Wageningen Centre for Food Science (WCFS, Wageningen, The Netherlands) and was purified according to the previously described method [40]. The powder contains 93.6 % w/w proteins (N x 6.38). Lysozyme from chicken egg-white (L6876) was obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands) and used without further purification. Polyoxyethylene sorbitan monolaurate (Tween 20) was purchased from Quest International (Zwijndrecht, The Netherlands), sodium dodecyl sulfate (SDS) from Merck (Schuchardt, Germany) and cetyltrimethylammonium bromide (CTAB) from BHD Chemicals Ltd. (Poole, England). Diacetyl tartaric acid ester of monoglyceride (Panodan) was kindly provided by Danisco (Grindstad, Denmark). Sunflower oil (Reddy, Vandemoortele, The Netherlands) was purchased from a local retailer; BCA^{TM} Protein Assay Kit from Pierce Biotechnology Inc. (Rockford, IL, USA) and sodium azide were obtained from Merck (Schuchardt, Germany).

Saliva collection and handling

Whole human unstimulated saliva was collected for 30 minutes from 11 (5 females and 6 males) healthy non-medicated volunteers, age ranged from 20 to 45 years, from 8.30 to 10.30 a.m. After optionally having breakfast and brushing their teeth, donors refrained from eating and drinking, with the exception of water, for two hours before donation. After rinsing their mouths with water, saliva was collected with closed lips for a couple of minutes and then expectorated into ice-chilled vessels. The first mL of saliva was discarded. The samples were kept constantly on ice during both donation and handling. Saliva was pooled and gently mixed. The amount of 3 grams was loaded in ice-chilled centrifuge tubes (Nalgene Oak Ridge centrifuge tubes, PC 10 mL, Nalge Nunc International, Rochester, USA) and centrifuged at 10000 g for 30 minutes at 4 °C to remove cellular debris (Beckman, model Avanti ^{TM} J-25 I, rotor JA-21, Beckman Coulter B.V. Mijdrecht, The Netherlands).

After centrifugation, saliva supernatant was pooled, gently mixed and frozen in liquid nitrogen in aliquots of 1 mL (Cryogenic Vials Nalgene tubes of 2 mL, Nalge Nunc International, Rochester, USA). Saliva supernatant, from now on indicated with the term saliva, was then stored at –80 °C and used within 6 weeks. After collection, about 2 hours

were needed for the completion of the handling procedure. Precipitation of salivary proteins was not detected after thawing. The pH of saliva was measured before and after storage and ranged from 6.8 to 7.1. Storage did not significantly affect the pH. Protein content determination of saliva was performed according to the BCA method of Pierce, with bovine serum albumin as standard, and varied from 1.2 mg mL^{-1} to 1.6 mg mL^{-1}.

Preparation and characterization of O/W emulsion stabilized by different emulsifiers

β-Lg and lysozyme solutions were prepared by dissolving protein powder respectively in demineralised water and 10 mM NaCl solution overnight at 4°C. SDS, Tween 20 and CTAB were dissolved in demineralised water and stirred for 2 hours at room temperature while Panodan was dissolved in oil at 75°C. Pre-emulsions were prepared using an Ultra-Turrax T 25 Basic (IKA-Werke). Subsequently they were homogenized at room temperature by 10 passes through a Delta Lab-scale homogenizer (Delta Instruments) operating at a pressure of 70 bar or, in case of lysozyme emulsion, 100 bar. The different pressure was used to obtain similar droplet sizes. The Panodan pre-emulsion was homogenized at 75°C.

Emulsions stabilized respectively by β-lg, SDS, Tween 20 and CTAB at pH 6.7, contained 40 % w/w sunflower oil and 1 % w/w emulsifier. β-lg emulsion with a pH of 3.0 was prepared by lowering the pH after homogenization. Lysozyme emulsion (pH 6.7, 1 % w/w protein and 10 mM NaCl) contained 20 % w/w sunflower oil while Panodan emulsion (pH 3.0, 2.5 % w/w surfactant, 10 mM NaCl) contained 37.5 % w/w sunflower oil. Sodium azide 0.02 % w/w (harmful to humans when swallowed) was added to the emulsions to prevent microbial growth.

The droplet-size distribution and volume-over surface average droplet diameter, d_{32}, were measured by laser diffraction with the Mastersizer Hydro 2000S (Malvern Instruments, Southborough, UK). Dilution with demineralised water was applied during the measurements.

Zeta (ζ) potential measurements of emulsions were performed using an Electroacoustic Spectrometer model DT 1200 (Dispersion Technology Inc, Bedford Hills, NY 10507 USA). 100 mL of each emulsion containing 10 % w/w oil and 10 mM NaCl were used and ζ-potential values were obtained as average of 3 measurements.

Sample preparation for flocculation experiments

Emulsion/saliva mixtures contained 10 % w/w oil phase and 0.6 mg mL^{-1} of salivary proteins (SPs). These concentrations have been chosen in order to compare our results with those reported earlier [2], where emulsions were mixed 1:1 with saliva. To eliminate possible problems related to the aging process in saliva, saliva was removed from –80°C and thawed at room temperature before each experiment. Mixtures were prepared at room temperature by adding thawed saliva to diluted emulsions containing 10 mM NaCl in the bulk phase. The pH of the mixtures was about 6.8 for emulsions made at pH 6.7 and about 4.3 for the Panodan and β-lg emulsions that were made at pH 3.0. However for simplification and unless specified otherwise, the pH in the text is referring to the pH of the emulsions.

Characterization of the flocculation

Flocculation was studied by light microscopy and particle size analysis. To evaluate the stability of the flocs and gaining insight into the mechanism, aliquots were analyzed at room temperature immediately after mixing and followed in time for 30 minutes. Light microscopy images were taken using an Olympus BX 60 Microscope equipped with an Olympus DP 70 camera (Olympus Nederland B.V., Zoeterwoude, The Netherlands). Size distribution analysis of the flocs was conducted by laser diffraction as previously indicated. However, due to the mathematical calculation model applied to the experimental data (the particles are considered as spheres) the obtained values provided only a rough estimate of the real dimensions of the flocs.

Demixing experiments

Demixing behavior was followed by measuring the intensity of a incident laser light scattered backwards along the height of an optical glass tube, using a Turbiscan MA 2000 apparatus (Ramonville St. Agne, France) as described before [41, 42]. The recorded profile gives a qualitative indication of the distribution of the oil droplets along the height of the tube, since the back scattering intensity varies mainly with the volume fraction of oil. The mixtures were prepared by loading the appropriate amount of saliva at the bottom of the tube and emulsion on top. After mixing, back scattering profiles were recorded at room temperature every minute for 90 minutes.

Rheology

The rheological behavior of emulsions upon addition of saliva was characterized by measuring the shear rate dependent viscosity. The mixtures were prepared as described for the flocculation experiments. Shear viscosity measurements were carried out in duplicate with Paar Physica MCR 300 (Anton Paar BVBA, Sint Martens Latem, Belgium) at 20°C with 10 minutes recovery time. Cone-and-plate geometry CP75-1, with an angle of 1º (0.0175 rad) and a gap width of 0.05 mm (at the tip) was used. The shear rate was initially increased from 1 to 1500 s^{-1} and then decreased to 1 s^{-1}. The viscosities of the emulsions for non-interacting oil droplets were calculated according to Batchelor [43] using Equation 1:

$$\eta_{emul} = \eta_0 (1+2.5\phi+6.2\phi^2) \quad \text{Eq.1}$$

where η_0 is the viscosity of the continuous phase and ϕ is the volume fraction of oil (0.1). η_0 was measured after centrifuging the emulsions at 120000 g for 30 minutes at 20°C to remove the oil phase (Beckman J-60, rotor SW 41, Beckman Coulter B.V. Mijdrecht, The Netherlands).

The theoretical viscosities of the mixtures were also calculated from equation 1 where the viscosity of the continuous phase is assumed to be the equal to the viscosity of saliva (η_{saliva}). We approximated that the viscosity of saliva in the mixture was, for each shear rate, the same of the measured one in absence of oil phase. η_{saliva} was measured at the concentration of 0.6 mg mL^{-1} SPs after dilution with 10 mM NaCl solution.

Results

Emulsion characteristics

Table 1 reports a summary of the emulsion properties, i.e. the emulsifiers, pH, d_{32} values and sign of the charge. d_{32} values of the emulsions ranged from 0.5 to 1 μm. The ζ-potentials of the emulsions are shown in Table 2. Briefly, negatively charged emulsions exhibit ζ-potential values ranging from -90 mV for SDS to -44 mV for β-lg pH 6.7. The uncharged Tween 20 emulsion showed, as expected, ζ-potential values close to zero. Positively charged emulsions displayed ζ-potential values that ranged from +21 mV for β-

lg stabilized emulsion at pH 4.3 (pH in the mixture with saliva) to +74 mV for CTAB emulsion pH 6.7.

Table 1 – Emulsion characteristics: emulsifiers, oil content, pH, d_{32} and charge sign of the emulsions.

Emulsifier type		Emulsifier (% w/w)	Oil (% w/w)	pH	d_{32}	Droplets Charge
Surfactants	SDS	1	40	6.7	0.5	-
	Panodan	2.5	37.5	3.0	0.7	-
	Tween 20	1	40	6.7	0.7	O
	CTAB	1	40	6.7	0.9	+
Proteins	β-lactoglobulin	1	40	6.7	0.8	-
	β-lactoglobulin	1	40	3.0	1.0	+
	Lysozyme	1	20	6.7	0.8	+

+ and – indicate positive and negative charge, respectively; O is used to indicate the neutrality of Tween 20.

Flocculation behavior in the presence of saliva

Figure 1 shows light microscopy images of emulsions (10 % w/w oil and 10 mM NaCl) immediately after mixing with saliva (0.6 mg mL^{-1} SPs). Highly negatively charged emulsions, stabilized respectively by SDS and Panodan, did not exhibit flocculation (Figure 1a, 1b). The neutral Tween 20 stabilized emulsion exhibited little flocculation (Figure 1c), whereas pronounced flocculation is seen for the weakly negatively charged (β-lg pH 6.7 Figure 1d) and the positively charged emulsions (β-lg pH 3.0, lysozyme and CTAB Figure 1e-g). Morphology differences are seen for the flocculated emulsions. The β-lg pH 6.7-saliva mixture presented homogenously dispersed flocs while larger densely packed structures were formed when β-lg pH 3.0 was mixed with saliva (compare Figure 1d and 1e). Both lysozyme and CTAB flocs appeared heterogeneous and contained both spherically shaped and long thread-like structures. The droplets seemed to be strongly packed in compact clumps (Figure 1f and 1g).

Particle size analysis was carried out on the mixtures to study the reversibility of the flocculation and to provide an indication of the size of the flocs. Unless differently specified, the term (ir)-reversible is used to describe the stability of the flocs upon dilution with water. Since the flocculation process occurred within seconds after mixing with saliva, it was only possible, in our experiments, to evaluate the effect of time on the stability of the flocs. Figure 2 shows the particle-size distribution of neutral and weakly negatively charged emulsions, i.e. Tween 20 and β-lg at pH 6.7 and their mixtures with saliva at t = 0 minutes, 5 minutes and 10 minutes after mixing.

In both cases the mixtures displayed the same droplet size distribution as the emulsions indicating that the flocculation process, detectable with light microscopy, was reversible upon dilution with water. No effect of time on the stability of the flocs was seen for these samples.

Figure 3 reports the size distribution profiles of the mixtures of saliva with positively charged emulsions, i.e. β-lg pH 3.0 and lysozyme pH 6.7, indicating irreversible flocculation with particle sizes ranging from 3 to 100 μm. Also the CTAB emulsion exhibited irreversible flocculation induced by saliva with particle sizes up to 100 μm (not shown). The influence of time on stability of the flocs is also reported in Figure 3. In case of β-lg stabilized emulsions made at pH 3.0, the size of the flocs changed during the first 10 minutes. After 10 minutes the effect of time was no longer detectable. For this positively charged emulsion, a shift in the pH (from pH 3.0 to 4.3) and in the ζ-potential (from + 69 mV to + 21 mV) occurred once mixed with saliva. At the pH value of the mixture, close to the isoelectric point of the protein, the net positive charge on the droplets is low. This might have a retarding influence on the formation of stable flocs.

Instead, the lysozyme and CTAB emulsion/saliva mixtures immediately flocculated into large structures. The overlapping of the size distribution for all the time intervals considered indicated no time dependency after the first observation.

For positively charged emulsion/saliva mixtures, the microscopic pictures showed different floc sizes than the particle size analysis. Flocs with dimensions > 100 μm were detected with light microscopy (Figure 1e, 1f, 1g) but not with the laser diffraction technique (Figure 2).

Possibly the larger flocs, observed with light microscopy, consist of weak aggregates of smaller flocs. The dilution involved in the laser diffraction may break these aggregates into smaller ones which are detected with this technique.

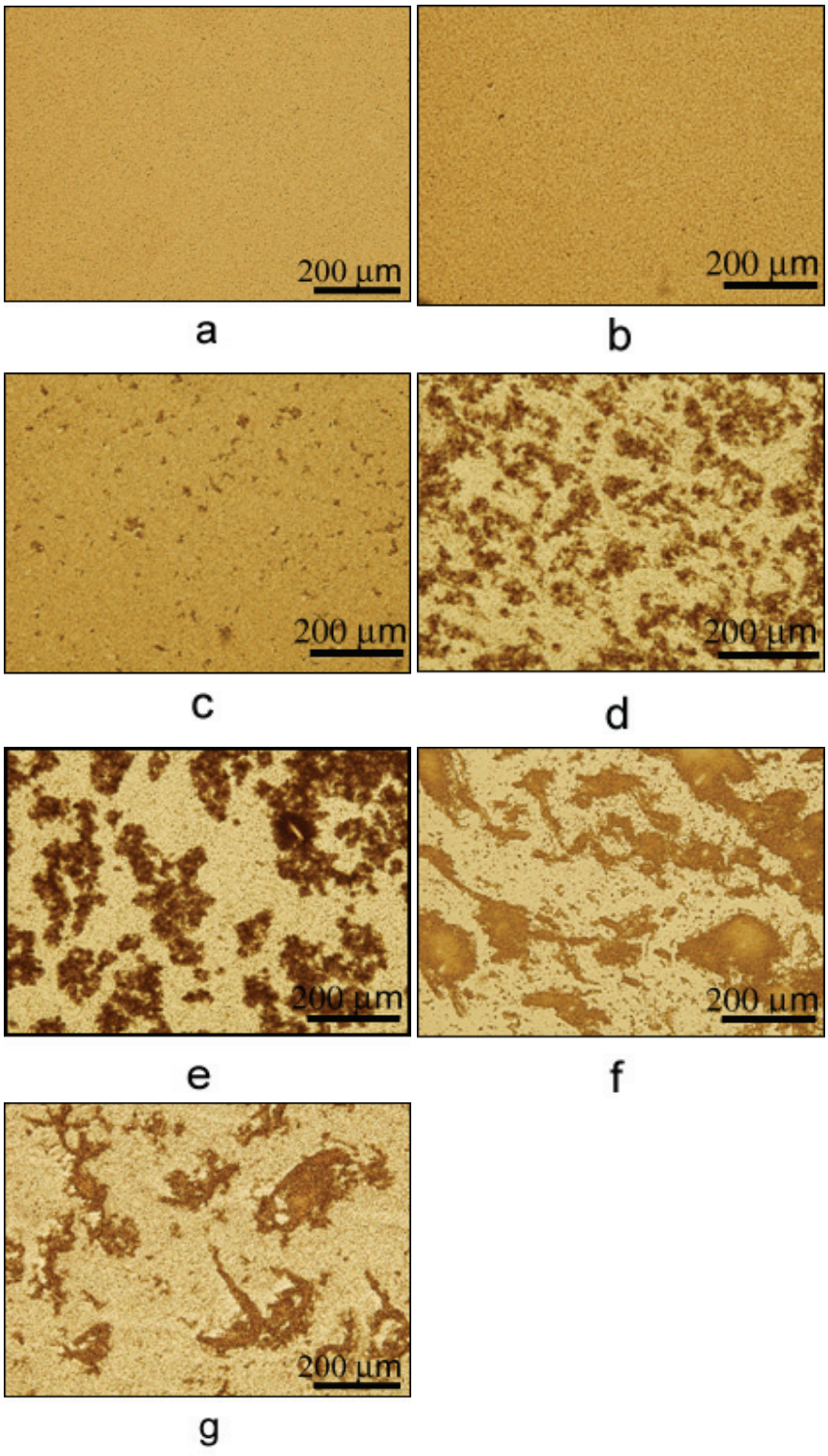

Figure 1 – Light microscopy photographs of saliva and emulsions containing 10 Mm NaCl immediately after mixing. (a) SDS pH 6.7, (b) Panodan pH 3.0, (c) Tween 20 pH 6.7, (d) β-lg pH 6.7, (e) β-lg pH 3.0, (f) lysozyme pH 6.7 and (g) CTAB pH 6.7. Scale bar of 200 µm.

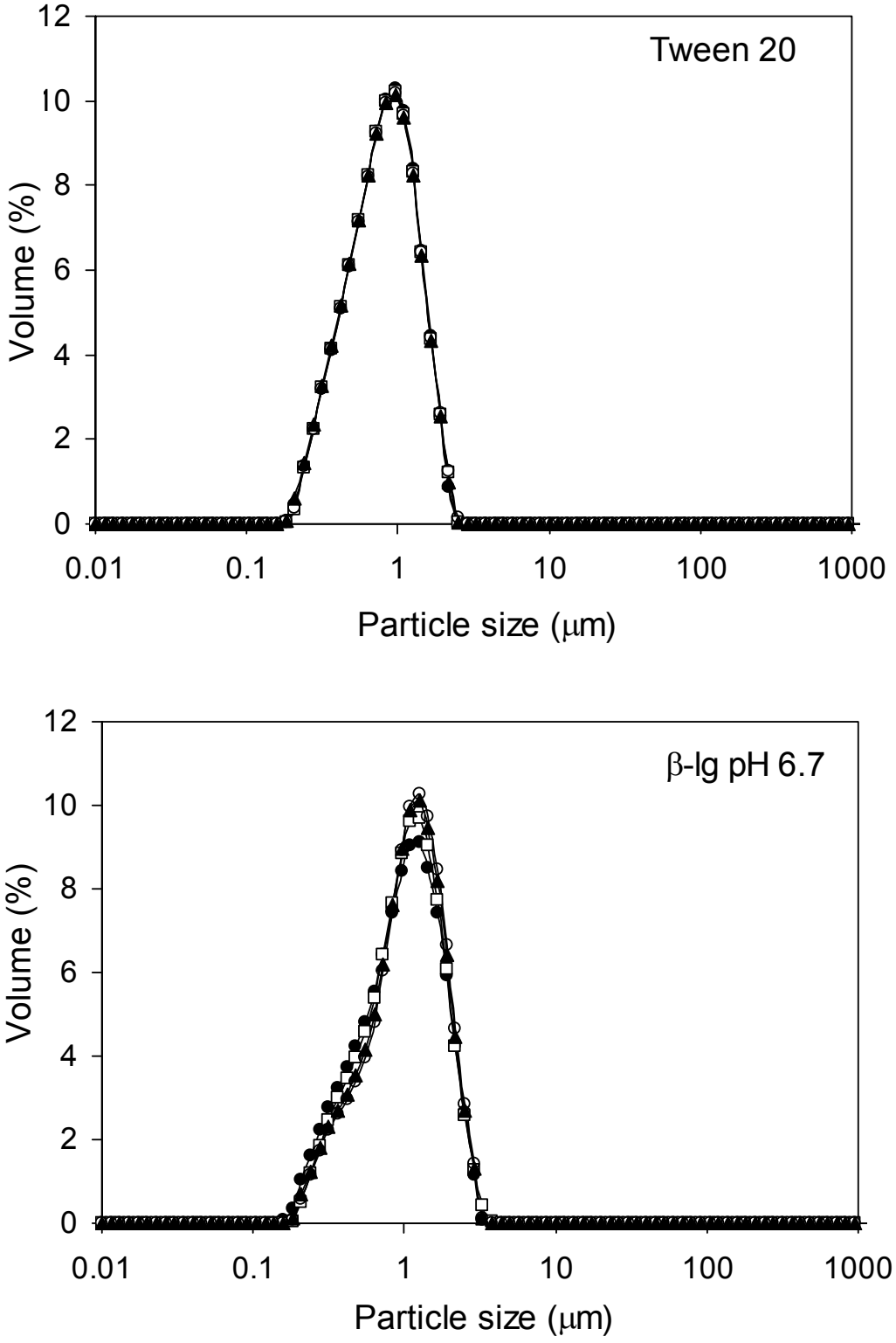

Figure 2 – Particle size analysis of saliva mixed with Tween 20 emulsion pH 6.7 (top) and β-lg emulsion pH 6.7 (bottom): (●) emulsion without saliva and mixtures at t = 0 minutes (□), t = 5 minutes (▲), t = 10 minutes (○) after mixing.

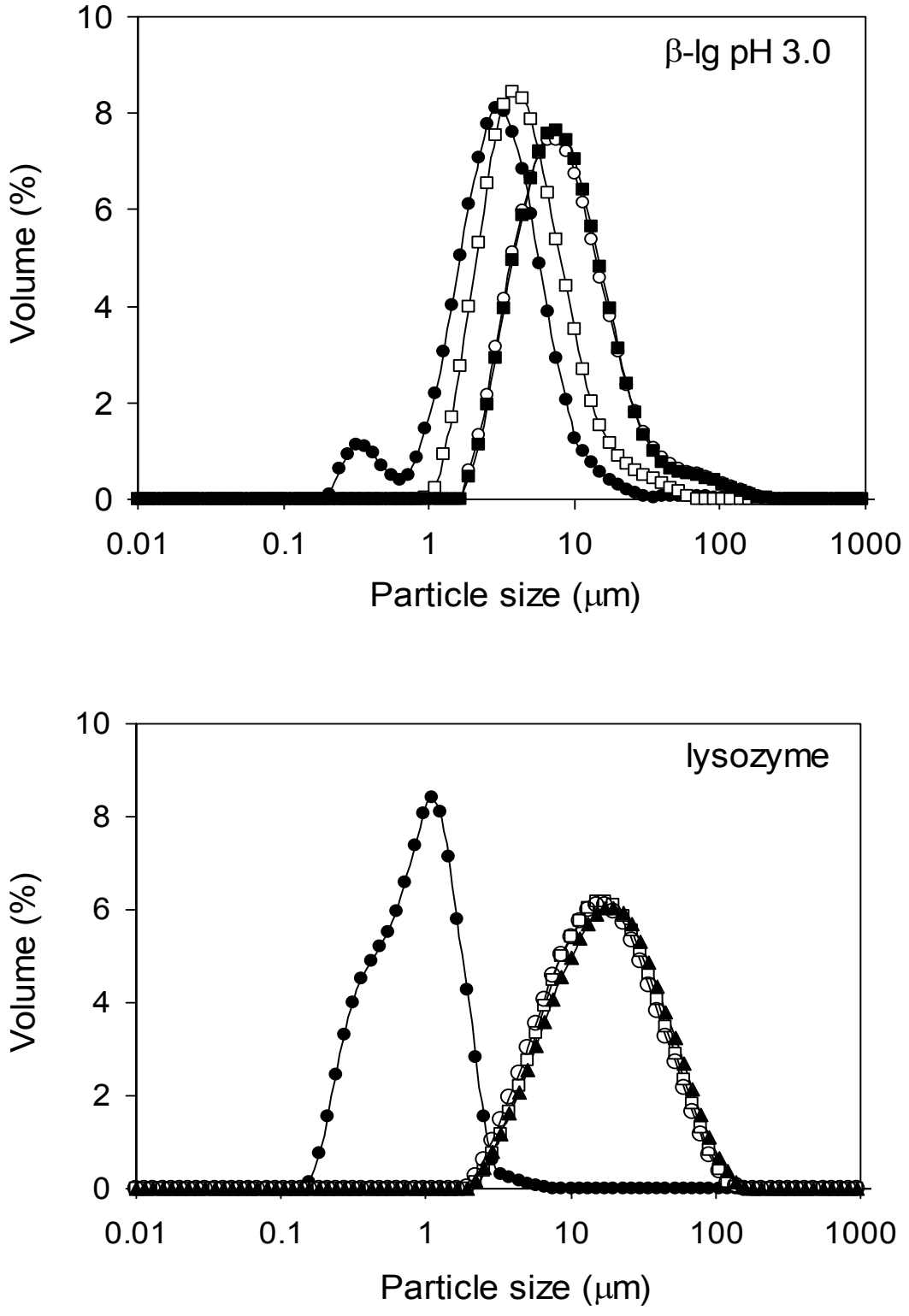

Figure 3 – Particle size analysis of saliva mixed with β-lg emulsion pH 3.0 (top) and lysozyme emulsion pH 6.7 (bottom) (●) emulsion without saliva and mixtures at t = 0 minutes (□), t = 5 minutes (▲), t = 10 minutes (○), t = 20 minutes (■) after mixing.

Demixing experiments

To gain insight into the effect of saliva on the macroscopic behavior of emulsions, mixtures were subjected to demixing experiments. In a typical demixing experiment the back scattering intensity, which correlates with the oil fraction in the sample, is recorded along the height of a tube providing a correspondent profile. An increased concentration of oil droplets at a certain height of the tube increases the back scattering intensity, thereby changing the recorded profile. Generally, flocculation also leads to variations in the back scattering profiles and to a change in the demixing behavior of emulsions in relation to the internal structure of the sample [36].

Figure 4 reports, as examples, the recorded back scattering profiles of Tween 20 and lysozyme emulsions at 0, 50 and 90 minutes after mixing with saliva. No variations in the profiles shape were detected for the Tween 20 emulsion/saliva mixture during the measurements (Figure 4a). This result is in line with the expectations considering the low degree of flocculation exhibited by the sample and the time scale of the experiment. Similar behavior was observed for the aggregated negatively charged β-lg emulsions (not shown).

In contrast, phase separation was observed for the positively charged emulsions with a sharp boundary between the serum layer and the emulsions as shown in Figure 4b for the lysozyme emulsion/saliva mixture. The upper movement of this boundary proceeded slowly for the first 20 minutes and then the velocity increased.

Figure 5 shows the different samples 24 hours after mixing. Serum formation is observed for β-lg pH 3.0 and the lysozyme emulsion. In case of lysozyme emulsion/saliva mixtures, the remaining emulsion phase after serum separation shows a structure that is congruent with the shape of the Eppendorf tube, indicating a collapse of a firmly aggregated structure.

Tween 20 and β-lg emulsions pH 6.7 exhibited instead cream layer formation, with a thicker layer formed for β-lg pH 6.7. The white arrows indicate the position of the two layers. The diversity in the layers thickness may be related to a faster creaming process of the mixture β-lg emulsion/saliva or to lower density of this layer. No cream layer or phase boundary could be seen for the SDS emulsion/saliva mixture and the Panodan emulsion/saliva mixture (not shown). These results are in line with the behavior exhibited by the mixtures in Figure 1a.

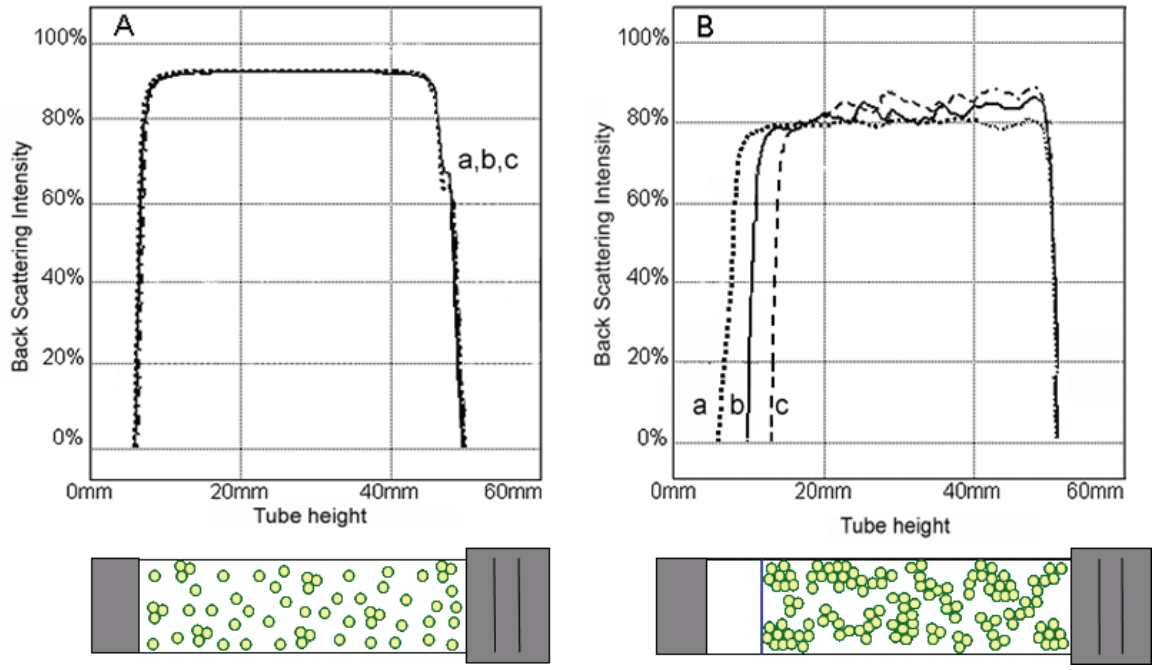

Figure 4 – Backscattering profiles of Tween 20 (A) and lysozyme (B) emulsions mixed with saliva (10 % w/w oil, 10 mM NaCl, 0.6 mg mL^{-1} SPs) (a) t = 0 minutes, (b) t = 50 minutes and (c) t = 90 minutes after mixing.

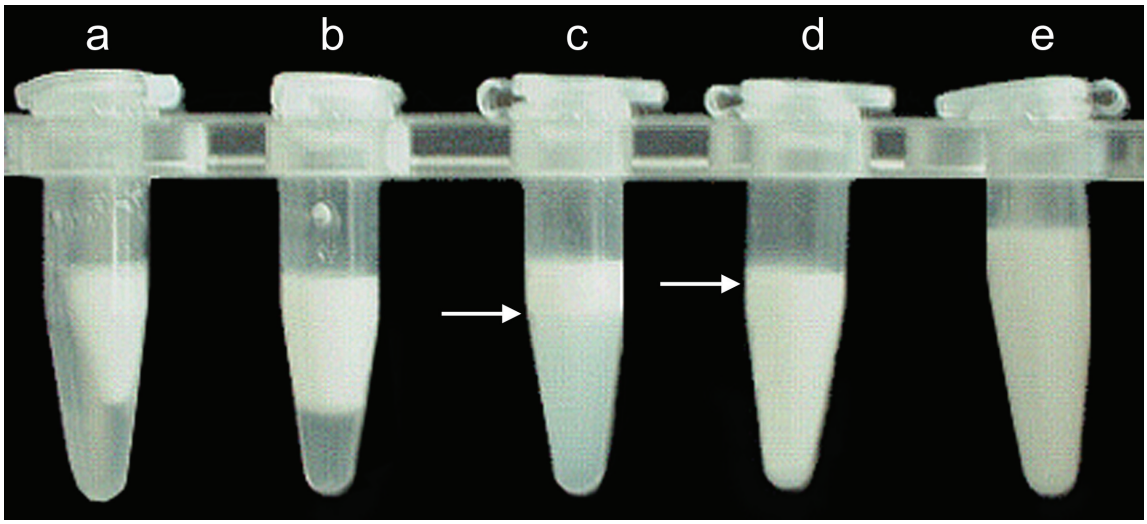

Figure 5 – Demixing behavior of differently charged emulsions (10 mM NaCl) 24 hours after mixing with saliva: (a) lysozyme pH 6.7, (b) β-lg pH 3.0, (c) β-lg pH 6.7, (d) Tween 20 pH 6.7 and (e) SDS pH 6.7.

Rheology

Figure 6 shows the shear rate dependent viscosity of saliva, β-lg emulsions at pH 3.0 and 6.7 at 10 mM NaCl and the mixtures. Saliva showed a weak shear-thinning behavior, in line with previous studies [44-47]. β-lg emulsions ($\phi = 0.1$) exhibit typical Newtonian behavior at both pH values. The measured viscosities are in good agreement with the calculated viscosities obtained from equation 1. The viscosities of the emulsion/saliva mixtures are shear rate dependent. The measured viscosity of the β-lg emulsion mixed with saliva at neutral pH (Figure 6, top), was higher than the theoretical at shear rate below 70 s^{-1}, indicating the presence of flocs. For shear rates above 70 s^{-1}, the curves overlapped indicating that there was no flocculation of the emulsions droplets at these shear rates. The results point to a weak flocculation of the emulsion/saliva mixture, which is disrupted upon shear.

In case of the mixture saliva/β-lg emulsion pH 3.0 (Figure 6, bottom), where irreversible flocculation upon dilution was observed, the measured viscosity of the mixture exhibited, at all shear rates, a higher viscosity compared to the theoretical curve. Even at high shear rate (i.e. 850 s^{-1}) the measured viscosity is almost twice the theoretical values. This indicates that the flocs of a β-lg emulsion pH 3.0 in presence of saliva are not completely disrupted by shear over the applied shear rate range, which is in line with the results obtained by microscopy and laser diffraction.

To compare the effect of saliva on the flocculation behavior of emulsion in relation to the ζ-potential, we evaluated the ratio η_{mix}/η_{emul}, where η_{mix} and η_{emul} are the viscosities of the mixture and the emulsion respectively, in function of the shear rates. The results of 4 different flocculating mixtures are shown in Figure 7.

All positively charged emulsion/saliva mixtures exhibited η_{mix}/η_{emul} ratios higher than negatively charged β-lg emulsion/saliva mixture. More positive ζ-potential values associated with higher η_{mix}/η_{emul} ratios. Also a small difference, as for example between the β-lg emulsion/saliva mixture pH 4.3 (+21 mV) and the lysozyme emulsion/saliva mixture (+30 mV), can still be distinguished. These results indicate that electrostatics also plays an important role in the increased viscosity of the positively charged emulsion/saliva mixtures.

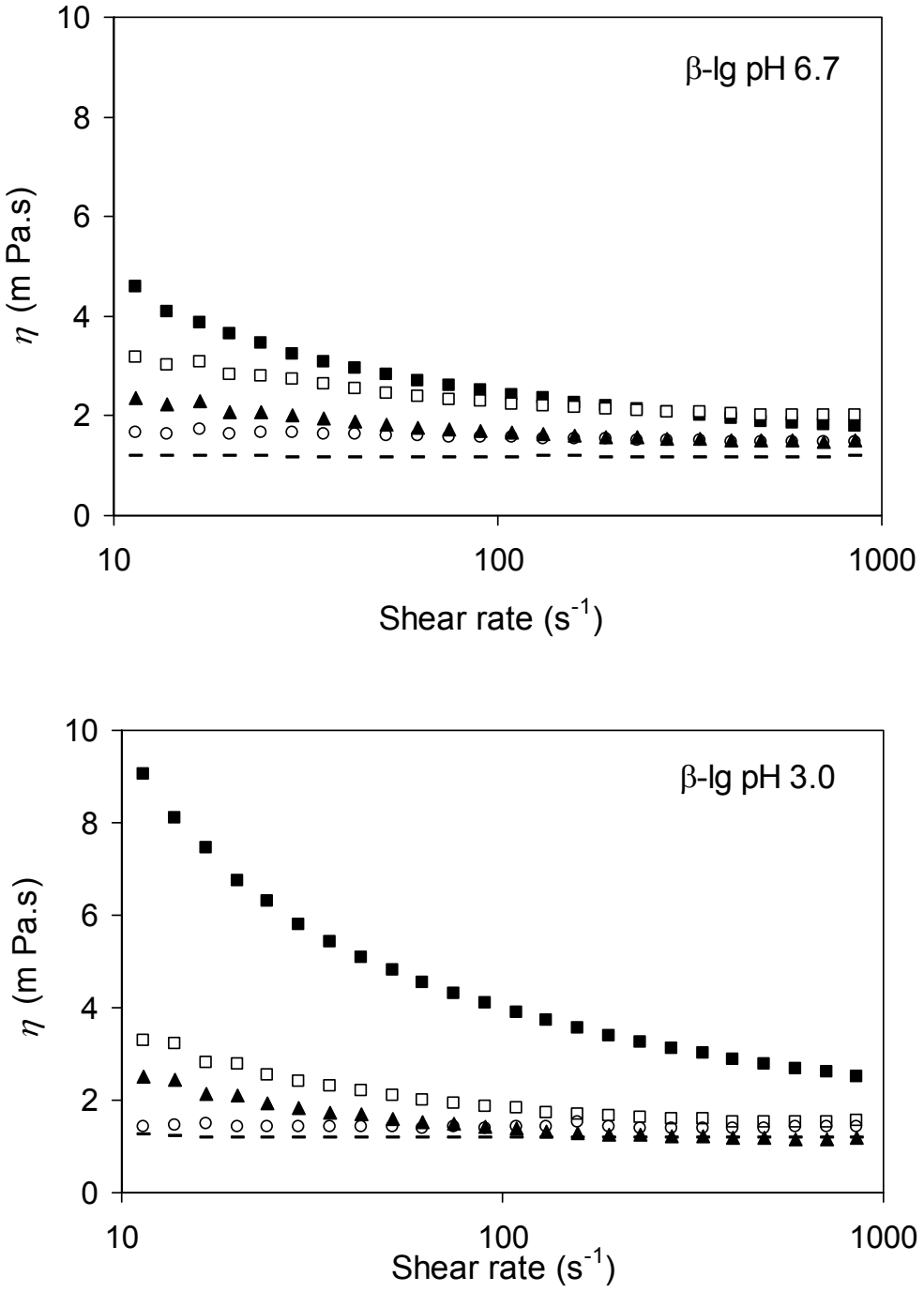

Figure 6 – Rheology measurements of emulsion viscosity (η) for β-lg pH 6.7 (top) and β-lg pH 3.0 (bottom) at 10 mM NaCl and their mixtures with saliva. (−) continuous phase of the emulsion, (○) emulsion, (▲) saliva, (■) mixture, (□) theoretical shear rate dependent viscosity of the mixture.

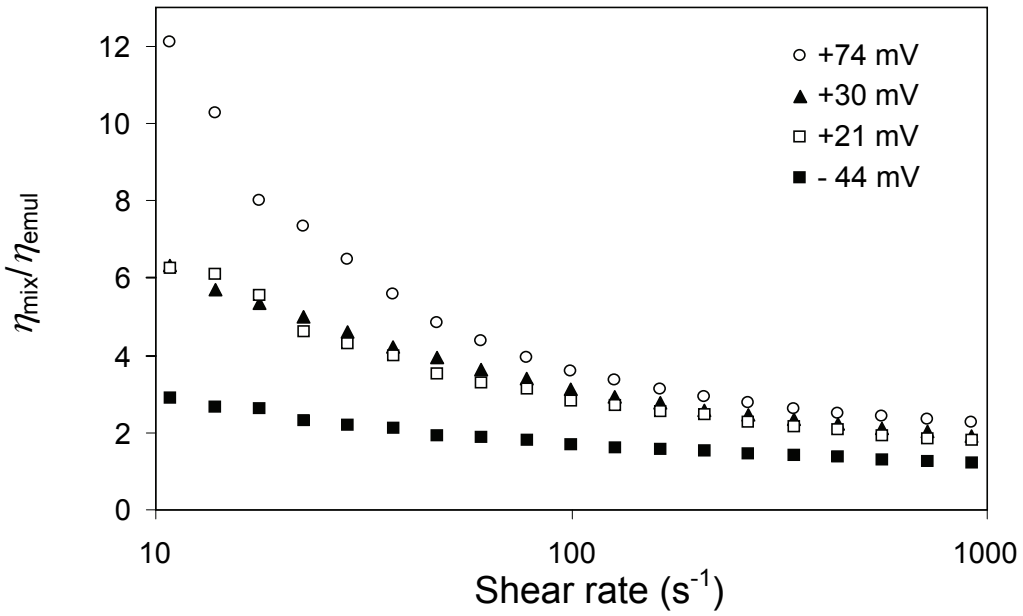

Figure 7 – η_{mix}/η_{emul} of differently charged emulsion/saliva mixtures: (○) CTAB pH 6.7 (+74 mV), (▲) lysozyme pH 6.7 (+30 mV), (□) β-lg at pH 4.3 (+21 mV) and (■) β-lg at pH 6.7 (-44 mV).

Discussion

The experimental results show that saliva-induced droplets flocculation depends on the ζ-potential values of the emulsion droplets. A summary of the findings obtained for all the studied systems, in relation to pH and ζ-potential on the droplets surface is reported in Table 2.

Effect of saliva on negatively charged and neutral emulsions

With highly negatively charged emulsions (SDS and Panodan stabilized emulsions), no flocculation was observed. The strong electrostatic repulsion between the droplets seems to overcome the van der Waals attraction and depletion forces which explain the non-flocculation behavior, as similarly found by Blijdenstein in emulsions containing dextran at concentration lower than 1 % w/w [36].

Weakly negatively charged and neutral emulsions, i.e. β-lg pH 6.7 and Tween 20, exhibited reversible flocculation meaning that the flocs are disrupted upon dilution. Such behavior was already described for β-lg and Tween 20 emulsions [2].

Table 2 – Summary of the ζ-potentials values of the different emulsions and corresponding flocculation behavior of the emulsions/saliva mixtures (10 % w/w oil, 0.6 mg mL^{-1} SPs).

Emulsifier	pH	NaCl (mM)	ζ-potential (mV)	Flocculation	Type of flocculation
SDS	6.7	10	- 90	No	-
Panodan	4.3(*)	10	-75	No	-
β-lg	6.7	10	- 44	Yes	Reversible [a]
Tween 20	6.7	10	- 2	Yes	Reversible [a]
β-lg	4.3(*)	10	+ 21	Yes	Irreversible [a]
Lysozyme	6.7	10	+ 30	Yes	Irreversible [a]
CTAB	6.7	10	+ 74	Yes	Irreversible [a]

(*)The pH and the corresponding measured ζ-potential values are referring to the pH of the mixtures and not to the pH of the emulsions after preparation; (-) not applicable; [a] with respect to dilution with water and shear.

It was suggested that the presence of the large negatively charged mucins in saliva is responsible for depletion flocculation in a similar way as other non-adsorbing biopolymers, e.g. methylcellulose, dextran or exocellular polysaccharide for which the effect on the stability of oil in water emulsion has been already reported [34-36]. Vingerhoeds et al. also suggested that other salivary components such as the salivary micelles could induce flocculation. Salivary micelles have been observed in samples of parotid and stimulated saliva. They appeared mostly in the size range of 40-400 nm with an overall net negative surface charge of -13 to -17 mV [24, 25, 48]. In our case, these components are of minor importance because of the low contribution of parotid saliva to whole unstimulated saliva [5, 9]. Instead it is likely that the large heterotypic complexes involving MUC5B and the small complexes of MUC7, present in submandibular and sublingual secretions of unstimulated saliva, contribute to the flocculation.

Effect of saliva on positively charged emulsions

In our study, irreversible flocculation was observed when positively charged emulsions were mixed with saliva. Rheological measurements confirmed the presence of structures

that do not completely break up into single droplets upon shear. This is in line with the expected results for a bridging mechanism where the bonds are relatively strong and irreversible. Furthermore, we also demonstrate that the effect of saliva on the viscosity of the mixtures increased with increasing the positive ζ-potential on the droplets surface. This reinforces the idea of electrostatic interaction between salivary components and positively charged emulsions. Attractive interaction between adsorbed proteins at an interface and the hydrocolloids present in the bulk phase is known to be responsible for bridging [49]. Bridging flocculation has been reported for example, for other food related systems, such as the mixture of β-lg emulsion and carboxy-methylcellulose at pH 3 [39] and for bovine serum albumin emulsions with ι-carrageenan [50]. We hypothesize that the bridging flocculation mechanism is driven by the binding of the negatively charged mucins to the adsorbed protein layer on the droplets surface. An earlier study on oral deposition conducted with pig gastric mucins also reported adsorption of positive chitosan emulsion onto the mucins film surface [29] indicating a role for the electrostatic interaction between mucins and positively charged emulsion droplets. Polymer solutions of cationic gelatin and chitosan have also shown strong interaction with pig gastric mucins at pH 5.5 [51]. Moreover most food proteins form complexes with anionic hydrocolloids in the pH region were the two macromolecules carry oppositely net charge [52]. Complexes of whey protein, β-lg and lysozyme solutions with gum arabic and/or carrageenan, as well as other food systems, have been extensively investigated [53-56].

Besides mucins, other components in saliva such as cystatins and serum albumin have a net negative charge at physiological pH as well [11]. These proteins could participate in the binding with lysozyme and CTAB emulsions although their concentration in whole unstimulated saliva is low [4]. Different isoforms of α-amylase, having pI values ranging from 5.9 to 7.2, could also interact with the positively charged droplets.

Conclusions

This study aimed to improve our understanding on the flocculation behavior of emulsions induced by saliva. We focused on the role of charge on the emulsion droplets and the contribution of electrostatics on the flocculation behavior of emulsion/saliva mixtures. The saliva/emulsion mixture behavior is regulated by a fine balance of forces including depletion, van der Waals forces and/or electrostatic interactions between emulsion droplets

and salivary proteins. Different scenarios have been shown to occur at the used saliva content. No flocculation was found for highly negatively charged emulsions where the electrostatic repulsion prevents close approach of the droplets. Emulsion flocculation occurred for weakly negatively charged, neutral and positively charged emulsions. For weakly negatively charged and neutral emulsions, reversible flocculation is induced probably by mucins alone or the salivary complexes present in saliva via depletion flocculation. Instead for positively charged emulsions a bridging mechanism, driven by electrostatic attraction between salivary proteins and emulsion droplets, is proposed.

Future research is dedicated to identify which saliva components are involved in the flocculation mechanism. However it seems plausible that mucins, individually or as complexes with other salivary proteins, play an important role in both bridging and depletion flocculation when emulsions are mixed with saliva.

Acknowledgements

The authors would like to thank Marcel van Eijk from Akzo Nobel (Arnhem, The Netherlands) for the assistance in the ζ-potential measurements and Wouter Jansen for the help with the rheological experiments. Ton van Vliet is acknowledged for the stimulating discussion on the rheological measurements.

References

1. van Aken, G.A., Vingerhoeds, M.H., and de Hoog, E.H.A., *Colloidal behavior of food emulsions under oral conditions*, in *Food colloids 2004: Interactions, microstructure and processing*, E. Dickinson, Editor. 2005, The Royal Society of Chemistry: Cambridge. p. 356.
2. Vingerhoeds, M.H., Blijdenstein, T.B.J., Zoet, F., and van Aken, G.A., *Emulsion flocculation induced by saliva and mucin.* Food Hydrocolloids, 2005. **19**: p. 915.
3. Zalewska, A., Zwierz, K., Zólkowski, K., and Gindzienski, A., *Structure and biosynthesis of human salivary mucins.* Acta Biochimica Polonica, 2000. **47**: p. 1067.
4. van Nieuw Amerongen, A., Veerman, E.C.I., and Vissink, A., *Speeksel, speekselklieren en mondgezondheid*. 2004, Houten: Bohn Stafleu Van Loghum. 384.
5. Humphrey, S.P. and Williamson, R.T., *A review of saliva: Normal composition, flow and function.* Journal of Prosthetic Dentistry, 2001. **85**: p. 162.
6. van Nieuw Amerongen, A. and Veerman, E.C.I., *Saliva - the defender of the oral cavity.* Oral Diseases, 2002. **8**: p. 12.
7. Bolscher, J.G.M., Groenink, J., van der Kwaak, J.S., van den Keijbus, P.A.M., van 't Hof, W., Veerman, E.C.I., and van Nieuw Amerongen, A., *Detection and quantification of MUC7 in submandibular, sublingual, palatine and labial saliva by anti-peptide antiserum.* Journal of Dental Research, 1999. **78**: p. 1362.
8. Troxler, R.F., Iontcheva, I., Oppenheim, F.G., Nunes, D.P., and Offner, G.D., *Molecular characterization of a major high molecular weight mucin from human sublingual gland.* Glycobiology, 1997. **7**: p. 965.
9. Veerman, E.C.I., van den Keijbus, P.A.M., Vissink, A., and van Nieuw Amerongen, A., *Human glandular salivas: Their separate collection and analysis.* European Journal of Oral Science, 1996. **104**: p. 346.
10. Vitorino, R., Lobo, M.J.C., Ferre-Correira, A.J., Dubin, J.R., Tomer, K.B., Domingues, P.M., and Amado, F.M.L., *Identification of human whole saliva protein components using proteomics.* Proteomics, 2004. **4**: p. 1109.
11. Yao, Y., Berg, E.A., Costello, C.E., Troxler, R.F., and Oppenheim, F.G., *Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches.* Journal of Biological Chemistry, 2003. **278**: p. 5300.
12. Hardt, M., Thomas, L.R., Dixon, S.E., Newport, G., Agabian, N., Prakobphol, A., Hall, S.C., Witkowska, H.E., and Fisher, S.J., *Toward defining the human parotid gland salivary proteome and peptidome: Identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry.* Biochemistry, 2005. **44**: p. 2885.
13. Fox, P.C., Bodner, L., Tabak, L.A., and Levine, M.J., *Quantitation of total human salivary mucins.* Journal of Dental Research, 1985. **64**: p. 327.
14. Bobek, L.A., Tsai, H., Biesbrock, A.R., and Levine, M.J., *Molecular cloning, sequence and specificity of expression of the gene encoding the low molecular weight salivary mucin (MUC7).* Journal of Biological Chemistry, 1993. **268**: p. 20563.
15. Veerman, E.C.I., van den Keijbus, P.A.M., Valentijn-Benz, M., and van Nieuw Amerongen, A., *Isolation of different high-M_r mucin species from human whole saliva.* Biochemical Journal, 1992. **283**: p. 807.
16. Thornton, D.J., Khan, N., Mehrotra, R., Howard, M., Veerman, E.C.I., Packer, N.H., and Sheehan, J.K., *Salivary mucin MG1 is comprised almost entirely of different glycosylated forms of the MUC5B gene product.* Glycobiology, 1999. **9**: p. 293.
17. Wickström, C., Davies, J.R., Eriksen, G.V., Veerman, E.C.I., and Carlstedt, I., *MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: Identification of glycoforms and c-terminal cleavage.* Biochemical Journal, 1998. **334**: p. 685.
18. Veerman, E.C.I., van den Keijbus, P.A.M., Nazmi, K., Vos, W., van der Wal, J.E., Bloemena, E., Bolscher, J.G.M., and van Nieuw Amerongen, A., *Distinct localization of MUC5B glycoforms in the human salivary glands.* Glycobiology, 2003. **13**: p. 363.
19. Thomsson, K.A., Prakobphol, A., Leffler, H., Reddy, M.S., Levine, M.J., Fisher, S.J., and Hansson, G.C., *The salivary mucin MG1 (MUC5B) carries a repertoire of unique oligosaccharides that is large and diverse.* Glycobiology, 2002. **12**: p. 1.
20. Reddy, M.S., Bobek, L.A., Haraszthy, G.G., Biesbrock, A.R., and Levine, M.J., *Structural features of the low-molecular-mass human salivary mucin.* Biochemical Journal, 1992. **287**: p. 639.

21. Iontcheva, I., Oppenheim, F.G., and Troxler, R.F., *Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins.* Journal of Dental Research, 1997. **76**: p. 734.
22. Soares, R.V., Lin, T., Siqueira, C.C., Bruno, L.S., Li, X., Oppenheim, F.G., Offner, G.D., and Troxler, R.F., *Salivary micelles: Identification of complexes containing MG2, sIgA, lactoferrin, amylase, glycolsylated proline-rich protein and lysozyme.* Archives of Oral Biology, 2004. **49**: p. 337.
23. Soares, R.V., Siqueira, C.C., Bruno, L.S., Oppenheim, F.G., Offner, G.D., and Troxler, R.F., MG*2 and lactoferrin form a heterotypic complex in salivary secretions.* Journal of Dental Research, 2003. **82**: p. 471.
24. Rykke, M., Smistad, G., Rolla, G., and Karlsen, J., *Micelle-like structures in human saliva.* Colloids and Surfaces B: Biointerfaces, 1995. **4**: p. 33.
25. Rykke, M., Young, A., Rølla, G., Devold, T., and Smistad, G., *Transmission electron microscopy of human saliva.* Colloids and Surfaces B: Biointerfaces, 1997. **9**: p. 257.
26. Prinz, J.F. and Lucas, P.W., *An optimization model for mastication and swallowing in mammals.* Proceedings of the Royal Society of London Series B-Biological Sciences, 1997. **264**: p. 1715.
27. van Ruth, S.M. and Roozen, J.P., *Influence of mastication and saliva on aroma release in a model mouth system.* Food Chemistry, 2000. **71**: p. 339.
28. Christensen, C.M., Brand, J.G., and Malamud, D., *Salivary changes in solution pH: A source of individual differences in sour taste perception.* Physiology & Behavior, 1987. **40**: p. 221.
29. Malone, M.E., Appelqvist, I.A.M., and Norton, I.T., *Oral behavior of food hydrocolloids and emulsions. Part 1. Lubrication and deposition considerations.* Food Hydrocolloids, 2003. **17**: p. 763.
30. Malone, M.E., Appelqvist, I.A.M., and Norton, I.T., *Oral behavior of food hydrocolloids and emulsions. Part 2. Taste and aroma release.* Food Hydrocolloids, 2003. **17**: p. 775.
31. Engelen, L., de Wijk, R.A., Prinz, J.F., and Bosman, F., *The relation between saliva flow after different stimulations and the perception of flavor and texture attributes in custard desserts.* Physiology & Behavior, 2003. **78**: p. 165.
32. de Roos, K.B., *Effect of texture and microstructure on flavour retention and release.* International Dairy Journal, 2003. **13**: p. 593.
33. Cao, Y., Dickinson, E., and Wedlock, D.J., *Creaming and flocculation in emulsions containing polysaccharide.* Food Hydrocolloids, 1990. **4**: p. 185.
34. Reiffers-Magnani, C.K., Cuq, J.L., and Watzkea, H.J., *Depletion flocculation and thermodynamic incompatibility in whey protein stabilised o/w emulsions.* Food Hydrocolloids, 2000. **14**: p. 521.
35. Tuinier, R. and de Kruif, C.G., *Phase separation, creaming, and network formation of oil-in-water emulsions induced by an exocellular polysaccharide.* Journal of Colloid and Interface Science, 1999. **218**: p. 201.
36. Blijdenstein, T.B.J., Hendriks, W.P.G., van der Linden, E., van Vliet, T., and van Aken, G.A., *Control of strength and stability of emulsion gels by a combination of long- and short-range interactions.* Langmuir, 2003. **19**: p. 6657.
37. Blijdenstein, T.B.J., van Vliet, T., van der Linden, E., and van Aken, G.A., *Suppression of depletion flocculation in oil-in-water emulsions: A kinetic effect of beta-lactoglobulin.* Food Hydrocolloids, 2003. **17**: p. 661.
38. Blijdenstein, T.B.J., Zoet, F.D., van Vliet, T., van der Linden, E., and van Aken, G.A., *Dextran-induced depletion flocculation in oil-in-water emulsions in the presence of sucrose.* Food Hydrocolloids, 2004. **18**: p. 857.
39. Blijdenstein, T.B.J., van Winden, A.J.M., van Vliet, T., van der Linden, E., and van Aken, G.A., *Serum separation and structure of depletion- and bridging-flocculated emulsions: A comparison.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2004. **245**: p. 41.
40. de Jongh, H.H.J., Groneveld, T., and de Groot, J., *Mild isolation procedure discloses new protein structural properties of beta-lactoglobulin.* Journal of Dairy Science, 2001. **84**: p. 562.
41. Mengual, O., Meunier, G., Cayré, I., Puech, K., and Snabre, P., *Characterisation of instability of concentrated dispersions by a new optical analyser: The turbiscan Ma 1000.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 1999. **152**: p. 111.
42. Mengual, O., Meunier, G., Cayré, I., Puech, K., and Snabre, P., *Turbiscan MA 2000: Multiple light scattering measurement for concentrated emulsion and suspension instability analysis.* Talanta, 1999. **50**: p. 445.
43. Batchelor, G.K., *Effect of brownian-motion on bulk stress in a suspension of spherical-particles.* Journal of Fluid Mechanics, 1977. **83**: p. 97.
44. van der Reijden, W.A., Veerman, E.C.I., and van Nieuw Amerongen, A., *Shear rate dependent viscoelastic behavior of human glandular salivas.* Biorheology, 1993. **30**: p. 141.

45. Vissink, A., Waterman, H.A., 's-Gravenmade, E.J., Panders, A.K., and Vermey, A., *Rheological properties of saliva substitutes containing mucin, carboxymethylcellulose or polyethylenoxide.* Journal of Oral Pathology, 1984. **13**: p. 22.
46. Schwartz, W.H., *The rheology of saliva.* Journal of Dental Research, 1987. **66**: p. 660.
47. Levine, M.J., Aguirre, A., Hatton, M.N., and Tabak, L.A., *Artificial salivas: Present and future.* Journal of Dental Research, 1987. **66**: p. 639.
48. Rykke, M., Young, A., Smistad, G., Rølla, G., and Karlsen, J., *Zeta potentials of human salivary micelle-like particles.* Colloids and Surfaces B: Biointerfaces, 1996. **6**: p. 51.
49. Healy, T.W. and Lamer, V.K., *Energetics of flocculation and redispersion by polymers.* Journal of Colloid Science, 1964. **19**: p. 323.
50. Dickinson, E. and Pawlowsky, K., *Influence of iota-carrageenan on flocculation, creaming and rheology of a protein-stabilized emulsion.* Journal of Agriculture and Food Chemistry, 1997. **45**: p. 3799.
51. Hassan, E.E. and Gallo, J.M., *A simple rheological method for the in vitro assessment of mucin-polymer bioadhesive bond strength.* Pharmaceutical Research, 1990. **7**: p. 491.
52. Dickinson, E., *Hydrocolloids at interfaces and the influence on the properties of dispersed systems.* Food Hydrocolloids, 2003. **17**: p. 25.
53. Schmitt, C., Sanchez, C., Lamprecht, A., Renard, D., Lehr, C.M., de Kruif, C.G., and Hardy, J., *Study of beta-lactoglobulin/acacia gum complex coacervation by diffusing-wave spectroscopy and confocal scanning laser microscopy.* Colloids And Surfaces B: Biointerfaces, 2001. **20**: p. 267.
54. Weinbreck, F. and de Kruif, C.G., *Complex coacervation of globular proteins and gum arabic.* Food colloids biopolymers and materials, ed. E. Dickinson and T. van Vliet. 2003, Cambridge, U.K.: Royal Society of Chemistry. 337.
55. Weinbreck, F., de Vries, R., Schrooyen, P., and de Kruif, C.G., *Complex coacervation of whey proteins and gum arabic.* Biomacromolecules, 2003. **4**: p. 293.
56. Weinbreck, F., Nieuwenhuijse, H., Robijn, G.W., and de Kruif, C.G., *Complexation of whey proteins with carrageenan.* Journal of Agricultural and Food Chemistry, 2004. **52**: p. 3550.

Chapter 3

Complex formation in mixtures of lysozyme stabilized emulsions and human saliva

E. Silletti, M.H. Vingerhoeds, W. Norde, and G.A. van Aken

Journal of Colloids and Interface Science 313 (2007) 485-493

Abstract

In this paper, we studied the interaction between human unstimulated saliva and lysozyme stabilized oil in water emulsions (10 % w/w oil phase, 10 mM NaCl, pH 6.7), to reveal the driving force for flocculation of these emulsions.

Confocal Laser Scanning Microscopy showed formation of complexes between salivary proteins and lysozyme adsorbed at the oil-water interface and lysozyme in solution as well.

To assess the electrostatic nature of the interaction in emulsion/saliva mixtures, laser diffraction and rheological measurements were conducted in function of the ionic strength by adding NaCl to the mixture in the range between 0 and 168 mM. Increasing the ionic strength reduced the ability of saliva to induce emulsion flocculation as shown by the decreased floc size and the effect on the viscosity.

Turbidity experiments with varying pH (between 3 and 7) and ionic strength also showed decreased complex formation in mixtures between saliva and lysozyme in solution upon NaCl addition up to 200 mM. Decreasing the pH increased the turbidity, in line with the increase of the positive net charge on the lysozyme molecule.

We conclude that electrostatic attraction is the main driving force for complex formation between saliva components and lysozyme adsorbed at the oil droplets and in solution.

Introduction

Human saliva is a biological fluid [1-3], secreted by different salivary glands and susceptible to variation depending on diet, gender and many other parameters [4]. Although a lot is known about saliva, the exact composition and structural features are still under investigation. Parameters such as charge density, molecular mass and structure of the protein, as well as protein concentration exhibits strong variation. The presence of different electrolytes (Ca^{2+}, Na^{+}, K^{+}, Mg^{2+}, PO_4^{2-}, SO_4^{2-}, NH_4^{+}, CO_3^{2-}) determines the ionic strength, which varies typically between 20 to 200 mM depending on the secretion flow rate and type of saliva (e.g. unstimulated vs. stimulated) [4-6].

According to recent literature, saliva contains more than 1050 different proteins [7] with molecular mass varying from a few kDa of the small peptides to > 1000 kDa of the large secreted polymeric glycoprotein MUC5B [8]. Despite their differences in isoelectric points (pI), at physiological pH most of the proteins carry a net negative charge. Therefore, to some degree of approximation, saliva may be considered as a colloidal dispersion of mainly negatively charged biopolymers. Several levels of protein organization, e.g. the presence of the network originated by MUC5B or the so-called "salivary micelles", which are protein globular complexes of 40-400 nm [9-11] add to the structural complexity of saliva [12-14].

During consumption, food emulsions are exposed to a range of processes, including mixing with saliva. The knowledge of the interaction with saliva is important to understand the oral behavior of emulsions in relation to sensory perception. Recently, an investigation on the physical-chemical effect of saliva on protein-stabilized food emulsions was initiated showing the ability of saliva to induce flocculation of emulsion droplets [15]. A clear role of the droplets' charge in affecting the stability of emulsions/saliva mixtures has been found (Chapter 2)[16]. Strongly negatively charged emulsions do not flocculate in presence of saliva, whereas weakly negatively charged emulsion droplets flocculate likely by depletion flocculation, since the flocculation was reversible with respect to dilution and shear. Saliva-induced flocculation of positively charged emulsions, e.g. stabilized by lysozyme, was irreversible upon dilution and shear instead.

Lysozyme is a small ellipsoid protein molecule of 129 amino acids (pI ~ 10.5), containing 18 amino acids with a basic side chain (11 arginines, 6 lysines and 1 histidine) [17, 18] resulting in a net positive charge at neutral pH. It forms electrostatically driven complexes with various negatively charged compounds, e.g. polysaccharides such as gum arabic and

κ-carrageenan [19, 20], phospholipids vesicles [21], α_s- and β-caseins, casein stabilized emulsion droplets and casein micelles [22].

Attractive interaction between oppositely charged compounds, which can be of both synthetic and biological origin, plays an important role in complex formation. Synthetic polymers offer the advantage of well-defined and controllable molecular characteristics as, for example, charge distribution and/or charge density. Although molecules of biological origin (e.g. protein) or present in biological system (e.g. saliva) cannot be easily modified according to experimental requirements, several examples of electrostatically driven complex formation (i.e. histones binding to the DNA molecule), can be found in literature [19, 23-28].

In this paper, we have investigated the interaction between a colloidal dispersion of oil in water and saliva. Our system, a lysozyme stabilized emulsion mixed with saliva, serves as a model for studying the behavior of a positively charged food emulsion in the mouth.

Materials and methods

Materials

Lysozyme from chicken egg-white (L 6876, lot 051K7028) was purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands) and used without further purification. Sunflower oil (Reddy, Vandemoortele, The Netherlands) was purchased from a local retailer. Oregon Green™ 488 (Lot 34A1-6) was purchased from Molecular Probes Inc. (The Netherlands) while Rhodamine B and Nile-Red were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). The BCA™ Protein Assay Kit was from Pierce Biotechnology Inc. (Rockford, IL, USA) and sodium azide was obtained from Merck (Schuchardt, Germany).

Collection and storage of saliva

Whole human unstimulated saliva was collected according to the procedure described in Chapter 2 [16]. The pH of saliva was measured after thawing and ranged from 6.7 to 7.0. Salivary protein content was determined according to the BCA method of Pierce [29] using bovine serum albumin as standard and varied from 1.1 mg mL^{-1} to 1.3 mg mL^{-1}. The

required amount of saliva was removed from –80 ^{o}C and thawed at room temperature before each experiment.

Preparation of lysozyme stabilized emulsion and its mixture with saliva

Lysozyme protein powder was dissolved in 10 mM NaCl for two hours at room temperature. Pre-emulsions were prepared using an Ultra-Turrax T 25 Basic (IKA-Werke GmbH & Co. KG, Staufen, Germany) and subsequently homogenized at room temperature by 10 passes through a Delta Lab-scale homogenizer (Delta Instruments, Drachten, The Netherlands) operating at a pressure of 100 bar. Lysozyme stabilized emulsions, containing 1 % w/w lysozyme, 20 % w/w sunflower oil, 10 mM NaCl, were prepared at pH 6.7 and are further indicated as emulsions. Sodium azide 0.02 % w/w (harmful to humans when swallowed) was added to the emulsions to prevent microbial growth. Droplet-size distribution and the volume-over surface average droplets diameter (d_{32}) were measured by laser diffraction with the Mastersizer Hydro 2000S (Malvern Instruments, Southborough, UK) as described in Chapter 2 [16]. Emulsion/saliva mixtures were prepared by mixing a required amount of emulsions (20 % w/w oil content and 10 mM NaCl in the emulsion continuous phase) with saliva to obtain 10 % w/w oil and 0.6 mg mL^{-1} salivary proteins (SPs) in the mixtures. The necessary volume adjustments were performed by adding 10 mM NaCl. The emulsion/saliva mixtures therefore contained the NaCl present in the emulsion continuous phase and the saliva's electrolytes. To study the effect of ionic strength, extra NaCl, from a concentrated NaCl solution, was added to the mixtures so that the added concentration varied between 0 mM and 168 mM. The conductivity of saliva and the emulsion/saliva mixtures was measured at 20 ^{o}C with a Schott Glas conductometer (Schott Glas, Mainz, Germany) in presence and absence of extra NaCl. The viscosity of emulsions and saliva, used as reference in ionic strength dependency experiments, was measured on prepared samples that have the same conductivity as those of the mixtures. Addition of 20 mM NaCl to the emulsion, whereafter a NaCl-based conductivity calibration curve was made, mimicked saliva conductivity. This value is in line with the ionic strength reported in literature for unstimulated saliva [5, 6].

Confocal Laser Scanning Microscopy, laser diffraction, and rheology of lysozyme emulsion/saliva mixtures

Confocal Laser Scanning Microscopy (CLSM) was performed with a LEICA TCS SP confocal laser scanning microscope used in the single-photon fluorescence mode. The set-up was configured with an inverted microscope (model LEICA DM IRBE) and an Ar/Kr laser. The objective lens used was a 63X/UV/1.20NA/water immersion/PL APO. Oregon Green^{TM} 488, simply referred as Oregon Green, has been used to stain saliva while Nile-Red was used to label the emulsions oil phase. Oregon Green was excited at 488 nm and the emission fluorescence was detected between 500 and 560 nm. Nile-Red was excited at 568 nm while the emission was detected between 580 and 700 nm.

To covalently label proteins with Oregon Green, 2.5 mg of the dye was dissolved in 249.5 mg dimethylformamide by stirring for 2 hours at room temperature. 4 mg Oregon Green-dimethylformamide solution was added to 2 g of the sample to be labeled. The sample solution was gently stirred overnight at 4 ^{o}C avoiding exposure to light.

A 0.01 % w/v Nile-Red solution in polyethylene glycol-glycerol was prepared by dissolving 10 mg Nile-Red in 50 mL polyethylene glycol by stirring at 90°C for 1 hour. In the meantime, 5 mL water was added to 45 mL glycerol and stirred until a homogeneous mixture was obtained. The water-glycerol mixture was added to the Nile-Red solution, cooled to room temperature and stirred to obtain a homogeneous solution. The Nile-Red solution was stored at 4 ^{o}C and added to the emulsions directly before CLSM experiments.

Laser diffraction was performed on emulsion/saliva mixtures with and without 30 mM NaCl as described above.

The viscosity of emulsions, saliva and the mixtures was measured using a Physica MCR 301 rheometer (Anton Paar BVBA, Sint Martens Latem, Belgium) at 20°C, using a cone-and-plate geometry CP 75-1 with an angle of 1º (0.0175 rad) and a gap-width of 0.05 mm at the tip. The shear rate was logarithmically increased over 20 minutes from 0.1 to 1500 s^{-1} and then reversed to 0.1 s^{-1}. Hysteresis, i.e. the viscosity on increasing the shear rate is usually higher than on decreasing the shear rate, was noted. This observation points to irreversible break up of the flocs upon shear. Discussing this topic lies beyond the scope of this article, therefore only the data relating to the increasing shear rates are shown. Prior to analysis, the lysozyme stabilized emulsions and saliva were kept separately in a water bath at 20°C for 15 minutes and then mixed. To correspond better to the real in-mouth situation,

the shear rate dependent viscosity was measured in duplicate, with no waiting time after bringing the sample in the measuring geometry.

In general, the viscosity of an emulsion, η_{emul}, is

$$\eta_{emul} = \eta_0 F(\phi_{part}) \qquad \text{Eq.1}$$

where the viscosity of the continuous phase (η_0) was measured after centrifuging the emulsions at 120,000 g for 30 minutes at 20 ^{o}C to remove the oil phase (Beckman J-60, rotor SW 41, Beckman Coulter B.V. Mijdrecht, The Netherlands). In principle, any function ($F(\phi_{part})$) that describes the way the viscosity of a dispersion of spherical particles with hard sphere interaction in a Newtonian medium varies with the particle's volume fraction can be used. Examples are the well-known Einstein equation $F(\phi) = 1+5/2\phi$, the Batchelor equation $F(\phi) = 1 + 5/2\phi + 6.25\phi^2$ and the Krieger-Dougherty equation $F(\phi) = \left(1-\phi/\phi_{cp}\right)^{-\frac{5}{2}\phi_{cp}}$, where ϕ_{cp} is the volume fraction of (random) closed packing of the particles [30, 31]. In this article, ϕ_{part} is used to indicate the oil-volume fraction of the lysozyme stabilized emulsions, both in case of non-interacting droplets and in case of emulsions flocculated by salt. Therefore, in presence of salt, ϕ_{part} corresponds to the volume fraction of the aggregates, which is larger than the sum of the volumes of the individual droplets within the floc because continuous liquid is entrained within the volume of the flocs. Theoretical models for the description of rheological behavior of aggregated dispersions have been developed by several authors [32, 33]. For example, to calculate the effective oil-volume fraction of the aggregates, Potanin, introduced parameters that relate to the volume of droplet aggregates or the interaction forces between the particles [32]. Because the above-mentioned parameters are currently unknown in emulsion/saliva mixtures we were not able to use this theoretical model. Therefore, analogously to equation 1, we express the theoretical viscosities of lysozyme stabilized emulsion flocculated by saliva by

$$\eta_{mix} = \eta_{saliva} F(\phi_{flocc}) \qquad \text{Eq.2}$$

where the viscosity of the continuous phase in emulsion/saliva mixture is approximated by η_{saliva}, assuming that η_{saliva} in the mixture is the same as in absence of the oil phase for each

shear rate and salt concentration. $F(\phi_{flocc})$ describes the dependency between the viscosity of a dispersion of flocculated droplets, upon mixing with saliva, and the volume fraction of the flocs. We use this approach to estimate the changes in the viscosity of the emulsion upon saliva-induced flocculation.

In view of equation 1 and 2, we have therefore derived the ratio

$$\eta_{mix}/\eta_{emul} = \eta_{saliva}/\eta_0 * F(\phi_{flocc})/ F(\phi_{part}) \qquad \text{Eq.3}$$

to illustrate the factorial increase in viscosity of the emulsion due to the additional flocculation caused by saliva.

Preparation, CLSM and turbidity measurements of lysozyme/saliva mixtures

For detection of complex formation between saliva and lysozyme by CLSM, 10 mg mL^{-1} lysozyme protein stock solution was prepared as indicated in the emulsion preparation paragraph. The mixture with saliva contained a final concentration of 5 mg mL^{-1} lysozyme labeled with Oregon Green and 0.6 mg mL^{-1} SPs. CLSM was also performed on saliva samples for structure analysis using the non-covalently label Rhodamine B. The Rhodamine solution was prepared by dissolving 50 mg Rhodamine B in a total weight of 100 g water and was kept at 4 ^{o}C until use. One drop of Rhodamine solution was added to few mL of saliva shortly before the analysis.

For turbidity measurements, 5 mg mL^{-1} lysozyme stock solution was prepared by dissolving the protein powder into 4 mM NaCl solution and stirring for 2 hours at room temperature. The pH was adjusted to 7 by addition of small amounts of 1M NaOH. To study the influence of ionic strength on complex formation between SPs and lysozyme, mixtures containing 0.6 mg mL^{-1} SPs and 0.5 mg mL^{-1} lysozyme have been prepared at different salt concentration. The lysozyme stock solution was therefore diluted with NaCl solution and then thawed saliva was added. The added NaCl concentration was varied between 0 mM and 200 mM.

The ionic strength influence on complex formation was evaluated at different pH by slow acidification by means of glucono-δ-lactone (GDL), which is an internal ester. Once added to the mixture, GDL dissolves and hydrolyses slowly to form gluconic acid (GH), a weak acid, and further dissociation leads to the formation of G^- and H^+ [34]. To cover the desired

pH range, the amount of 1.5 % w/w of the total sample volume was used. Before adding GDL, lysozyme solution and saliva were kept separately in a water bath at 25°C. After mixing, GDL was added and stirred for 1 minute to allow a homogeneous dissolution of GDL. As control, saliva and lysozyme were measured at the same condition as in the mixture. Measurements were taken in duplicate every minute until pH 3 was reached. Turbidity (τ) measurements on lysozyme solution, saliva and the mixtures were carried out in a spectrophotometer (Cary 1E, Varian, USA) at a wavelength of 514 nm at 25 °C and was defined as:

$$\tau = -\ln (I/I_0) \qquad \text{Eq.4}$$

where I is the intensity of the light that passes through a volume of solution in a 1 cm path length cuvette and I_0 the incident light intensity.

Results

Microscopic structure of the flocculated lysozyme stabilized emulsion/saliva mixture

CLSM images of lysozyme stabilized emulsions before and after mixing with saliva are shown in Figure 1. To distinguish the different components in the mixtures, Nile-Red and Oregon Green were used to label the oil phase and the SPs, respectively. Using only Nile-Red, the emulsions droplets appeared in a light color against a dark background (Figure 1a and 1b). As expected in case of a non-flocculating emulsion, e.g. without addition of saliva, the emulsion droplets, ranging from 0.1 to 5 μm in size (d_{32}=0.8 μm) appeared homogeneously dispersed in the sample (Figure 1a). Upon saliva addition, in line with previous results [16], flocculation occurred (Figure 1b). By means of a covalently labeling dye to stain saliva, i.e. Oregon Green, SPs can be observed as well. Figure 1c is an example of a CLSM picture of an emulsion/saliva mixture obtained by overlapping the Nile-Red and Oregon Green signals. The emulsion droplets and SPs are colored in black and white, respectively, against a grey background. The displayed image is not meant to provide information on the dimensions of the flocs but only to highlight the flocs structure. The two dyes were, in fact, better distinguishable in small flocs and therefore Figure 1c is taken at higher magnification. The CLSM picture demonstrated that the emulsions droplets are in close proximity of SPs, as indicated by the layer around the droplets. Furthermore, we

observed saliva-induced flocculation in washed emulsions as well (not shown). These findings suggest that SPs induce emulsion flocculation by forming complexes with the lysozyme molecules adsorbed at the oil-water interface of the droplets.

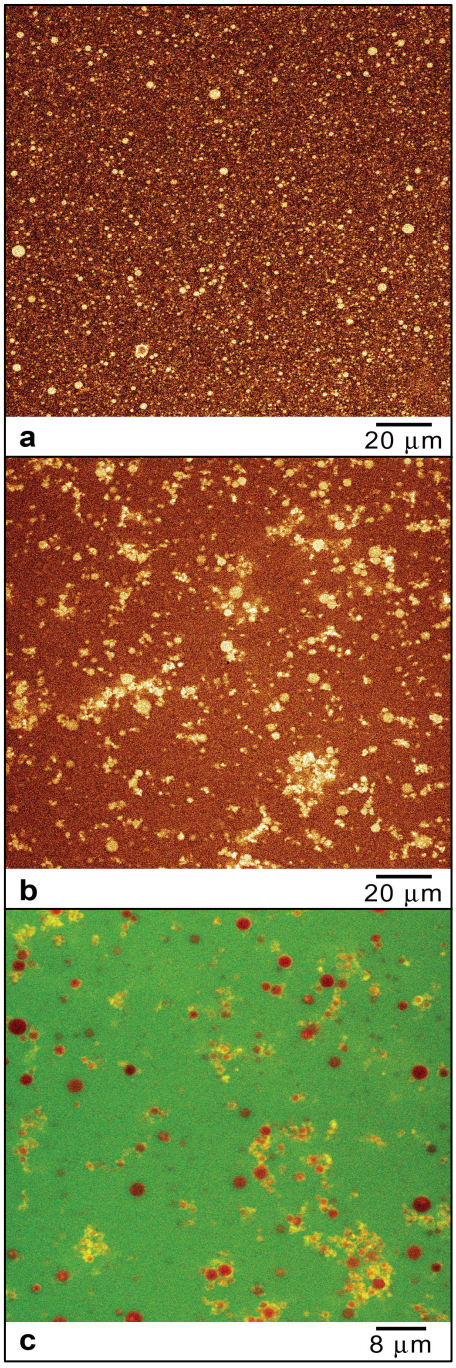

Figure 1 – CLSM images of (a) lysozyme stabilized emulsion (10% w/w oil) stained with Nile-Red, (b) mixture of lysozyme stabilized emulsion/saliva (10% w/w oil; 0.6 mg mL^{-1} SPs) stained with only Nile-Red and (c) lysozyme stabilized emulsion/saliva mixture stained with both Nile-Red (oil phase) and Oregon Green (SPs).

Complex formation between saliva and lysozyme molecules in solution

Figure 2 shows CLSM images of lysozyme in solution, upon mixing with saliva. When saliva is added to the Oregon Green labeled-lysozyme solution, macroscopic complexes up to 10 µm formed instantly, as illustrated in Figure 2a. To prove that the observed structures resulted from the complex formation between saliva and lysozyme, lysozyme solution and saliva have also been separately analyzed. Lysozyme molecules, which have been covalently labeled with Oregon Green, could not be detected with this technique resulting in a diffuse orange image (Figure 2b). Also saliva, which upon CLSM examination with the non-covalent label Rhodamine B, showed protein structures larger than 20 µm, did not exhibit any visible structural element when Oregon Green was added (Figure 2c). Therefore the observed complexes are due to the interaction between salivary proteins and lysozyme.

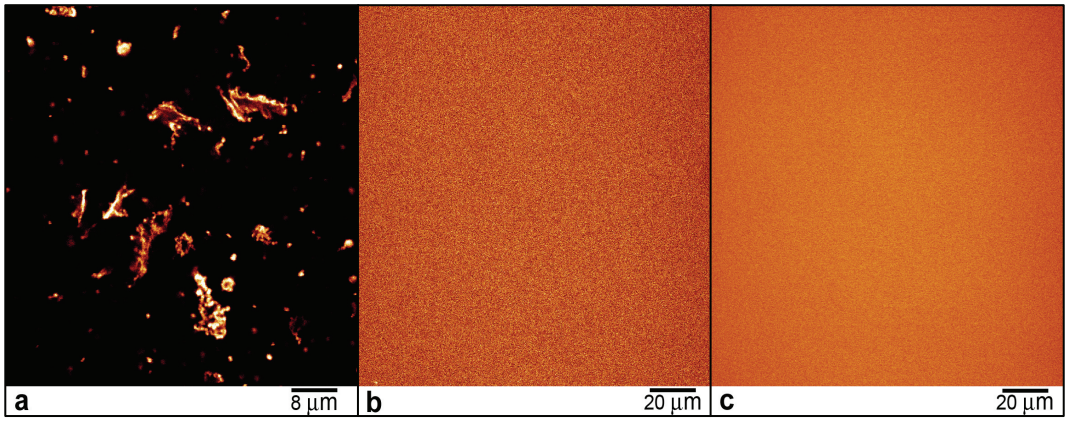

Figure 2 – CLSM pictures of (a) complex formation between lysozyme-salivary proteins (5 mg mL^{-1} lysozyme protein solution; 0.6 mg mL^{-1} SPs), (b) lysozyme protein solution (5 mg mL^{-1}) covalently labeled with Oregon Green and (c) salivary proteins 5 minutes after mixing with Oregon Green.

Complex formation was also studied by means of turbidity experiments during acidification of the lysozyme solution, saliva and the mixture (Figure 3). No turbidity signal was observed for the lysozyme solution in the considered pH range, while the turbidity of saliva increased rapidly below pH 4.5. Due to complex formation, the saliva/lysozyme mixtures

exhibited turbidity in the whole pH range with turbidity values that slowly increased as the pH decreased. However at pH < 4.5, the turbidity signal reached a plateau which is probably largely due to the turbidity of saliva itself.

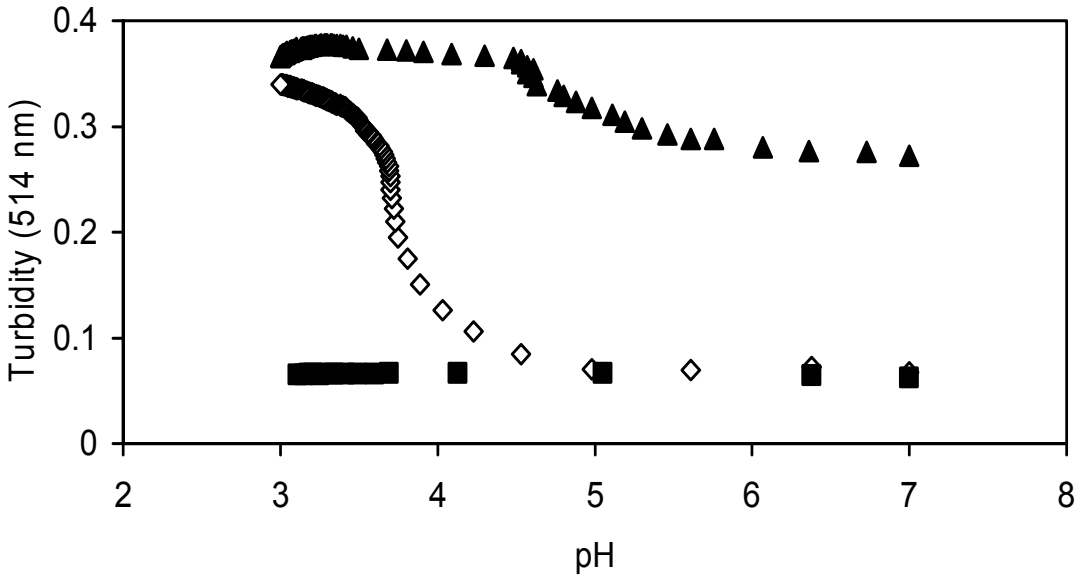

Figure 3 – Turbidity measurements during GDL acidification of lysozyme solution (0.5 mg mL^{-1}) (■), saliva (0.6 mg mL^{-1} SPs) (◊) and their mixture (0.6 mg mL^{-1} SPs; 0.5 mg mL^{-1} lysozyme) (▲).

Influence of ionic strength on complex formation between salivary proteins and lysozyme molecules adsorbed on emulsion droplets and in solution.

We studied the influence of ionic strength on lysozyme stabilized emulsions after mixing with saliva by performing laser diffraction and rheology measurements.

The particle-size distribution of emulsion/saliva mixtures is shown in Figure 4. Irreversible flocculation, which occurred when the emulsion was mixed with saliva (Chapter 2) [16], led to a particle-size distribution ranging from 1 to 200 μm. The addition of 30 mM NaCl to the mixture changed the size distribution and a reduction of the dimensions of the flocs was observed (i.e. shift of the profile towards lower sizes and increase of the particle fraction between 0.1 and 1 μm). However, the dilution step necessary to conduct laser diffraction measurements is a serious drawback in this technique, since it affects flocculation of the samples by breaking the flocs and reducing their particle size. Therefore, to fully evaluate the effect of ionic strength on emulsion/saliva mixture, rheology measurements were

preferred. Typical shear rate dependent viscosity measurements over the applied shear rate are illustrated in Figure 5a for saliva, emulsions and the mixtures.

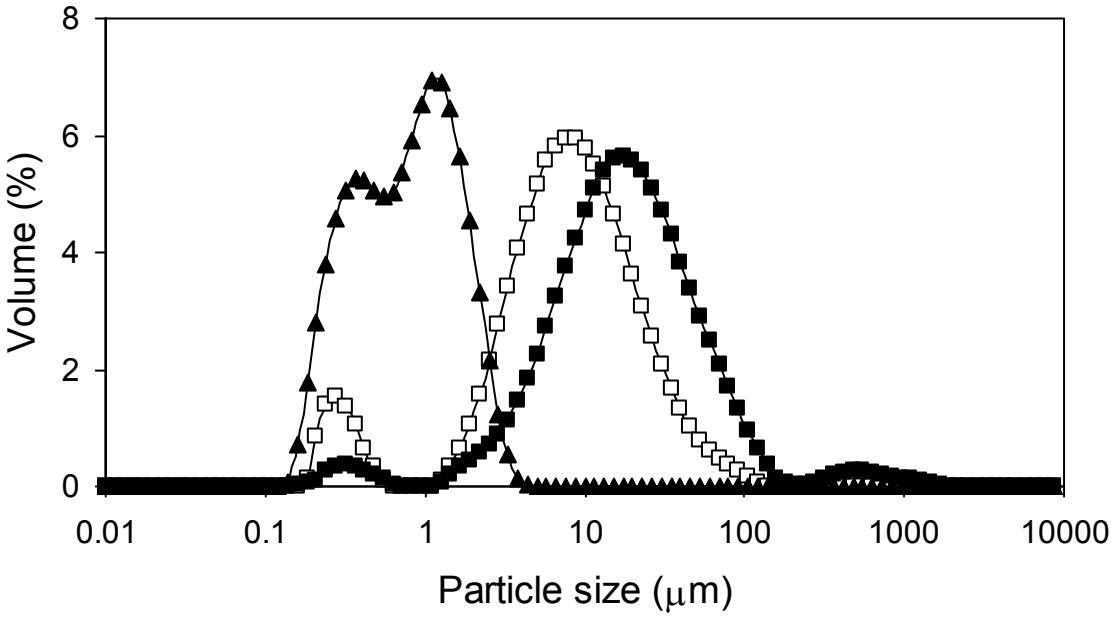

Figure 4 – Particle size analysis of lysozyme stabilized emulsion (▲), lysozyme stabilized emulsion/saliva mixture without (■) and with (□) addition of 30 mM NaCl.

The emulsion/saliva mixture exhibited a shear thinning behavior, with viscosity values larger than the viscosity of the emulsion even at high shear rate, e.g. 5 times at 560 s^{-1}. The oral shear rate is largely dependent on the flow characteristics of the food [35] and on the gap between the oral surfaces, which varies in time during consumption and throughout the oral cavity. Shear rates ranging from 10 s^{-1} to 1000 s^{-1} have been applied in literature to mimic the *in vivo* shearing of food products [35-37]. As the observed behavior was similar at the different shear rates, we presented the results at one shear rate, i.e. 100 s^{-1}. Figure 5b reports an example of the rheological behavior of saliva, emulsions and the mixtures as function of the added [NaCl] at 100 s^{-1}. NaCl was added to the prepared emulsion before mixing with saliva. The emulsions and saliva used as references had the same conductivity as the mixtures.

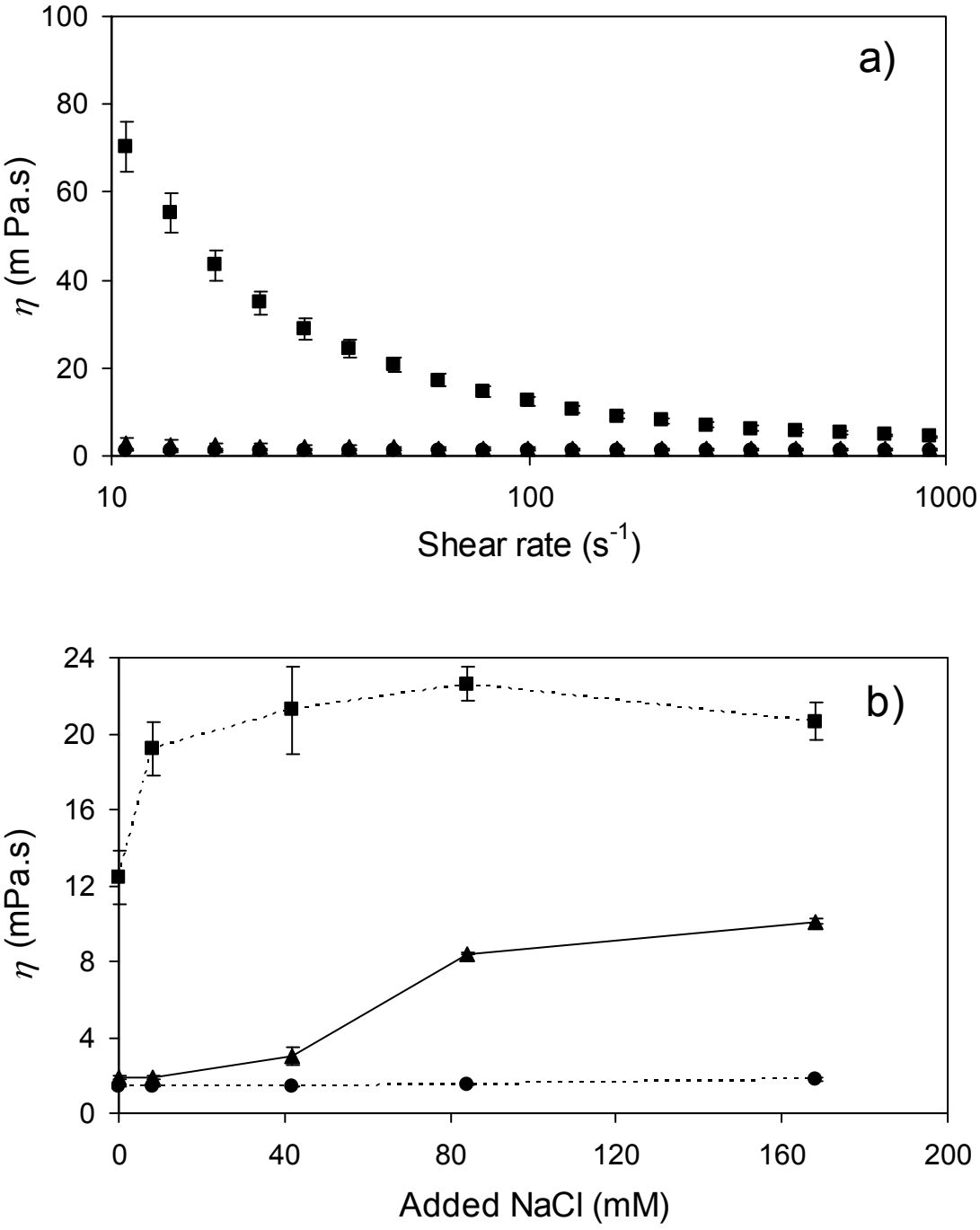

Figure 5 – Experimental shear rate dependent viscosity (η) obtained in absence of NaCl between 10 and 1000 s^{-1} (a) and after addition of NaCl at 100 s^{-1} (b) for lysozyme stabilized emulsion/saliva mixtures (■), lysozyme stabilized emulsion (▲) and saliva (●). Lines are drawn to guide the eye. Error bars indicate the standard deviation.

The increased ionic strength significantly affected the viscosity of the emulsions and the viscosity of emulsion/saliva mixtures, which flocculated at all the added [NaCl]. However, for [NaCl] >10 mM, no further viscosity increase was observed in the mixture.

The ratio $F(\phi_{flocc})/F(\phi_{part})$, calculated from equation 3, as a function of the added [NaCl], is shown in Figure 6. Here the viscosity of the saliva-salt-continuous phase was approximated by the viscosity of saliva samples, which were measured in presence of different [NaCl].

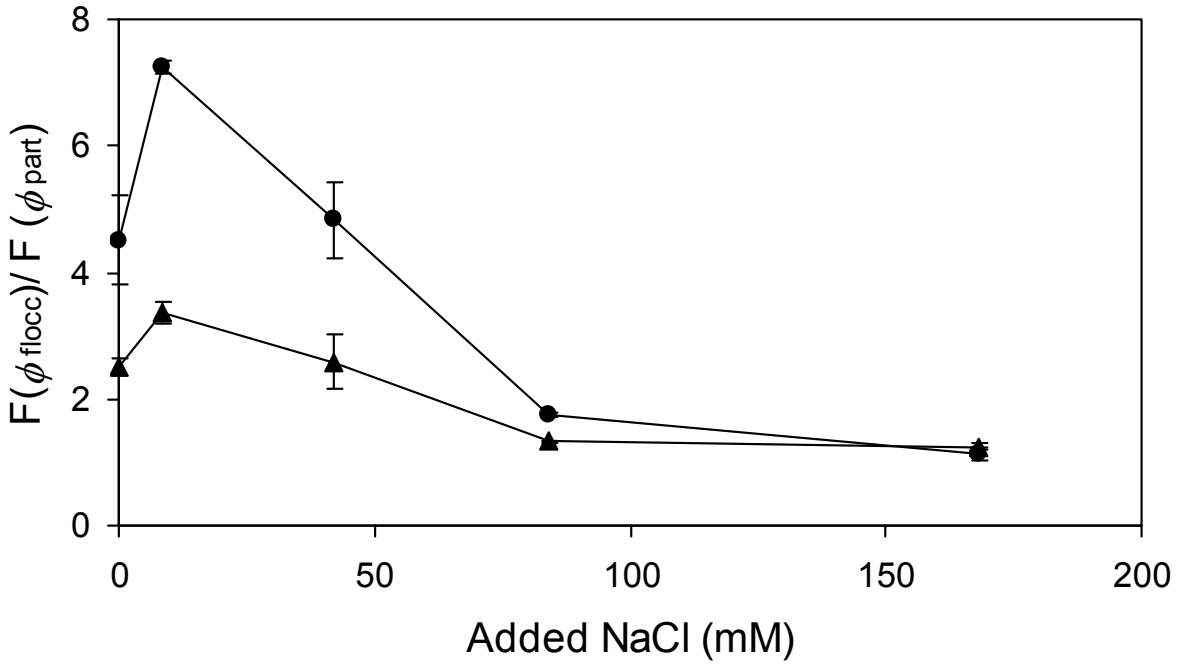

Figure 6 – $F(\phi_{flocc})/F(\phi_{part})$ calculated according to equation 3 for lysozyme stabilized emulsion mixed with saliva (10 % w/w oil; 0.6 mg mL^{-1} SPs) at 100 s^{-1} (●) and 560 s^{-1} (▲) after addition of NaCl. Lines are drawn to guide the eye. Error bars indicate the standard deviation.

After an initial augment, which might be caused by other salivary electrolytes, $F(\phi_{flocc})/F(\phi_{part})$ decreased. Values of about 1 are reached for added [NaCl] ≥ 80 mM indicating that the influence of saliva in inducing emulsion flocculation is negligible or absent at these salt concentrations. This experimental behavior was observed at all the applied shear rates and also when NaCl was added to the emulsion after mixing with saliva (not shown).

In parallel to the above-described results on emulsion/saliva mixtures, to study the nature of the complex formation between lysozyme molecules in solution and saliva, turbidity experiments have been carried out both varying the pH and the NaCl concentration. NaCl

was added to lysozyme aqueous solution before mixing with saliva and then the mixtures were acidified with GDL. The results are illustrated in Figure 7. The increase of the ionic strength had no effect on the turbidity of lysozyme solution in the pH range considered, while it largely influenced the turbidity of both saliva and the mixtures (see Figure 7a-7d). Upon increasing NaCl concentration ([NaCl]< 200 mM), saliva showed a turbidity decrease below pH 4.5, while above this pH the turbidity of saliva remained unaffected. At [NaCl] = 200 mM, saliva samples were fully transparent in the whole pH range (not shown). In case of the lysozyme solution/saliva mixtures, addition of salt induced a turbidity decrease in the pH range considered. A large reduction was especially observed for [NaCl] < 50 mM (Figure 7a and 7b). For [NaCl] ≥ 50 mM, the mixtures showed no turbidity between pH 4 and pH 7, while below pH 4, the turbidity was comparable to that of saliva (Figure 7c and 7d).

Discussion

To characterize the observed saliva-induced flocculation of lysozyme stabilized emulsions, which resembles bridging flocculation [38-41], we examined the emulsion/saliva mixtures using different techniques. CLSM images of the saliva-induced emulsion flocs revealed the presence of salivary proteins surrounding the emulsion droplets surface (Figure 1c). The observation that washed emulsions flocculated upon mixing with saliva, suggests that flocculation is due to complexes formed between SPs and lysozyme molecules adsorbed to the oil-droplets surface. In view of similar positive charge densities on lysozyme stabilized emulsion droplets and lysozyme molecules, emulsion/saliva mixtures may be considered, as a first approximation, analogous to lysozyme in solution mixed with saliva. Moreover, lysozyme molecules are also present in the aqueous continuous phase of the emulsions. The finding of lysozyme-salivary proteins complex formation in solution, which was detected with CLSM (Figure 2) and turbidity measurements (Figure 3), strongly suggests that complex formation between lysozyme adsorbed at the oil-water interfaces and SP is causing emulsion flocculation. Whether and to what extent SPs-lysozyme complexes in the continuous phase of emulsion/saliva mixture participate in the emulsion flocculation is not clear and requires further research.

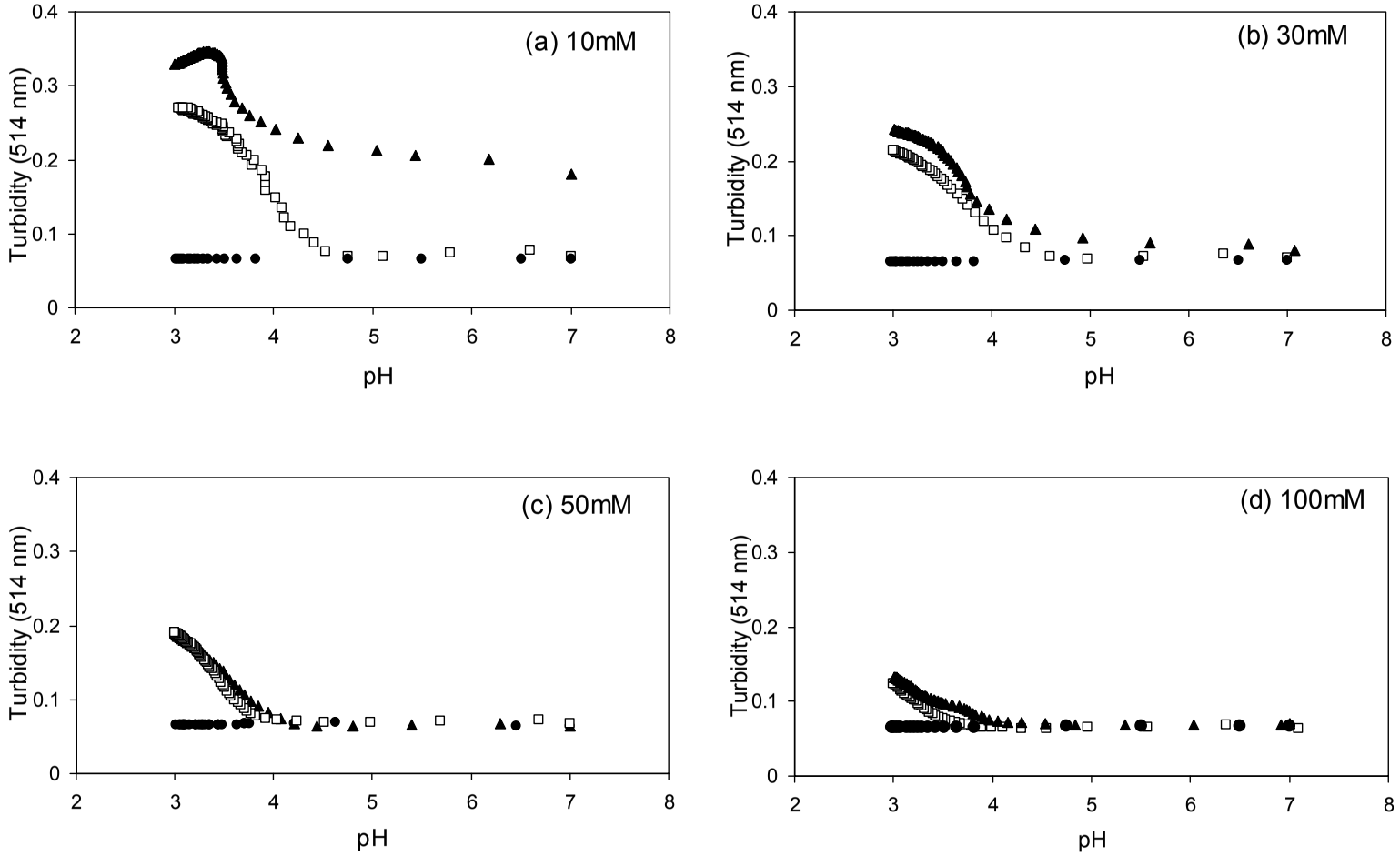

Figure 7 – Turbidity measurements during acidification with GDL of (▲) saliva/lysozyme protein solution mixture (0.6 mg mL^{-1} SPs; 0.5 mg mL^{-1} lysozyme), (□) saliva (0.6 mg mL^{-1} SPs) and (●) lysozyme (0.5 mg mL^{-1}) after addition of 10 mM NaCl (a), 30 mM NaCl (b), 50 mM NaCl (c) and 100 mM NaCl (d).

Numerous examples are reported in literature of complexes formed between proteins and oppositely charged molecules [19, 26, 27, 42-45]. Main parameters affecting electrostatic interaction are the pH and the ionic strength of the solution [46-51]. The pH influences the interaction potential between oppositely charged molecules by varying the degree of protonation and, hence, the molecular charge. The presence of ions, instead, screens the charges thereby reducing the electrostatic potential and hence the electrostatic forces between interacting components. Consequently, at high ionic strength the complex formation is reduced or, depending on the nature and the concentration of the salt, completely suppressed [52-54].

In our system, lysozyme stabilized emulsion droplets, as well as lysozyme molecules in an aqueous solution on the one hand, and the salivary proteins on the other, are oppositely charged. To identify whether the observed flocculation and complex formation were electrostatically driven phenomena, we studied the influence of salt addition to lysozyme stabilized emulsions and lysozyme solution upon mixing with saliva. At neutral pH, addition of NaCl to lysozyme emulsion/saliva mixtures weakened the attraction between lysozyme stabilized droplets and salivary proteins thereby reducing the saliva-induced flocculation. This is reflected by the decrease of the particle size of the mixture (Figure 4) and of the ratio $F(\phi_{\text{flocc}})/F(\phi_{\text{part}})$ (Figure 6) upon increasing the NaCl concentration. By means of turbidity we monitored the influence of pH and ionic strength on lysozyme in solution mixed with saliva (Figures 3 and 7). Such measurements, with detection wavelength in the visible spectrum, are commonly used to study the effect of pH and ionic strength on complex formation between oppositely charged polymers in solution [55, 56]. Lowering the pH adds to the complex formation in lysozyme solution/saliva mixture (Figure 3). This can be explained by the charge increase on the lysozyme molecules. In fact, when considering the amino acids pK values as equivalent to those of the isolated residues, the lysozyme theoretical net charge is +8 at pH 7 and +13 at pH 4.5. On the other hand, variations in pH also influence the net charge on salivary proteins and their interaction in saliva samples. Macroscopic complexes seem to be induced at acid pH, as suggested by the increased turbidity at pH < 4.5, which is in line with the acid precipitation conditions used by Soares and co-workers to isolate salivary micelles for studying their protein composition [57]. Therefore, below this pH the interpretation of the turbidity data in terms of complex formation between salivary proteins and lysozyme could be erroneous. However, the electrostatic nature of the complex formation in the pH range 4.5-7 is also supported by the

behavior of lysozyme/saliva mixtures at different ionic strength (Figure 3 and Figure 7a-7d). The addition of NaCl decreased the turbidity indicating reduced complex formation. At added NaCl concentration > 50 mM complex formation seems to be completely prevented in the pH range considered (Figure 7d).

Besides helping to understand saliva-induced flocculation of lysozyme stabilized emulsions, our results may point to the existence of other molecular complexes in saliva as, for example, the so-called "salivary micelles". These complexes, with dimensions between 40 and 400 nm, as estimated by photon correlation spectroscopy and electron microscopy [11, 57] are composed of salivary lysozyme, lactoferrin, α-amylase, glycosilated prolin-rich protein and MUC7 [57]. Calcium ions seem to be important for the maintenance of the globular structure of these micelles, while addition of pyrophosphate causes structure disintegration [9, 10]. However, so far no other detailed mechanistic studies have been conducted on these micelles.

Besides MUC7 or salivary micelles, it is also possible that lysozyme interacts in a non-covalent manner with MUC5B as suggested in a saliva macromolecular organization study conducted by Wickström [58]. MUC7 and MUC5B belong to the mucin family. The members of this family are characterized, among other features, by their large dimensions and molecular mass, viscoelastic properties, glycosylation and presence of negative charges [59]. MUC5B is composed of disulphide-linked monomers, which contain heavily glycosylated domains carrying a negative charge due to the presence of sialic acid residues (pK~ 2.6) and sulfate groups [60, 61]. MUC5B and pig gastric mucin have shown strong interaction with cationic macromolecules such as gelatin and chitosan [62-64]. Lindh and co-workers also showed that MUC5B and lactoperoxidase (pI = 8.3) could be used to build layer-by-layer mucus assembly in vitro at pH 7. However, the same authors also reported failure of the multilayer technique for combinations of MUC5B and other positively charged proteins such as lysozyme, lactoferrin and histatin 5, respectively [65]. Moreover, it is known that the centrifugation step performed in the procedure to remove bacteria and other debris from saliva, also reduced the amount of salivary mucins. Nevertheless complex formation was observed, which suggests that besides MUC5B and MUC7, other salivary proteins might contribute as well. For example, other components in saliva such as cystatins and serum albumin have a net negative charge at physiological pH as well [66]. These proteins could participate in the binding to the lysozyme adsorbed at the emulsions oil-water interface although their concentration in whole unstimulated saliva is low [4].

Conclusions

Saliva-induced flocculation of lysozyme stabilized emulsions was studied as a model for understanding the oral behavior of positively charged food emulsions. Experimental results showed that molecular complex formation between saliva and emulsion droplets leads to emulsion flocculation. To elucidate the interaction mechanism occurring at the oil droplets surface in a lysozyme stabilized emulsion/saliva mixture, we studied the interaction between lysozyme in aqueous solution and saliva. Complex formation was clearly observed and it supports our suggestion that similar complexation is occurring between saliva and lysozyme adsorbed at the oil-water interface.

The nature of saliva-lysozyme and saliva-oil droplets interactions was addressed by studying the influence of pH and ionic strength. Decreasing the pH of a lysozyme/saliva mixture induced an increase in the turbidity, as expected considering the increased positive surface charges on the lysozyme molecule. Addition of NaCl reduced both complex formation and flocculation which were completely suppressed at high ionic strength. These results, in line with the influences of pH and ionic strength on complexation in mixtures of oppositely charged colloids, point to electrostatic attraction as a driving force for saliva-lysozyme complex formation, which is likely to be responsible for the irreversible saliva-induced flocculation of lysozyme stabilized emulsion.

Acknowledgments

We thank Jan Klok (NIZO Food Research B.V. Ede, The Netherlands) for the assistance with the CLSM, the students Catia da Silva and Dimitris Nitsas for their help during the measurements. Ton van Vliet and Renko de Vries (Wageningen University and Research Centre, Wageningen, The Netherlands) are acknowledged for the stimulating discussion.

References

1. Schwartz, W.H., *The rheology of saliva.* Journal of Dental Research, 1987. **66**: p. 660.
2. Waterman, H.A., Blom, C., Holterman, H.J., 's-Gravenmade, E.J., and Mellema, J., *Rheological properties of human saliva.* Archives of Oral Biology, 1988. **33**: p. 589.
3. Vissink, A., Waterman, H.A., 's-Gravenmade, E.J., Panders, A.K., and Vermey, A., *Rheological properties of saliva substitutes containing mucin, carboxymethylcellulose or polyethylenoxide.* Journal of Oral Pathology, 1984. **13**: p. 22.
4. van Nieuw Amerongen, A. and Veerman, E.C.I., *Saliva - the defender of the oral cavity.* Oral Diseases, 2002. **8**: p. 12.
5. Jensdotti, T., Nauntofte, B., Buchwald, C., and Bardow, A., *Effect of sucking acid candy on whole-mouth saliva composition.* Caries Research, 2005. **39**: p. 468.
6. Neyraud, E., Prinz, J., and Dransfield, E., *NaCl and sugar release, salivation and taste during mastication of salted chewing gum.* Physiology & Behavior, 2003. **79**: p. 731.
7. Hu, S., Loo, J.A., and Wong, D.T., *Human saliva proteome analysis*. Annals of the New York Academy of Sciences, 2007. **1098**: p. 323
8. Raynal, B.D.E., Hardingham, T.E., Thornton, D.J., and Sheehan, J.K., *Concentrated solutions of salivary MUC5B mucin do not replicate the gel-forming properties of saliva.* Biochemical Journal, 2002. **362**: p. 289.
9. Rykke, M., Devold, T.G., Smistad, G., and Vegarud, G.E., *Comparison of bovine casein micelles and human salivary micelle-like structures.* International Dairy Journal, 1999. **9**: p. 365.
10. Rykke, M., Smistad, G., Rolla, G., and Karlsen, J., *Micelle-like structures in human saliva.* Colloids and Surfaces B: Biointerfaces, 1995. **4**: p. 33.
11. Rykke, M., Young, A., Rolla, G., Devold, T., and Smistad, G., *Transmission electron microscopy of human saliva.* Colloids and Surfaces B: Biointerfaces, 1997. **9**: p. 257.
12. Glantz, P.O., Natiella, J.R., Vaughan, C.D., Meyer, A.E., and Baier, R.E., *Structural studies of human saliva.* Acta Odontolologica Scandinavica, 1989. **47**: p. 17.
13. Glantz, P.O., Friberg, S.E., Wirth, S.M., and Baier, R.E., *Thin-section transmission electron microscopy of human saliva.* Acta Odontolologica Scandinavica, 1989. **47**: p. 111.
14. Glantz, P.O., *Interfacial phenomena in the oral cavity.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 1997. **123-124**: p. 657.
15. Vingerhoeds, M.H., Blijdenstein, T.B.J., Zoet, F., and van Aken, G.A., *Emulsion flocculation induced by saliva and mucin.* Food Hydrocolloids, 2005. **19**: p. 915.
16. Silletti, E., Vingerhoeds, M.H., van Aken, G., and Norde, W., *The role of electrostatics in saliva-induced emulsion flocculation.* Food Hydrocolloids, 2007. **21**: p. 596.
17. Canfield, R.E., *The amino acid sequence of egg white lysozyme.* Journal of Biological Chemistry, 1963. **238** p. 2698.
18. Jolles, J., Jauregui, A.J., Bernieri, I., and Jolles, P., *The chemical structure of hen's egg-white lysozyme: Detailed study.* Biochimica et Biophysica Acta, 1963. **13**.
19. Weinbreck, F., de Vries, R., Schrooyen, P., and de Kruif, C.G., *Complex coacervation of whey proteins and gum arabic.* Biomacromolecules, 2003. **4**: p. 293.
20. Yang, C.C., Chen, C.C., and Chang, H.M., *Separation of egg white lysozyme by anion polysaccharides.* Journal of Food Science, 1998. **63**: p. 962.
21. Zschornig, O., Paasche, G., Thieme, C., Korb, N., and Arnold, K., *Modulation of lysozyme charge influences interaction with phospholipid vesicles.* Colloids and Surfaces B: Biointerfaces, 2005. **42**: p. 69.
22. de Roos, A.L., Walstra, P., and Geurts, T.J., *The association of lysozyme with casein.* International Dairy Journal, 1998. **8**: p. 319.
23. Belmont, A. and Nicolini, C., *Polyelectrolyte theory and chromatin-DNA quaternary structure: Role of ionic strength and Hl histone.* Journal of Theoretical Biology, 1981. **90**: p. 169.
24. Clark, D.J. and Kimura, T., *Electrostatic mechanism of chromatin folding.* Journal of Molecular Biology, 1990. **211**: p. 883.
25. Schmitt, C., Sanchez, C., Despond, S., Renard, D., Thomas, F., and Hardy, J., *Effect of protein aggregates on the complex coacervation between beta-lactoglobulin and acacia gum at pH 4.2.* Food Hydrocolloids, 2000. **14**: p. 403.
26. Schmitt, C., Sanchez, C., Thomas, F., and Hardy, J., *Complex coacervation between [beta]-lactoglobulin and acacia gum in aqueous medium.* Food Hydrocolloids, 1999. **13**: p. 483.

27. Weinbreck, F., Tromp, R.H., and de Kruif, C.G., *Composition and structure of whey protein/gum arabic coacervates.* Biomacromolecules, 2004. **5**: p. 1437.
28. Weinbreck, F. and Wientjes, R.H.W., *Rheological properties of whey protein/gum arabic coacervates.* Journal of Rheology, 2004. **48**: p. 1215.
29. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C., *Measurement of protein using bicinchoninic acid.* Analytical Biochemistry. , 1985. **150**: p. 76.
30. Batchelor, G.K., *Effect of brownian-motion on bulk stress in a suspension of spherical-particles.* Journal of Fluid Mechanics, 1977. **83**: p. 97.
31. Krieger, I.M., *Rheology of monodisperse latices.* Advances in Colloidal and Interface Sciences, 1972. **3**: p. 111.
32. Potanin, A.A., de Rooij, R., van den Ende, D., and Mellema, J., *Microrheological modeling of weakly aggregated dispersion.* Journal of Chemical Physics, 1995. **102**: p. 5845.
33. Wolthers, W., Duits, M.H.G., van den Ende, D., and Mellema, J., *Shear history dependence of the viscosity of aggregated colloidal dispersions.* Journal of Rheology, 1996. **40**: p. 799.
34. de Kruif, C.G., *Skim milk acidification.* Journal of Colloids and Interface Science, 1997. **185**: p. 19.
35. Shama, F. and Sherman, P., *Identification of stimuli controlling the sensory evaluation of viscosity. II. Oral methods.* Journal of Texture Studies, 1973. **4**: p. 111.
36. Cutler, A.N., Morris, E.R., and Taylor, L.J., *Oral perception of viscosity in fluid foods and model systems.* Journal of Texture Studies, 1983. **14**: p. 337.
37. Skriver, A., Holstborg, J., and Qvist, K.B., *Relation between sensory texture analysis and rheological properties of stirred yogurt.* Journal of Dairy Research, 1999. **66**: p. 609.
38. Healy, T.W. and Lamer, V.K., *Energetics of flocculation and redispersion by polymers.* Journal of Colloid Science, 1964. **19**: p. 323.
39. Blijdenstein, T.B.J., van Winden, A.J.M., van Vliet, T., van der Linden, E., and van Aken, G.A., *Serum separation and structure of depletion- and bridging-flocculated emulsions: A comparison.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2004. **245**: p. 41.
40. Dickinson, E. and Pawlowsky, K., *Influence of iota-carrageenan on flocculation, creaming and rheology of a protein-stabilized emulsion.* Journal of Agriculture and Food Chemistry, 1997. **45**: p. 3799.
41. Dickinson, E. and Pawlowsky, K., *Rheology as a probe of protein-polysaccharide interactions in oil in water emulsions* Gum and stabilizers for the food industry ed. D.J.W.P.A.W. G.O. Phillips. Vol. 8. 1996, Oxford, UK: IRL Press.
42. Sanchez, C. and Renard, D., *Stability and structure of protein-polysaccharide coacervates in the presence of protein aggregates.* International Journal of Pharmaceutics, 2002. **242**: p. 319.
43. Turgeon, S.L., Beaulieu, M., Schmitt, C., and Sanchez, C., *Protein-polysaccharide interactions: Phase-ordering kinetics, thermodynamic and structural aspects.* Current Opinion in Colloid & Interface Science, 2003. **8**: p. 401.
44. Weinbreck, F., Nieuwenhuijse, H., Robijn, G.W., and de Kruif, C.G., *Complex formation of whey proteins: Exocellular polysaccharide EPS B40.* Langmuir, 2003. **19**: p. 9404.
45. Weinbreck, F., Nieuwenhuijse, H., Robijn, G.W., and de Kruif, C.G., *Complexation of whey proteins with carrageenan.* Journal of Agricultural and Food Chemistry, 2004. **52**: p. 3550.
46. Burgess, D.J., *Practical analysis of complex coacervate systems.* Journal of Colloid and Interface Science, 1990. **140**: p. 227.
47. Burgess, D.J., Kwok, K.K., and Megremis, P.T., *Characterization of albumin-acacia complex coacervation.* Journal of Pharmaceutical Pharmacology, 1991. **43**: p. 232.
48. Mattison, K.W., Dubin, P.L., and Brittain, I.J., *Complex formation between bovine serum albumin and strong polyelectrolytes: Effect of polymer charge density.* Journal of Physical Chemistry B, 1998. **102**: p. 3830
49. Girard, M., Turgeon, S.L., and Gauthier, S.F., *Interbiopolymer complexing between [beta]-lactoglobulin and low- and high-methylated pectin measured by potentiometric titration and ultrafiltration.* Food Hydrocolloids, 2002. **16**: p. 585.
50. Galazka, V.B., Smith, D., Ledward, D.A., and Dickinson, E., *Complexes of bovine serum albumin with sulphated polysaccharides: Effects of pH, ionic strength and high pressure treatment.* Food Chemistry, 1999. **64**: p. 303.
51. Galazka, V.B., Smith, D., Ledward, D.A., and Dickinson, E., *Complexes of ovalbumin with sulphated polysaccharides: Effects of pH, ionic strength, heat and high pressure treatment.* Food Hydrocolloids, 1999. **13**: p. 81.
52. Bungenberg de Jong, H.G., *Complex colloids systems*. Colloids science, ed. H.R. Kruyt. Vol. 2. 1949, Amsterdam: Elsevier Publishing Company 335.

53. Overbeek, J.T.G. and Voorn, M.J., *Phase separation in polyelectrolytes solution. Theory of complex coacervation.* Journal of Cellular and Comparative Physiology, 1957. **49**: p. 7.
54. Schmitt, C., Sanchez, C., Desobry-Banon, S., and Hardy, J., *Structure and technofunctional properties of protein-polysaccharide complexes: A review.* Critical Reviews in Food Science and Nutrition, 1998. **38**: p. 689.
55. Plashchina, I.G., Mrachkovskaya, T.A., Danilenko, A.N., Kozhevnikov, G.O., Starodubrovskaya, N.Y., Braudo, E.E., and Schwenke, K.D., *Complex formation of faba bean legumin with chitosan: Surface activity and emulsion properties of complexes*, in *Food colloids, fundamentals and formulation.* E. Dickinson and R. Miller, Editors. 2001, Royal Society of Chemistry: Cambridge. p. 292.
56. Sanchez, C., Mekhloufi, G., Schmitt, C., Renard, D., Robert, P., Lehr, C.M., Lamprecht, A., and Hardy, J., *Self-assembly of beta-lactoglobulin and acacia gum in aqueous solvent: Structure and phase-ordering kinetics.* Langmuir, 2002. **18**: p. 10323.
57. Soares, R.V., Lin, T., Siqueira, C.C., Bruno, L.S., Li, X., Oppenheim, F.G., Offner, G.D., and Troxler, R.F., *Salivary micelles: Identification of complexes containing MG2, sIgA, lactoferrin, amylase, glycolsylated proline-rich protein and lysozyme.* Archives of Oral Biology, 2004. **49**: p. 337.
58. Wickström, C., Christersson, C., Davies, J.R., and Carlstedt, I., *Macromolecular organization of saliva: Identification of 'insoluble' MUC5B assemblies and non-mucin proteins in the gel phase.* Biochemical Journal, 2000. **351**: p. 421.
59. van Nieuw Amerongen, A., Bolscher, J., and Veerman, E.C.I., *Salivary proteins: Protective and diagnostic value in cariology?* Caries Research, 2004. **38**: p. 247.
60. Thornton, D.J., Khan, N., Mehrotra, R., Howard, M., Veerman, E.C.I., Packer, N.H., and Sheehan, J.K., *Salivary mucin MG1 is comprised almost entirely of different glycosylated forms of the MUC5B gene product.* Glycobiology, 1999. **9**: p. 293.
61. Thomsson, K.A., Prakobphol, A., Leffler, H., Reddy, M.S., Levine, M.J., Fisher, S.J., and Hansson, G.C., *The salivary mucin MG1 (MUC5B) carries a repertoire of unique oligosaccharides that is large and diverse.* Glycobiology, 2002. **12**: p. 1.
62. Dédinaite, A. and Bastardo, L., *Interactions between mucin and surfactants at solid-liquid interfaces.* Langmuir, 2002. **18**: p. 9383.
63. Hassan, E.E. and Gallo, J.M., *A simple rheological method for the in vitro assessment of mucin-polymer bioadhesive bond strength.* Pharmeutical Research, 1990. **7**: p. 491.
64. Svensson, O., Lindh, L., Cárdenas, M., and Arnebrant, T., *Layer-by-layer assembly of mucin and chitosan—influence of surface properties, concentration and type of mucin.* Journal of Colloid and Interface Science 2006. **299** p. 608.
65. Lindh, L., Svendsen, I.E., Svensson, O., Cárdenas, M., and Arnebrant, T., *The salivary mucin MUC5B and lactoperoxidase can be used for layer-by-layer film formation.* Journal of Colloid and Interface Science 2007. **310**: p. 74.
66. Yao, Y., Berg, E.A., Costello, C.E., Troxler, R.F., and Oppenheim, F.G., *Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches.* Journal of Biological Chemistry, 2003. **278**: p. 5300.

Chapter 4

Rheological behavior of food emulsions mixed with saliva: effect of oil content, salivary protein content and saliva type

E. Silletti, M.H. Vingerhoeds, G.A. van Aken and W. Norde

Submitted

Abstract

In this paper we studied the effect of saliva on the rheological properties of β-lactoglobulin and lysozyme stabilized emulsions, prepared at pH=6.7, in relation to variation of emulsions- and saliva related parameters. The effect of oil-volume fraction (2.5% w/w to 10% w/w), salivary protein concentration (0.1 to 0.8 mg mL^{-1}) and the use of both stimulated and unstimulated saliva was investigated. Viscosity, storage- and loss moduli were measured before (η_{emul}, G'_{emul} and G''_{emul}) and after addition of saliva (η_{mix}, G'_{mix} and G''_{mix}). To better estimate the changes due to saliva-induced flocculation of the emulsions, the ratios η_{mix}/η_{emul}, G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul} were calculated. Increasing the oil-volume fraction and salivary protein concentration resulted in an increase in η_{mix}/η_{emul}, G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul}.

When compared with unstimulated saliva, mixing β-lactoglobulin stabilized emulsions with stimulated saliva led to a reduction in η_{mix}/η_{emul}, G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul}. In case of lysozyme stabilized emulsions, compared to unstimulated, stimulated saliva increased both G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul}. The effect of stimulated saliva on the η_{mix}/η_{emul} of lysozyme emulsion/saliva mixture is similar to that of unstimulated saliva. These results indicate that the effect of stimulated saliva on the rheological parameters of emulsion/saliva mixtures largely depends on the type of emulsions. To conclude, our findings demonstrate that the rheological behavior of emulsions upon mixing with saliva is greatly affected by both saliva and emulsion properties.

Introduction

Human saliva is involved in several functions such as maintaining oral health, protection of the teeth and mucosal surfaces and eating [1-4]. Proteomics revealed that saliva contains more than 1050 different proteins and peptides [5] with molecular mass varying from a few kDa to > 1000 kDa, e.g. for the large secreted polymeric mucin MUC5B [6]. Mucins, which are the main constituents of the mucous secretion throughout the body, are a family of highly glycosilated molecules, carrying at physiological pH a net negative charge due to the presence of sialic acid residues and sulphate groups [7, 8]. Several levels of protein organization, e.g. the presence of the network composed of MUC5B and the so-called "salivary micelles" [9-11], add to the structural complexity of saliva [12-14] and influence its rheological properties. In particular the type of mucin and its origin appear to play a dominant role in the viscoelastic properties of saliva [15]. Saliva is secreted by different glands and is susceptible to variation depending on many factors as type of the salivary gland or stimuli [16, 17]. Unstimulated saliva is mainly secreted from sublingual and submandibular glands, while the parotid gland contributes for about 80% of the total stimulated saliva production. Parotid saliva does not contain mucins and has a shear rate independent viscosity slightly higher than water [15, 18]. Sublingual saliva, instead, shows a clear shear-thinning behavior [15] and plays an important role in the prevention of oral dryness because of its high viscosity and elasticity. Submandibular saliva exhibits lower elasticity than sublingual saliva. This characteristic is important for the lubrication during speaking and swallowing, and for bolus formation [15].

The consumer's perception of food products is becoming increasingly important for the food industry in relation to product design and evaluation. Attempts have been made to correlate sensory perceived attributes with physical parameters of the products, as for example the in-mouth thickness (perceived thickness) of fluids and semisolid foods with the shear viscosity [19] or the shear stress [20, 21]. Moreover, several authors reported the influence of saliva properties, such as flow, composition and lubrication, on sensory perception and flavour release [22-25]. It is becoming evident that knowledge on the interaction of food products with saliva is important for understanding oral processing of food since often perception cannot be directly related to the texture of the products before consumption. For example, the correlation between shear-thinning behavior of different polysaccharide solutions and mouth-feel sliminess, which was formulated over 30 years

ago [26], is not clearly established, as no correlation was observed in subsequent investigations [27]. Therefore, investigations of the dynamic processes occurring in the mouth, which are affecting the food structure, were initiated. In our research, we focused on liquid food emulsions stabilized by proteins which undergo flocculation after mixing with saliva [28, 29]. A clear role of droplet charge on the emulsion stability and viscosity upon mixing with saliva was established by using differently charged surfactants and proteins as emulsifiers (Chapter 2) [30]. Strongly negatively charged emulsions did not flocculate in the presence of saliva, whereas weakly negatively charged emulsion droplets (i.e. stabilized by β-lactoglobulin) reversibly flocculated with respect to dilution and shear. Saliva-induced flocculation of positively charged emulsions, e.g. stabilized by lysozyme, was instead irreversible upon dilution and shear. In particular, for lysozyme, complex formation between lysozyme stabilized emulsion droplets and salivary proteins was demonstrated (Chapter 3) [31].

Rheological measurements are widely used to evaluate emulsion behavior under different applied conditions [32-34], to assess long term stability [35] or to characterize flocculation [36]. Several parameters, such as oil-volume fraction and characteristics of the continuous phase, contribute to the rheological properties of emulsions. It is known that if the effects of colloidal interparticle interactions are negligible, emulsions with oil-volume fraction < 0.5 behave Newtonian [37]. In case of flocculation, rheology depends on the type and strength of the interaction between the droplets. Flocculated emulsions exhibit a higher viscosity (η), as a result of the increase in the effective volume fraction, and a shear-thinning behavior [38]. Storage (G') and loss moduli (G'') of these emulsions are usually also evaluated by means of oscillatory measurements [32, 34-36, 38]. Flocculation is frequently accompanied by a rapid increase in the storage modulus [35] with irreversibly flocculated emulsions showing larger storage moduli than reversibly flocculated emulsions [34].

In this paper we studied the influence of different parameters on the rheological properties, i.e. η, G' and G'', of a negatively charged emulsion stabilized by β-lactoglobulin and a positively charged emulsion stabilized by lysozyme after mixing with saliva. The aim of this work was to illustrate how oil-volume fraction, type of saliva and salivary protein content influence flocculation behavior and consequently the rheological properties of emulsion/saliva mixtures.

Materials and methods

Materials

Freeze-dried β-lactoglobulin (β-lg) was provided by Wageningen Centre for Food Science (WCFS, Wageningen, The Netherlands) and was purified as described previously [39]. The powder contains 93.6% w/w proteins (N x 6.38). Lysozyme from chicken egg-white (L6876 batch 051K7028) was obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands) and used without further purification. Lysozyme from chicken egg-white from the batch number 016K1189 (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) was instead desalted on a Sephadex G10 (flow = 40 ml min^{-1} and pressure 0.4 mPa, eluted with water de-gassed with He). The eluent was freeze dried and stored at –20 ^{o}C until further use. Without this treatment, lysozyme solutions obtained from this batch number had an acid pH and could not be used to make stable emulsions according to the method indicated in the following paragraph. Sunflower oil (Reddy, Vandemoortele, The Netherlands) was purchased from a local retailer; BCA^{TM} Protein Assay Kit from Pierce Biotechnology Inc. (Rockford, IL, USA) and sodium azide was obtained from Merck (Shuchardt, Germany).

Collection and handling of saliva

Unstimulated saliva: Whole human unstimulated saliva was collected according to the procedure described in Chapter 2 [30]. Briefly, whole human unstimulated saliva was collected from 8.30 to 10.30 a.m. from ten healthy non-medicated volunteers. After rinsing their mouths with water, saliva was collected with closed lips for a couple of minutes and then expectorated into ice-chilled vessels. The first mL of saliva was discarded.

Stimulated saliva: Whole human stimulated saliva was collected from the same group of volunteers from 8.30 to 10.30 a.m. In line with our protocol for unstimulated saliva donation, after optionally having breakfast and brushing their teeth, donors refrained from eating and drinking, with the exception of water, for two hours before donation. After rinsing their mouths with water, the volunteers chewed a piece of parafilm of 5 x 5 cm in dimension for 6 minutes. During this time, stimulated saliva was expectorated into ice-chilled vessels every 30 seconds. Also in this case, the first mL of saliva was discarded**.**

Handling: During collection and handling, the samples were constantly kept on ice. Both unstimulated and stimulated saliva were separately pooled and centrifuged at 10,000 g for

30 min at 4 ^{o}C to remove cellular debris (Beckman, model Avanti ^{TM} J-25 I, rotor JA-21, Beckman Coulter B.V. Mijdrecht, The Netherlands). The supernatants were frozen in liquid nitrogen, stored at –80 ^{o}C and used within six weeks. SELDI-TOF-MS experiments conducted to test the effect of different treatments and storage conditions on the low molecular weight salivary proteins have shown that the used handling conditions have little effect on saliva when compared with fresh saliva [40]. No precipitation of salivary proteins was observed after thawing. The pH of unstimulated saliva ranged from 6.7 to 7.0, while the pH of stimulated saliva was about 8. The salivary protein (SP) content both of stimulated and unstimulated saliva was determined according to the BCA method of Pierce using bovine serum albumin as standard. The protein content varied from 1.1 to1.3 mgmL^{-1} for unstimulated saliva and about 0.7 mg mL^{-1} for stimulated saliva. In this paper, with the term saliva we indicate pooled unstimulated saliva after handling procedure.

Preparation and characterization of O/W emulsions

β-lactoglobulin- and lysozyme solutions were prepared by dissolving the protein powder (1% w/w) overnight at 4 ^{o}C in demineralised water and 10 mM NaCl solution (59 mL), respectively. Pre-emulsions were prepared using an Ultra-Turrax T 25 Basic (IKA-Werke GmbH & Co. KG, Staufen, Germany) and subsequently stock emulsions were homogenized at room temperature by 10 passes through a Delta Lab-scale homogenizer (Delta Instruments B.V., Drachten, the Netherlands). The operating pressure was 70 bar for β-lg stabilized emulsions and 100 bar in case of lysozyme emulsions. β-lg stabilized stock emulsions made at pH 6.7, contained 40% w/w sunflower oil and 1% w/w emulsifier while lysozyme stabilized stock emulsions (pH 6.7, 1% w/w protein and 10 mM NaCl) contained 20% w/w sunflower oil. Sodium azide (0.02% w/w) was added to the emulsions to prevent microbial growth.

Light microscopy images were taken using an Olympus BX 60 Microscope equipped with an Olympus DP 70 camera (Olympus Nederland B.V., Zoeterwoude, The Netherlands). Droplet-size distribution and the volume-over surface average droplets diameter (d_{32}) were measured by laser diffraction with the Mastersizer Hydro 2000S (Malvern Instruments, Southborough, UK) as described in Chapter 2 [30].

Sample preparation for rheology experiments

Emulsion/saliva mixtures were prepared at room temperature by adding thawed saliva to diluted β-lg and lysozyme stabilized emulsions containing 10 mM NaCl in the bulk phase. To reduce proteolytic activity, which is naturally present in saliva, samples were thawed at room temperature shortly before each experiment. The effect of oil-volume fraction (ϕ) was studied by varying the oil phase between 2.5% w/w and 10% w/w in emulsion/saliva mixtures containing 0.6 mg mL^{-1} SPs, in line with a previous paper where emulsions were mixed 1:1 with saliva [28]. The influence of salivary protein content on the emulsion/saliva mixture has been determined at two different oil contents (2.5% w/w and 10% w/w). Salivary protein content was therefore varied between 0.1 and 0.6 mg mL^{-1} in a mixture containing a 10% w/w oil phase and between 0.1 and 0.8 mg mL^{-1} in a mixture containing 2.5% w/w oil phase. Lastly, the effect of saliva type (stimulated vs unstimulated) was determined in two mixtures containing 0.6 mg mL^{-1} SPs and 5% w/w oil and 0.4 mg mL^{-1} SPs and 10% w/w oil, respectively.

Rheology

The shear rate dependent viscosity of emulsions, saliva and the mixtures was measured in duplicate, using a Physica MCR 301 rheometer (Anton Paar BVBA, Sint Martens Latem, Belgium) at 20°C, using a cone-and-plate geometry CP 75-1 with an angle of 1º (0.0175 rad) and a gap-width of 0.05 mm at the tip. The shear rate was logarithmically increased over 20 minutes from 0.1 to 1500 s^{-1}. Viscoelasticity measurements were carried out on emulsions, saliva and their mixtures using the Vilastic-3 viscoelasticity analyzer (Vilastic Scientific Inc., Austin, Texas, USA). This instrument is mostly used in measurements of low viscosity fluids, in particular blood [41-43] but also saliva [15]. Measurements were performed in duplicate using oscillatory flow in a vertical capillar with the dimension of 63.97 mm in length and 5.04 mm in diameter. The pressure drop and volume flow across the tube are measured with an accuracy of 2%. The pressure and flow are related to the shear stress, -strain, -rate and viscoelasticity of the fluid as described by Thurston for the oscillation of a viscoelastic fluid in a circular tube [44, 45]. According to the manufacturer's protocol, a frequency of 2 Hz was selected. The storage- and loss moduli were consequently measured in the shear strain (γ) range between 0.002 and 19 and shear viscosity in the shear rate range between 0.01 and 250 s^{-1}. The results from the viscoelasticity analyzer were in line with

those obtained with the cone-and-plate geometry measurements but covered a smaller shear rate range. For this reason, in case of shear viscosity, we show only the findings from the Physica MCR 301 rheometer measurements.

As a tool to determine the increased viscosity due to saliva-induced flocculation, independently from the emulsion viscosity under experimental conditions, we calculated the ratio η_{mix}/η_{emul}, where the viscosity of the mixtures (η_{mix}) is normalized by the viscosity of the emulsions (η_{emul}) (Chapter 2) [30]. To evaluate saliva influence on viscoelastic parameters, we determined the ratio G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul}, where G'_{mix} and G''_{mix} are the storage- and loss moduli of the mixtures and G'_{emul} and G''_{emul} the storage- and loss moduli of the emulsions. As the observed trends were independent of the applied strain, we chose to present the results at a deformation of 1.1.

Results

Emulsion and saliva characterization

Microscopic images of the prepared emulsions revealed that emulsion droplets were homogenously dispersed throughout the sample and emulsion flocculation was not observed (not shown). Table 1 summarizes the droplet sizes, expressed as volume-over surface average droplets diameter (d_{32}), and the related measured rheological parameters, i.e. viscosity (η), storage- and loss moduli (G' and G'', respectively) of the prepared emulsions at different oil contents. Droplet diameters were similar for β-lg and lysozyme stabilized emulsions. In line with previous measurements (Chapter 2) [30], β-lg and lysozyme stabilized emulsions exhibited Newtonian behavior and displayed similar viscosity values (Table 1). Storage- and loss moduli were strain independent at all studied oil contents, with loss moduli about 10 times higher than storage moduli (Table 1). A small but detectable increase in η, G' and G'' was observed upon increasing the oil content (2.5% w/w vs. 10% w/w).

Rheological parameters of unstimulated and stimulated saliva are also reported in Table 1. Viscosities of saliva (1.15 mPa.s for stimulated and 1.18 mPa.s for unstimulated saliva) are in line with the values reported in literature [15, 46]. G' and G'' were strain independent for both unstimulated and stimulated saliva with G' and G'' of unstimulated saliva being slightly higher than those of stimulated saliva.

Table 1 – Volume-over surface average droplet diameter (d_{32}), viscosity (η), storage modulus (G') and loss modulus (G'') of β-lactoglobulin and lysozyme stabilized emulsions at different oil content (% w/w). η, G' and G'' of unstimulated and stimulated saliva for a SPs concentration of 0.6 mg mL^{-1} is reported as well.

Sample	**Oil (%w/w)**	d_{32} **(µm)**	η **(mPas)***	η **(mPas)****	***G'* (mPa)****	***G''* (mPa)****
β-Lactoglobulin	2.5	0.98 (0.01)	1.11 (0.01)	1.10 (<0.01)	1.17 (0.13)	14.10 (0.21)
	5	1.10 (0.01)	1.34 (0.01)	1.18 (<0.01)	1.18 (0.13)	14.80 (0.10)
	10	1.12 (0.01)	1.43 (<0.01)	1.44 (<0.01)	1.55 (0.16)	17.10 (0.04)
Lysozyme	2.5	1.14 (0.07)	1.07 (0.06)	1.07 (<0.01)	1.50 (0.02)	13.51 (0.09)
	5	0.98 (0.03)	1.28 (0.17)	1.28 (0.19)	1.53 (0.05)	17.81 (5.50)
	10	1.10 (0.08)	1.39 (0.03)	1.27 (<0.01)	1.74 (0.07)	16.01 (0.08)
Unstimulated saliva	-	-	1.18 (0.32)	1.23 (<0.01)	1.94 (0.17)	16.09 (0.19)
Stimulated saliva	-	-	1.15 (0.01)	1.16 (0.01)	1.34 (0.07)	14.70 (0.03)

** Values measured with cone-and-plate geometry at 100 s^{-1}; ** Values measured with capillary set-up at 95 s^{-1} and $\gamma = 1$. Standard deviation is shown in parenthesis.*

Effect of oil content

The effect of oil content on the rheological properties of β-lg and lysozyme stabilized emulsions after mixing with saliva was analyzed as a function of the shear rate and deformation. Since the two emulsions showed a similar trend in saliva-induced viscosity changes (η_{mix}/η_{emul}), we report our findings only for β-lg emulsion/saliva mixtures (Figure 1). Increasing the oil content led to an increase of η_{mix}/η_{emul} at all applied shear rates. Although not easily visible, small differences in η_{mix}/η_{emul} are still present at high shear rate (> 560 s^{-1}).

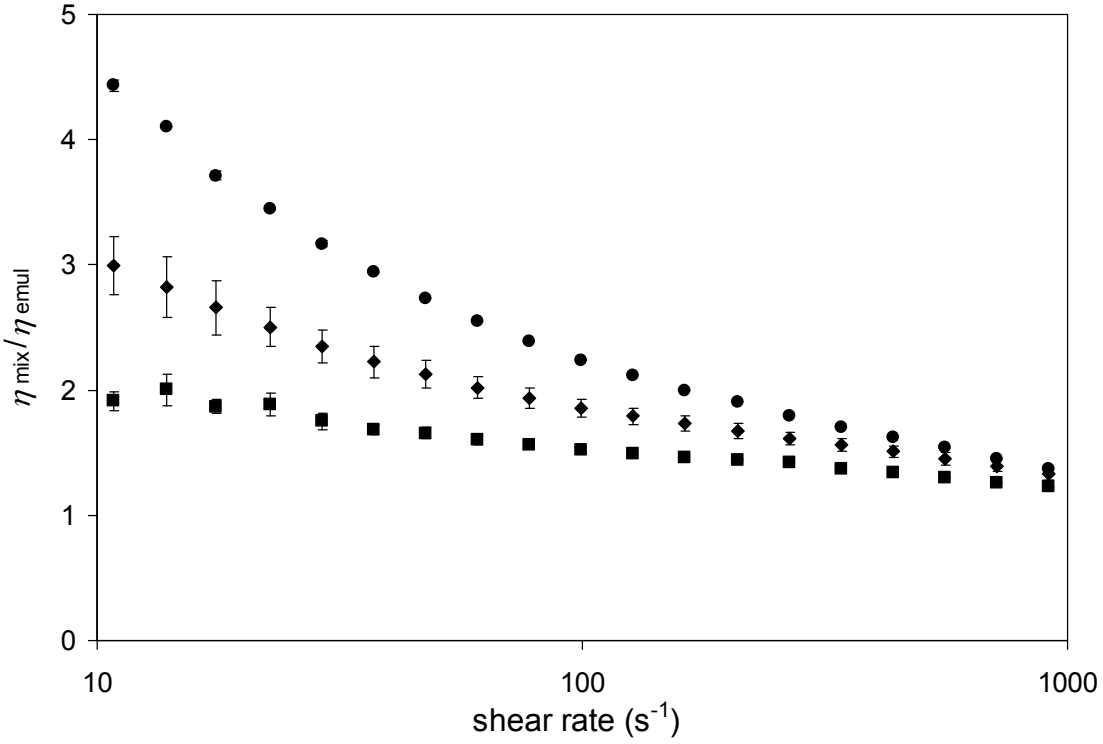

Figure 1 – Effect of oil content on the shear rate dependent η_{mix}/η_{emul} for β-lg stabilized emulsions after mixing with saliva (0.6 mg mL^{-1} SPs) at 2.5% w/w (■), 5% w/w (⧫) and 10% w/w (●) oil phase. Error bars represent the standard deviation.

The influence of the oil-volume fraction on G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul} is exemplified in Figure 2 for both emulsions. Increasing the oil content led to an augment of G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul}. Large differences in the ratios between the mixtures of saliva with the two emulsions have been seen, especially at high volume fraction oil. At 10% w/w, the lysozyme stabilized emulsion/saliva mixture showed a 3-fold higher value of G'_{mix}/G'_{emul} than the β-lg emulsion/saliva mixture. By lowering the oil content, the difference between the two mixtures became smaller until it disappeared at 2.5% w/w oil content. Moreover as shown in Figure 2, G'_{mix}/G'_{emul} was found to be higher than G''_{mix}/G''_{emul} at all evaluated oil contents and applied strains, indicating that mixing with saliva induced larger changes in the storage modulus than in the loss modulus. This feature, i.e. G'_{mix}/G'_{emul} larger than $G''_{mix}/G''_{emul,}$ was a common characteristic of emulsions upon mixing with unstimulated saliva.

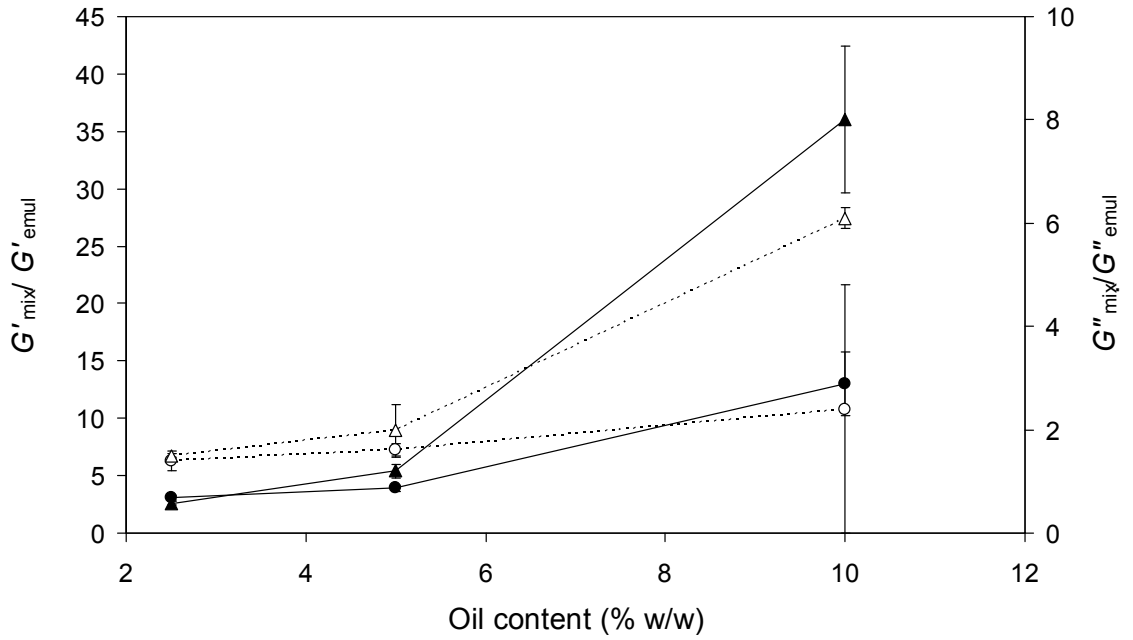

Figure 2 – Effect of oil content on the G'_{mix}/G'_{emul} (closed symbols) and G''_{mix}/G''_{emul} (open symbols) measured at γ = 1.1 for β-lg (●/○) and lysozyme (▲/Δ) stabilized emulsions after mixing with saliva (0.6 mg mL^{-1} SPs). Error bars represent the standard deviation.

Influence of salivary protein content and type of saliva

Figure 3 summarizes the effect of salivary protein (SPs) concentration on the saliva-induced viscosity increase for β-lg and lysozyme stabilized emulsions at two different oil contents (2.5% w/w and 10% w/w). Typically, increasing the SPs concentration in the mixtures resulted in an augment of the ratio η_{mix}/η_{emul}. This effect is most pronounced at 10% w/w, for both emulsion types. In addition, in line with previous results (Chapter 2) [30], lysozyme stabilized emulsion/saliva mixtures show a larger viscosity increase compared to β-lg stabilized emulsion/saliva mixtures prepared at the same oil-volume fraction. It is worth to note that comparable viscosity ratios could be obtained, for example, when 0.4 mg mL^{-1} SPs was added to a β-lg stabilized emulsion (10% w/w) and a lysozyme stabilized emulsion (2.5% w/w). Clearly a combination of both SPs concentration and oil-volume fraction is affecting the saliva-induced flocculation behavior of emulsions and therewith the ratio η_{mix}/η_{emul}.

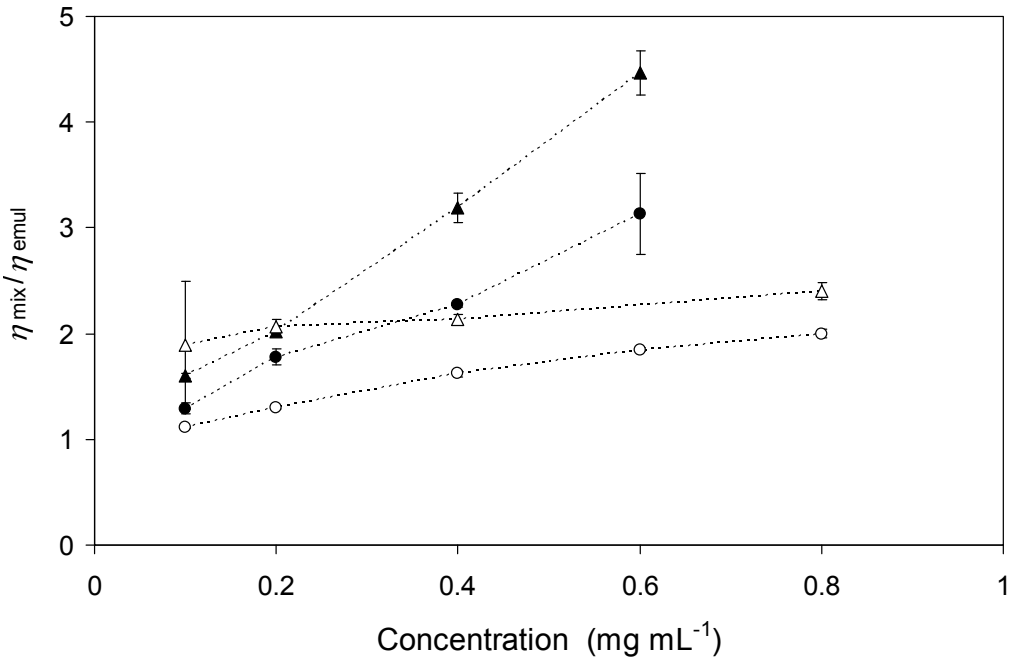

Figure 3 – Effect of salivary protein concentration on η_{mix}/η_{emul} at 100 s^{-1} for β-lg stabilized emulsions (●/○) and lysozyme stabilized emulsions (▲/Δ) after mixing with saliva at 2.5% w/w oil content (open symbols) and 10% w/w oil content (closed symbols). Error bars represent the standard deviation. Lines are intended to guide the eye.

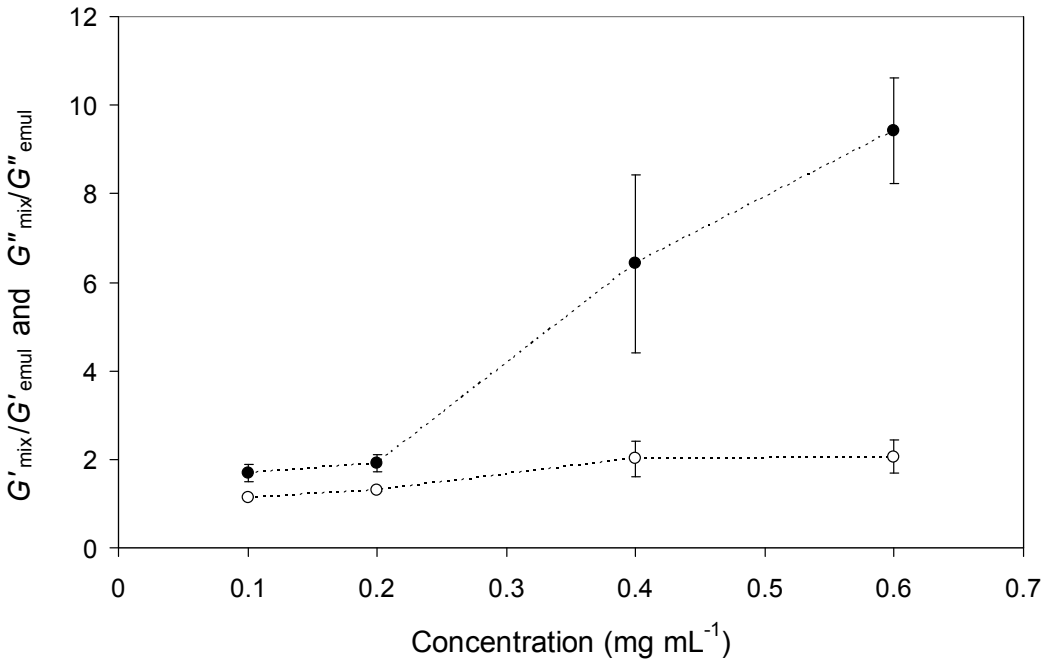

Figure 4 – Effect of SPs concentration on the strain dependent G'_{mix}/G'_{emul} (closed symbols) and G''_{mix}/G''_{emul} (open symbols) for β-lg stabilized emulsions (10% w/w) after mixing with saliva at γ = 1.1. Error bars represent the standard deviation. Lines are intended to guide the eye.

To conclude the analysis of the influence of SPs concentration on the rheological parameters of emulsion/saliva mixtures, we illustrate in Figure 4 the G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul} values for β-lg stabilized emulsion/saliva mixtures at 10% w/w oil. An increase in G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul} as function of the SPs concentration is observed, with the largest effect on G'_{mix}/G'_{emul}. Similar behavior has been observed for the other studied oil-volume fractions, as well as for lysozyme stabilized emulsion/saliva mixtures.

The different effect of unstimulated and stimulated saliva on the shear rate dependent η_{mix}/η_{emul} of β-lg and lysozyme stabilized emulsions is shown in Figure 5. Since the protein concentration in stimulated saliva was lower than in unstimulated saliva, we prepared emulsion/saliva mixtures at 10% w/w and 0.4 mg mL^{-1} instead of the usually used concentration of 0.6 mg mL^{-1}. The type of saliva had a major effect on β-lg stabilized emulsions, since as shown, stimulated saliva induced a lower ratio η_{mix}/η_{emul} compared to unstimulated saliva, at shear rates below 300 s^{-1}. A minor effect of the type of saliva on η_{mix}/η_{emul} has been seen for lysozyme stabilized emulsion/saliva mixtures.

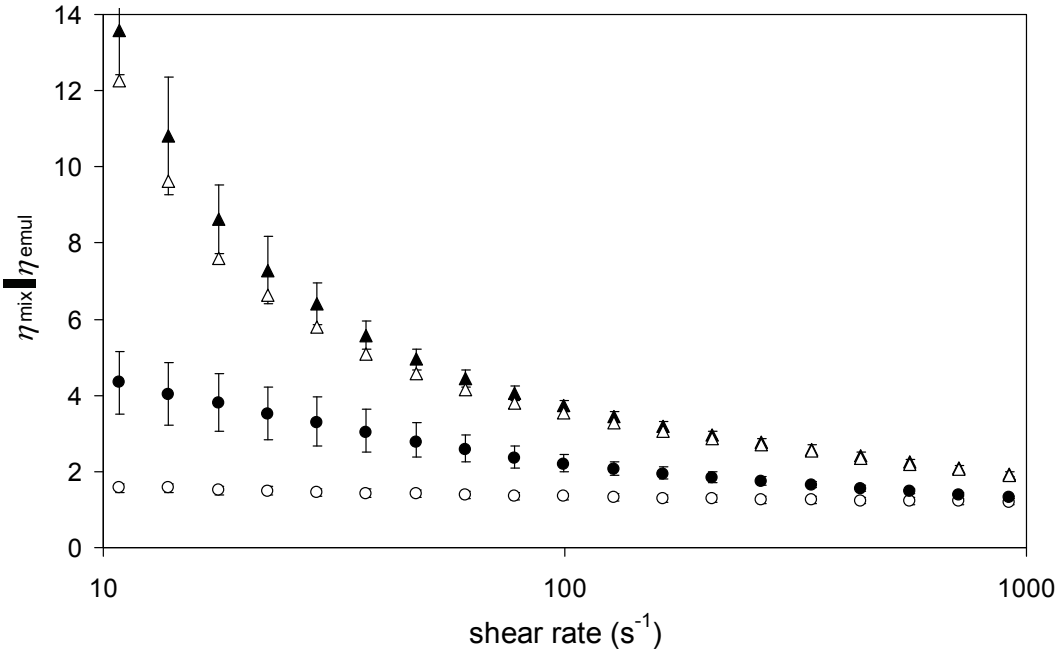

Figure 5 – Effect of stimulated (open symbols) and unstimulated (closed symbols) saliva on the shear rate dependent η_{mix}/η_{emul} for β-lg (●/○) and lysozyme stabilized emulsions (▲/Δ) after mixing with saliva (10% w/w oil phase; 0.4 mg mL^{-1} SPs). Error bars represent the standard deviation.

In line with the effect on the viscosity (Figure 5), the use of stimulated saliva in β-lg stabilized emulsion/saliva mixtures resulted in smaller G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul} compared to unstimulated saliva (Figure 6a).

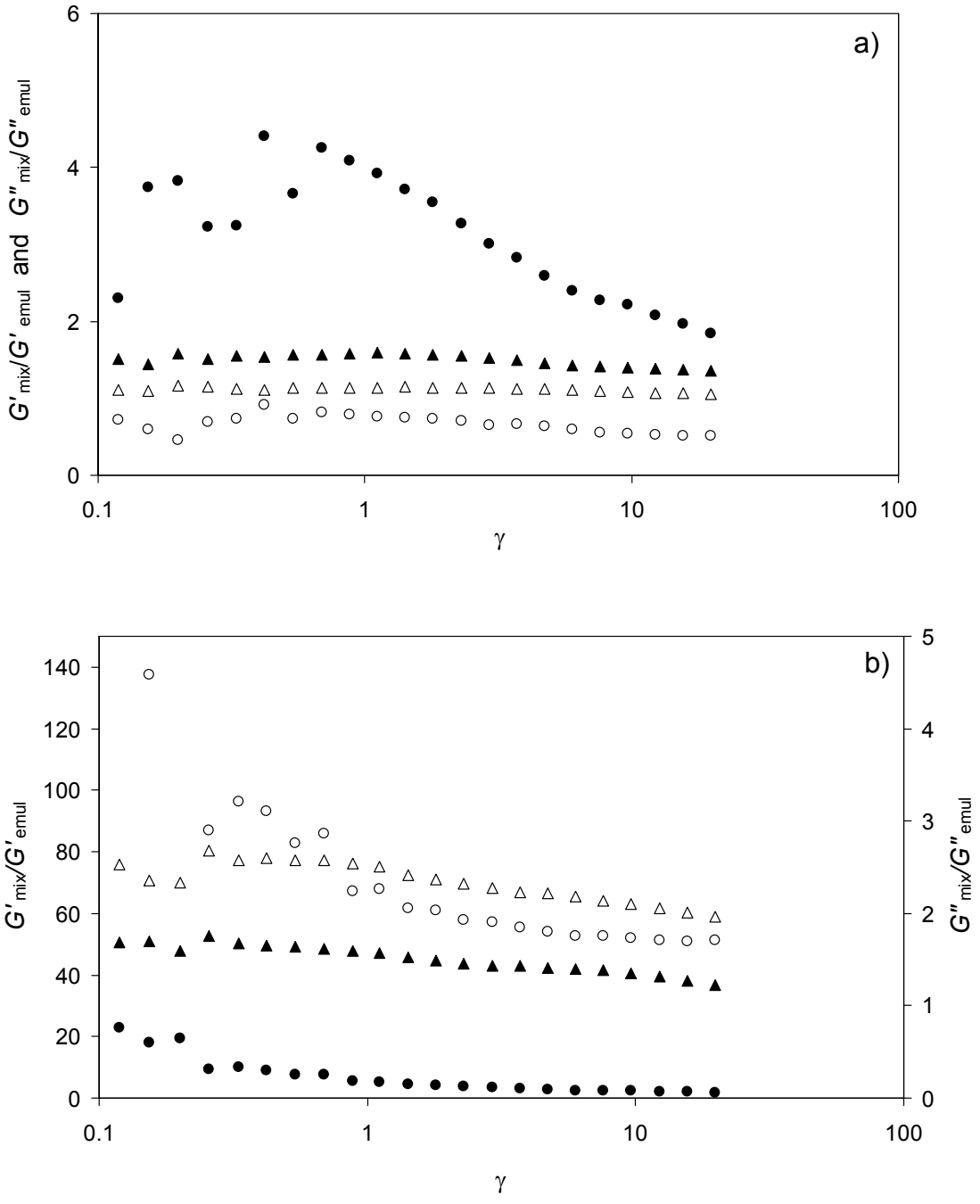

Figure 6 – Effect of stimulated (open symbols) and unstimulated (closed symbols) saliva on G'_{mix}/G'_{emul} (●;○) and G''_{mix}/G''_{emul} (▲;Δ) for β-lg stabilized emulsions (Figure 6a) and lysozyme stabilized emulsions (Figure 6b) after mixing with saliva (5% w/w oil phase; 0.6 mg mL^{-1} SPs).

Moreover, it is noted that the storage modulus of the mixture was lower compared to the G' values of the emulsions, which resulted in the illustrated $G'_{mix}/G'_{emul} < 1$ (Figure 6a). Since the G'_{mix} values were obtained in the measurable range of the instrument provided by the manufacturer, we cannot provide a plausible explanation for this result. Figure 6b illustrates G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul} for lysozyme stabilized emulsions after addition of saliva. Remarkably, stimulated saliva induced higher G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul} at all applied deformations than unstimulated saliva.

Discussion

In this chapter, we studied the effect of different parameters on saliva-induced flocculation for emulsions stabilized by β-lg (pI~ 4.9) and by lysozyme (pI~ 10.5) at neutral pH. As we have previously reported, emulsions flocculated upon addition of saliva [28, 30], which enhanced the viscosity, storage- and loss moduli. The increase of these parameters by saliva, presented as the ratios η_{mix}/η_{emul}, G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul}, can be of relevance for understanding the oral perception of liquid emulsions. Identification of measurable physical properties to predict perceived texture and mouth-feel has been the focus of several studies. A number of attempts has been made to establish correlations between sensory and rheological properties as well as to determine the exact conditions, e.g. shear stress and shear rate, in the mouth [19-21, 47]. It is generally accepted that viscosity enhancement plays an important role in oral sensory perception of fluid and semi-solid foods [48, 49]. Our study shows that saliva generally affects the viscoelasticity of the emulsions upon mixing by mainly increasing the elastic component, i.e. G'_{mix}/G'_{emul} is larger than G''_{mix}/G''_{emul}. Correlations between increased storage modulus, loss modulus and viscosity with sensory perception of emulsions are beyond the scope of this chapter. Nevertheless, our results indicate the importance of including the contribution of saliva to emulsion rheology when attempting to clarify the relation between emulsion properties and sensory perception.

One of the main factors affecting emulsion rheology is the volume fraction of the dispersed phase. Several studies demonstrated the effect of volume fraction by illustrating the relationship between the emulsion relative viscosity and ϕ (up to 0.6-0.8) or by reporting the influence of ϕ on the stability of a flocculated emulsion [50-52]. In non-flocculating diluted emulsions, the viscosity can be theoretically calculated by using, for example, the

Batchelor equation [53], where the viscosity of the emulsion depends on the viscosity of the continuous phase and the oil-volume fraction of the droplets (up to $\phi = 0.2$) [36]. In flocculated emulsions, as obtained by the addition of salivary components, the volume fraction of the flocculated droplets is higher than ϕ, since the continuous phase is also included in the floc structure. These emulsions exhibit a higher viscosity and a shear-thinning behavior [28, 51, 54]. As expected from literature, raising the oil content up to 10% w/w increases the ratios η_{mix}/η_{emul}, G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul} as a consequence of the enhanced saliva-induced flocculation occurring in the system.

The addition of polymers to the continuous phase and its effect on the stability of emulsions is extensively discussed in the emulsion literature in relation to creaming [51, 54-56], flocculation [57-59] and rheological characteristics [32, 60]. By modulating the concentration of added polymer, which induces changes in the internal structure of the emulsions, the interaction between the droplets can be controlled. Although, in some cases, the experiments have been carried out at different emulsion volume fractions, it has been generally observed that viscosity, shear stress, storage- and loss moduli increase with increasing polymer concentration [32, 60, 61].

In view of its composition, saliva can be considered as a colloidal dispersion of highly structured biopolymers, e.g. salivary mucins, and micelles. Secreted salivary mucins, MUC5B and MUC7, can be considered as negatively charged polysaccharides, since, as reported by Zalewska [62], 40-80% of the mass of such mucins consist of O-linked oligosaccharides. Sialic acid and sulphate groups, as well as aspartic and glutamic acids, provide the molecule with a negative charge [62]. The effect of salivary protein content was investigated in emulsion/saliva mixtures at 2.5% w/w and 10% w/w oil content. Similarly to the oil content observations and in line with results reported in literature for other polysaccharides, rising the SPs concentration increased η_{mix}/η_{emul}, G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul}. In view of the fact that a liquid emulsion remains in the mouth generally less than 10 seconds before being swallowed, it is likely that the emulsions are mixed with saliva in a heterogeneous way. Therefore *in vivo,* the physical-chemical features of a mixture may depend on the relative amounts of both saliva and oil and may vary with the oral movements which may cause inhomogeneous mixing of the sample. We anticipate that the combination of both these factors, i.e. salivary protein concentration as well as oil content, might be of extreme relevance for understanding the real behavior of an emulsion in the mouth.

As saliva is secreted by different glands and its properties are affected by stimulation, we compared the effect of stimulated saliva, obtained by chewing on a piece of parafilm, with that of unstimulated saliva. Saliva stimulation influences the protein composition, in particular the type of secreted proteins and their concentration [1, 17, 63]. Unstimulated saliva is composed of several proteins including α-amylase, serum albumin, immunoglobulin and mucin, which make up for 20-30% of the protein content [64]. Stimulated saliva contains, among others, α-amylase, proline-rich protein and a lower amount of mucin (MUC5B and MUC7). MUC7 was found in whole stimulated saliva and parotid saliva as one of the components of the salivary micelles, together with lysozyme, lactoferrin, α-amylase, glycosilated prolin-rich protein [65]. Salivary micelles are globular structures with sizes in the range 40-500 nm [9, 66] with negative surface potential at physiological pH [66, 67].

As observed in Figure 5, stimulated saliva induced lower viscosity in β-lg stabilized emulsion/saliva mixtures than unstimulated saliva. This is in line with the previous hypothesis [28] that mucins were causing the flocculation of β-lg emulsions by depletion. In fact the concentration of salivary mucins is lower in stimulated saliva than in unstimulated saliva, since up to 80% of saliva during stimulation is secreted by the parotid glands. The observed flocculation of β-lg stabilized emulsions upon mixing with stimulated saliva is likely induced by both salivary mucins and salivary micelles, as previously suggested [28].

In case of lysozyme stabilized emulsions, we showed in Chapter 3 that complex formation between emulsion and salivary proteins was responsible for the flocculation of these emulsions [31]. In particular, MUC5B is likely to play a role in this process, since pig gastric mucin, which is often used as model for the MUC5B, has shown strong interaction with cationic macromolecules such as gelatins and chitosan [68]. Besides MUC5B, other salivary proteins could also interact with the positively charged droplets, explaining therefore the small difference observed when the two different types of saliva were used. The relative amount and type of proteins produced with the secretion of stimulated saliva might strengthen the complex formation between saliva and lysozyme stabilized emulsion droplets and this could explain the observed augment in G'_{mix}/G'_{emul} and G''_{mix}/G''_{emul} and the small effect on the η_{mix}/η_{emul}.

Conclusions

This study aimed to elucidate the effect of several parameters, i.e. oil-volume fraction, salivary protein concentration and type of saliva, on the rheological properties of saliva-induced flocculated emulsions. We showed that saliva increased emulsion viscosity, storage- and loss moduli. In particular, a larger increase in the storage modulus was observed in both β-lg and lysozyme stabilized emulsions upon mixing with unstimulated saliva, compared to loss modulus. Moreover, increasing the oil-volume fraction and the amount of salivary proteins increased the viscosity, storage- and loss moduli. Lastly, the effect of the type of saliva was analyzed. The results indicated that stimulated saliva causes a smaller increase of the viscosity of β-lg stabilized emulsion compared to unstimulated saliva. A decrease in storage modulus and loss modulus was observed upon mixing stimulated saliva with β-lg stabilized emulsions. In case of the lysozyme stabilized emulsion/saliva mixtures, compared to unstimulated, stimulated saliva increased the storage- and loss moduli, but did not significantly affect the viscosity of this mixture.

With this study, we demonstrated that saliva has a great influence on emulsion properties and that the rheological behavior is determined by both emulsion properties and saliva characteristics. Therefore, we advise to include the contribution of saliva in studies which aim to understand the oral perception of food emulsions.

Acknowledgments

The authors would like to thank Catia Da Silva and Eefjan Timmerman (TNO, Zeist, The Netherlands) for their assistance with the measurements.

References

1. van Nieuw Amerongen, A. and Veerman, E.C.I., *Saliva - the defender of the oral cavity.* Oral Diseases, 2002. **8**: p. 12.
2. Schwartz, W.H., *The rheology of saliva.* Journal of Dental Research, 1987. **66**: p. 660.
3. Waterman, H.A., Blom, C., Holterman, H.J., 's-Gravenmade, E.J., and Mellema, J., *Rheological properties of human saliva.* Archives of Oral Biology, 1988. **33**: p. 589.
4. Vissink, A., Waterman, H.A., 's-Gravenmade, E.J., Panders, A.K., and Vermey, A., *Rheological properties of saliva substitutes containing mucin, carboxymethylcellulose or polyethylenoxide.* Journal of Oral Pathology, 1984. **13**: p. 22.
5. Hu, S., Loo, J.A., and Wong, D.T., *Human saliva proteome analysis*. Annals of the New York Academy of Sciences, 2007. **1098**: p. 323
6. Raynal, B.D.E., Hardingham, T.E., Thornton, D.J., and Sheehan, J.K., *Concentrated solutions of salivary MUC5B mucin do not replicate the gel-forming properties of saliva.* Biochemical Journal, 2002. **362**: p. 289.
7. Slomiany, B.L., Murty, V.L.N., Piotrowski, J., and Slomiany, A., *Salivary mucins in oral mucosal defense.* General Pharmacology, 1996. **27**: p. 761.
8. van Nieuw Amerongen, A., Bolscher, J.G.M., and Veerman, E.C.I., *Salivary mucins: Protective functions in relation to their diversity.* Glycobiology, 1995. **5**: p. 733.
9. Rykke, M., Smistad, G., Rolla, G., and Karlsen, J., *Micelle-like structures in human saliva.* Colloids and Surfaces B: Biointerfaces, 1995. **4**: p. 33.
10. Rykke, M., Devold, T.G., Smistad, G., and Vegarud, G.E., *Comparison of bovine casein micelles and human salivary micelle-like structures.* International Dairy Journal, 1999. **9**: p. 365.
11. Young, A., Rykke, M., and Rølla, G., *Quantitative and qualitative analyses of human salivary micelle-like globules.* Acta Odontologica Scandinavica, 1999. **57**: p. 105.
12. Glantz, P.O., Friberg, S.E., Wirth, S.M., and Baier, R.E., *Thin-section transmission electron microscopy of human saliva.* Acta Odontologica Scandinavica, 1989. **47**: p. 111.
13. Glantz, P.O., Natiella, J.R., Vaughan, C.D., and Baier, R.E., *Structural studies of human saliva.* Acta Odontologica Scandinavica, 1989. **47**: p. 17.
14. Glantz, P.O., *Interfacial phenomena in the oral cavity.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 1997. **123-124**: p. 657.
15. van der Reijden, W.A., Veerman, E.C.I., and van Nieuw Amerongen, A., *Shear rate dependent viscoelastic behaviour of human glandular salivas.* Biorheology, 1993. **30**: p. 141.
16. Veerman, E.C.I., van den Keybus, P.A.M., Vissink, A., and van Nieuw Amerongen, A., *Human glandular salivas: Their separate collection and analysis.* European Journal of Oral Science, 1996. **104**: p. 346.
17. Humphrey, S.P. and Williamson, R.T., *A review of saliva: Normal composition, flow and function.* Journal of Prosthetic Dentistry, 2001. **85**: p. 162.
18. Veerman, E.C.I., Valentijn-Benz, M., and van Nieuw Amerongen, A., *Viscosity of human salivary mucins: Effect of pH and ionic strength and role of sialic acid.* Journal de Biologie Buccale, 1989: p. 297.
19. Morris, E.R., *Polysaccharide rheology and in-mouth perception (chapter 16)*, in *Food polysaccharides and their applications*, A.M. Stephen, Editor. 1995, Marcel Dekker, Inc.: New York. p. 517.
20. Kokini, J.L., Kadane, J.B., and Cussler, E.L., *Liquid texture perceived in the mouth.* Journal of Texture Studies, 1977. **8**: p. 195.
21. Terpstra, M., Janssen, A.M., Prinz, J.F., de Wijk, R.A., Weenen, H., and van der Linden, E., *Modeling the thickness for semisolid food.* Journal of Texture Studies, 2005. **36**: p. 213.
22. Malone, M.E., Appelqvist, I.A.M., and Norton, I.T., *Oral behaviour of food hydrocolloids and emulsions. Part 1. Lubrication and deposition considerations.* Food Hydrocolloids, 2003. **17**: p. 763.
23. Malone, M.E., Appelqvist, I.A.M., and Norton, I.T., *Oral behaviour of food hydrocolloids and emulsions. Part 2. Taste and aroma release.* Food Hydrocolloids, 2003. **17**: p. 775.
24. Engelen, L., de Wijk, R.A., Prinz, J.F., and Bosman, F., *The relation between saliva flow after different stimulations and the perception of flavor and texture attributes in custard desserts.* Physiology & Behavior, 2003. **78**: p. 165.
25. de Roos, K.B., *Effect of texture and microstructure on flavour retention and release.* International Dairy Journal, 2003. **13**: p. 593.

26. Szcenesniak, A. and Farkas, E., *Objective characterization of the mouth-feel of gum solutions.* Journal of Food Science, 1962. **27**: p. 381.
27. Richardson, R.K., Morris, E.R., Ross-Murphy, S.B., Taylor, L.J., and Dea, I.C.M., *Characterization of the perceived texture of thickened systems by dynamic viscosity measurements.* Food Hydrocolloids, 1989. **3**: p. 175.
28. Vingerhoeds, M.H., Blijdenstein, T.B.J., Zoet, F., and van Aken, G.A., *Emulsion flocculation induced by saliva and mucin.* Food Hydrocolloids, 2005. **19**: p. 915.
29. van Aken, G.A., Vingerhoeds, M.H., and de Hoog, E.H.A., *Colloidal behaviour of food emulsions under oral conditions*, in *Food colloids 2004: Interactions, microstructure and processing*, E. Dickinson, Editor. 2005, The Royal Society of Chemistry: Cambridge. p. 356.
30. Silletti, E., Vingerhoeds, M.H., Norde, W., and van Aken, G.A., *The role of electrostatics in saliva-induced emulsion flocculation.* Food Hydrocolloids, 2007. **21**: p. 596.
31. Silletti, E., Vingerhoeds, M.H., Norde, W., and van Aken, G.A., *Complex formation in mixtures of lysozyme stabilized emulsion s and human saliva.* Journal of Colloid and Interface Science, 2007. **313**: p. 485.
32. Ibanoglu, E., *Rheological behaviour of whey protein stabilized emulsions in the presence of gum arabic.* Journal of Food Engineering, 2002. **52**: p. 273.
33. Dickinson, E. and Golding, M., *Influence of calcium ions on creaming and rheology of emulsions containing sodium caseinate.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 1998. **144**: p. 167.
34. Blijdenstein, T.B.J., van Winden, A.J.M., van Vliet, T., van der Linden, E., and van Aken, G.A., *Serum separation and structure of depletion- and bridging-flocculated emulsions: A comparison.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2004. **245**: p. 41.
35. Tadros, T., *Application of rheology for assessment and prediction of the long-term physical stability of emulsions.* Advances in Colloid and Interface Science, 2004. **108-109**: p. 227.
36. Tadros, T.H., *Fundamental principles of emulsion rheology and their applications.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 1994. **91**: p. 39.
37. Dickinson, E., *Milk protein interfacial layers and the relationship to emulsion stability and rheology.* Colloids and Surfaces B: Biointerfaces, 2001. **20**: p. 197.
38. Dickinson, E., Golding, M., and Povey, M.J.W., *Creaming and flocculation of oil-in-water emulsions containing sodium caseinate.* Journal of Colloid and Interface Science, 1997. **185**: p. 515.
39. de Jongh, H.H.J., Groneveld, T., and de Groot, J., *Mild isolation procedure discloses new protein structural properties of beta-lactoglobulin.* Journal of Dairy Science, 2001. **84**: p. 562.
40. Schipper, R., Loof, A., de Groot, J., Harthoorn, L., Dransfield, E., and van Heerde, W., *SELDI-TOF-MS of saliva: Methodology and pre-treatment effects.* Journal of Chromatography B, 2007. **847**: p. 45.
41. Undar, A., *Effect of hypothermic cardiopulmonary bypass on blood viscoelasticity in pediatric cardiac patients.* ASAIO Journal, 2005. **51**: p. 522.
42. Henderson, N.M. and Thurston, G.B., *A new method for the analysis of blood and plasma coagulation.* Biomedical Sciences Instrumentation, 1993. **29**: p. 95.
43. Thurston, G.B., *The elastic yield stress of human blood.* Biomedical Sciences Instrumentation, 1993. **29**: p. 87.
44. Thurston, G.B., *Theory of oscillation of a viscoelastic fluid in a circular tube.* Journal of the Acoustical Society of America, 1969. **32**: p. 210.
45. Thurston, G.B., *The viscosity and viscoelasticity of blood in small diameter tubes.* Microvascular Research, 1976. **11**: p. 133.
46. Nordbö, H., Darwish, S., and Bhatnagar, R.S., *Rate of viscosity changes in five salivary protein fractions following pH alterations.* Scandinavian Journal of Dental Research, 1984. **92**: p. 302.
47. Kokini, J.L., *The physical basis of liquid food texture and texture-taste interactions.* Journal of Food Enginering, 1987. **6**: p. 51.
48. Kokini, J.L. and van Aken, G.A., *Discussion session on food emulsions and foams.* Food Hydrocolloids, 2006. **20**: p. 438.
49. Akhtar, M., Stenzel, J., Murray, B.S., and Dickinson, E., *Factors affecting the perception of creaminess of oil-in-water emulsions.* Food Hydrocolloids, 2005. **19**: p. 521.
50. Dickinson, E., Goller, M.I., and Wedlock, D.J., *Osmotic pressure, creaming, and rheology of emulsions containing non ionic polysaccharides.* Journal of Colloid and Interface Science, 1995. **172**: p. 192.
51. Tuinier, R. and de Kruif, C.G., *Phase separation, creaming, and network formation of oil-in-water emulsions induced by an exocellular polysaccharide.* Journal of Colloid and Interface Science, 1999. **218**: p. 201.

52. Starov, V.M. and Zhdanov, V.G., *Viscosity of emulsions: Influence of flocculation.* Journal of Colloid and Interface Science, 2003. **258**: p. 404.
53. Batchelor, G.K., *Effect of brownian-motion on bulk stress in a suspension of spherical-particles.* Journal of Fluid Mechanics, 1977. **83**: p. 97.
54. Dickinson, E., Semenova, M.G., Antipova, A.S., and Pelan, E.G., *Effect of high-methoxy pectin on properties of casein stabilized emulsion s.* Food Hydrocolloids, 1998. **12**: p. 425.
55. ten Grotenhuis, E., Paques, M., and van Aken, G.A., *The application of diffusing-wave spectroscopy to monitor the phase behavior of emulsion-polysaccharide systems.* Journal of Colloid and Interface Science, 2000. **227**: p. 495.
56. Gu, Y.S., Decker, E.A., and McClements, D.J., *Influence of pH and carrageenan type on properties of β-lactoglobulin stabilized oil-in-water emulsions.* Food Hydrocolloids, 2005. **19**: p. 83.
57. Euston, S.R., Finnigan, S.R., and Hirst, R.L., *Kinetics of droplet aggregation in heated whey protein stabilized emulsions: Effect of polysaccharides.* Food Hydrocolloids, 2002. **16**: p. 499.
58. Blijdenstein, T.B.J., Zoet, F.D., van Vliet, T., van der Linden, E., and van Aken, G.A., *Dextran-induced depletion flocculation in oil-in-water emulsions in the presence of sucrose.* Food Hydrocolloids, 2004. **18**: p. 857.
59. Blijdenstein, T.B.J., Veerman, C., and van der Linden, E., *Depletion-flocculation in oil-in-water emulsions using fibrillar protein assemblies.* Langmuir, 2004. **20**: p. 4881.
60. Manoj, P., Fillery-Travis, A.J., Watson, A.D., Hibberd, D.J., and Robins, M.M., *Characterization of a polydisperse depletion-flocculated emulsion.* Journal of Colloid and Interface Science, 2000. **228**: p. 200.
61. Dickinson, E., *Stability and rheological implications of electrostatic milk protein-polysaccharide interactions.* Trends in Food Science & Technology, 1998. **9**: p. 347.
62. Zalewska, A., Zwierz, K., Zólkowski, K., and Gindzienski, A., *Structure and biosynthesis of human salivary mucins.* Acta Biochimica Polonica, 2000. **47**: p. 1067.
63. Bardow, A., Nyvad, B., and Nauntofte, B., *Relationship between medication intake, complains of dry mouth, salivary flow rate and composition, and the rate of tooth demineralization in situ.* Archives of Oral Biology, 2001. **46**: p. 413.
64. Fox, P.C., Bodner, L., Tabak, L.A., and Levine, M.J., *Quantitation of total human salivary mucins.* Journal of Dental Research, 1985. **64**: p. 327.
65. Soares, R.V., Lin, T., Siqueira, C.C., Bruno, L.S., Li, X., Oppenheim, F.G., Offner, G.D., and Troxler, R.F., *Salivary micelles: Identification of complexes containing MG2, sIgA, lactoferrin, amylase, glycolsylated proline-rich protein and lysozyme.* Archives of Oral Biology, 2004. **49**: p. 337.
66. Rykke, M., Young, A., Rølla, G., Devold, T., and Smistad, G., *Transmission electron microscopy of human saliva.* Colloids and Surfaces B: Biointerfaces, 1997. **9**: p. 257.
67. Rykke, M., Young, A., Smistad, G., Rølla, G., and Karlsen, J., *Zeta potentials of human salivary micelle-like particles.* Colloids and Surfaces B: Biointerfaces, 1996. **6**: p. 51.
68. Hassan, E.E. and Gallo, J.M., *A simple rheological method for the in vitro assessment of mucin-polymer bioadhesive bond strength.* Pharmaceutical Research, 1990. **7**: p. 491.

Chapter 5

Analysis and identification of salivary peptides interacting with lysozyme and β-lactoglobulin stabilized emulsions by SELDI-TOF-MS and RP-HPLC coupled to MS-TOF-TOF

E. Silletti, R.M.P. Vitorino, J. de Groot, M.H. Vingerhoeds and F.M.L. Amado

Submitted

Abstract

In this chapter, we used two proteomics techniques, i.e. SELDI-TOF-MS and RP-HPLC coupled with MS, to study the interaction between emulsions and salivary proteins and peptides (SPs) of human unstimulated saliva in the molecular mass range between 800 and 20000 Da. This set-up combines the advantage of a rapid output and a general overview of the data provided by SELDI-TOF-MS with the identification and sequence characterization provided by RP-HPLC-MS. Lysozyme and β-lactoglobulin (β-lg) stabilized emulsions were mixed with saliva to obtain a final mixture containing 10% w/w emulsion oil content and 0.6 mg mL^{-1} SPs. Salivary components associated with emulsions droplets were recovered after treatment with a solution of 2% SDS and were subsequently analyzed. A total of 60 and 119 different SPs have been detected in the fractions associated with droplets of lysozyme and β-lg stabilized emulsions, respectively. 78 SPs were identified with RP-HPLC-MS alone in the sample fractions associated with both emulsion droplets. Hydrophilicity and hydrophobicity seems to play a role in the SPs < *m/z* 2000 that associated with lysozyme and β-lg stabilized emulsions, respectively. Moreover phosphorylated SPs have been found predominantly to associate with β-lg stabilized emulsions in the whole studied *m/z* range. Our results suggest that the adsorption/association of SPs onto the droplets surface is partially driven by specific interaction of SPs with exposed sequences of the emulsifier molecule at the emulsion oil-water interfaces.

Introduction

Human saliva is mainly constituted, with respect to protein composition, of glycoproteins (e.g., mucins, proline-rich glycoproteins) [1], enzymes (e.g. α-amylase, carbonic anhydrase) and a wide range of small proteins and peptides (e.g. cystatins, statherin, histatins, proline-rich proteins) which account for more than 75% in weight of the total salivary protein content [2-5]. Salivary proteins present an array of posttranslational modifications (PTM), including glycosylation and phosphorylation [6-10]. Increasing evidence suggests that proteolysis can be considered a common PTM of salivary proteins occurring prior to or during secretion [11, 12]. Most salivary proteins with molecular mass (M_r) < 40 kDa are posttranslationally cleaved into smaller fragments [13]. Proteolysis can be either complete, leaving no trace of the parent protein or partial, yielding a mixture of intact and cleavage products [14]. Examples of proteolytic activity as PTM include the members of the family of histatins, statherins [5, 15] and proline-rich proteins (PRP) [16]. In case of PRP, the complete cleavage of the product of the gene PRB1, PRB2 and PRB4 originates, for example, basic proline-rich peptide as P-D, P-E, P-F, II-2, to name a few. Therefore, it is not surprising that the low-molecular-weight fraction of human saliva is rich in peptides [11, 12, 16, 17]. Many of these cleavage products maintain their important biological functions, such as the antifungal activities of histatins [3]. The biological significance of proteolytic protein processing could be to increase the diversity of salivary proteins without the need for additional genes encoding for such proteins [13].

Proteins and peptides in saliva have been partially characterized by conventional biochemical strategies focused on individual molecules or specific groups of proteins [18-21]. A promising approach rapidly gaining interest is the analysis of human saliva using proteomic techniques. The exploration of the salivary proteome improved knowledge on oral physiology and -compositions, e.g. acquired enamel pellicle [22] and allowed detection and identification of novel proteins as well as the examination of changes in protein levels under different conditions [23, 24]. Several proteomic techniques are nowadays available, e.g., two-dimensional gel electrophoresis (2DE) followed by mass spectrometry (MS) [25-28] and the shotgun approach of digested proteins, where the peptides are separated by liquid chromatography (LC) and identified using MS [29]. LC methods coupled to MS/MS are particularly suitable for separation and identification of low-molecular-weight components and peptides [12, 16, 30-32]. Many small salivary proteins in the range 1-20

kDa, such as PRPs, statherin, histatins, and defensins were only identified by shotgun, whereas the smallest proteins identified by 2DE-MS were cystatins and immunoglobulin chains with a M_r equal or higher than 10 kDa.

A decade ago, surface enhanced laser desorption/ionization time-of-flight mass spectroscopy (SELDI-TOF-MS) has been developed [33]. This new proteomic technology combines matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) with surface chromatography utilizing sample chips displaying various chemically enriched and active surfaces to bind protein molecules. The technique enables rapid and high-throughput detection of proteins and peptides directly from crude mixtures without labor-intensive pre-processing. For this reason, SELDI-TOF-MS has been used in a large number of studies related to diagnostic screening, biomarkers discovery in clinical proteomics researches [34-38] as well as for studying protein-protein interaction [39] and food protein modifications [40, 41]. Recently, it has also been applied in saliva research in relation to the effects of sample treatment, e.g. storage condition, or for the discovery of biomarkers for Sjögren's syndrome [42-44].

In the last few years, it is becoming evident that knowledge on the interaction between saliva and food during oral processing is important to understand human response to food, including perception and consumption, as well as for example, perceived satiety [45]. Several authors reported the influence of saliva properties, such as flow, composition and lubrication, on sensory perception and flavour release [46-50]. However, most of the sensory studies addressing emulsion perception do not take into account the in-mouth role of saliva to support their results. In this context, proteomics approach is an extreme powerful tool to investigate protein-protein interaction occurring between food emulsions and human saliva.

Recently, it has been shown that emulsion droplets stabilized, for example, by chicken egg-white lysozyme and bovine β-lactoglobulin (β-lg), flocculated upon mixing with human unstimulated saliva (Chapter 2) [51, 52]. Lysozyme is a 129 amino acid residues enzyme with a M_r of approximately 14.3 kDa constituting about 3% of the total chicken egg-white proteins content. It is a small ellipsoid molecule containing 18 amino acids (aa) with basic side chain (11 arginines, 6 lysines and 1 histidine) and 9 residues with acid side chain (7 aspartic acids, 2 glutamic acids). At physiological pH, due to the high isolectric point (pI ~10.5) [53, 54] lysozyme possesses a positive net charge. Bovine β-lg is the predominant whey protein in milk (up to 50% of the total amount of whey proteins) [55, 56] and

therefore largely used as ingredient in the food industry. It consists of 162 amino acid residues with a $M_r \sim 18.2$ kDa and a pI of about 4.9. The molecule contains 10 aspartic acids, 16 glutamic acids, 14 lysines, 2 arginines and 1 histidine and a free thiol group in position 121. Furthermore, β-lg contains a hydrophobic cavity able to bind hydrophobic compounds such as retinol [57, 58] allowing, for example, the transfer of vitamin A from the maternal milk to the neonate.

A sensory investigation conducted on food emulsions stabilized by chicken egg-white lysozyme and whey proteins indicated that saliva-induced flocculation has a large impact on perception (Chapter 7) [59]. In particular, lysozyme stabilized emulsions were found to be related to attributes such dryness, roughness and astringency. According to literature, astringency has been related, in tannin-containing food products, to complex formation and precipitation of α-amylase, histatins and PRPs with tannins [60-67]. Although astringency has been also reported in studies evaluating sensory properties of dried food ingredients such as whey and soy proteins at high protein content [68-70], it has never been described in case of whey protein stabilized emulsions.

In chapter 3, using confocal laser scanning microscopy, we demonstrated complex formation between salivary proteins and chicken egg-white lysozyme molecules in aqueous solution as well as lysozyme adsorbed onto the oil-droplets surfaces, which led to the above-mentioned emulsion flocculation [71]. Therefore, our first goal of the current study was to identify salivary proteins and peptides (SPs) interacting (or associating) with lysozyme stabilized emulsions droplets by using both SELDI-TOF-MS and reverse-phase high performance liquid chromatography (RP-HPLC) couple to MS. Secondly, we studied the SPs associated to β-lg stabilized emulsion droplets in order to better understand flocculation behavior and sensory perception of these two emulsions.

Materials and methods

Materials

Freeze-dried β-lactoglobulin (β-lg) was provided by TI Food and Nutrition (TIFN, Wageningen, The Netherlands) and was purified according to the previously described method [72]. Lysozyme from chicken egg-white was kindly donated from Belovo (B1378, Belovo, Bastogne, Belgium) while sunflower oil (Reddy, Vandemoortele, The Netherlands) was purchased from a local retailer. BCA™ Protein Assay Kit was from Pierce

Biotechnology Inc. (Rockford, IL, USA), RCDC Protein Assay from Bio-Rad (500-0121, Bio-Rad Laboratories, USA), sodium azide and SDS were obtained from Merck (Shuchardt, Germany). Proteases inhibitor (Proteases inhibitor Cocktail P8340-5mL, 096K4130) was purchased from Sigma (Sigma, St Louis, MI, USA), sinapinic acid (SPA) from Bio-Rad (C70-00067, Bio-Rad Laboratories, USA). HPLC-grade acetonitrile (ACN) from Riedel (Riedel, Seelze, Germany) and trifluoroacetic acid (TFA) was from Fluka (Fluka, Buchs, Switzerland). Trypsin was bought from Promega (Madison, WI, USA). The calibrant mixtures for SELDI ProteinChip All-in-One Peptide Standard and ProteinChip All-in-One Protein Standard II were obtained from Bio-Rad (C10-00005 and C10-00007, Bio-Rad Laboratories, Nazareth Eke, Belgium). α-Cyano-4-hydroxycinnamic acid (α-CHCA) and the calibrant mixture for the 4800 Proteomics Analyser were from Applied Biosystems (Applied Biosystems, Foster City, CA, USA).

Collection and storage of saliva

Whole human unstimulated saliva was collected according to the procedure described in Chapter 2 [52]. Briefly, whole human unstimulated saliva was collected from 8.30 to 10.30 a.m. from 17 healthy non-medicated volunteers. After rinsing their mouths with water, saliva was collected with closed lips for a couple of minutes and expectorated into ice-chilled vessels. The samples were kept constantly on ice during both donation and handling. Saliva was centrifuged at 10000 g for 30 minutes at 4 ^{o}C to remove cellular debris and saliva supernatant was frozen in liquid nitrogen, stored at –80 ^{o}C. Salivary protein content, determined according to the BCA method of Pierce [73], varied from 1.1 mg mL^{-1} to 1.3 mg mL^{-1}.

Sample preparation

β-Lg and lysozyme stabilized emulsions, pH 6.7, contained 40% w/w and 20% w/w sunflower oil, respectively, 1% w/w protein, 10 mM NaCl and were prepared according to the procedure reported in Chapter 2 [52]. Sodium azide (0.02% w/w) was added to the emulsions to prevent microbial growth. Protein content in the emulsion continuous phase was determined with the BCA method after centrifugation of 10% w/w emulsion at 120000 g at 20 ^{o}C for 1 hour (Beckman LB-60 Ultracentrifuge, rotor SW 41, Beckman Coulter, Mijdrecht, The Netherlands).

Emulsion/saliva mixtures were prepared by mixing the required amount of emulsions with saliva, which was removed from –80 ^{o}C and thawed at room temperature shortly before each experiment. The SPs concentration and emulsion oil content in the mixtures were 0.6 mg mL^{-1} and 10% w/w, respectively. If necessary, the volume was adjusted by addition of a 10 mM NaCl solution. Proteases inhibitor was added to the mixtures to a final inhibitor concentration of 1 mM 4-(2-aminoethyl)benzene sulfonyl fluoride, 800 μM aprotinin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin and 14 μM E-64, according to Schipper et al [43]. Sample preparation is schematically represented in Figure 1. The mixtures were centrifuged at 15000 g for 30 minutes at 4 ^{o}C (2x) using a Beckman LB-60 Ultracentrifuge (rotor SW 41, Beckman Coulter, Mijdrecht, The Netherlands). After each centrifugation step, the aqueous phases containing proteins and peptides not associated with the emulsion droplets were collected and stored at -80 ^{o}C until further use. The emulsion oil droplets, present in the so-called cream layers at the top of the tube, were removed and gently suspended in 10 mM NaCl by using a Vortex Mix VM-300 (Gemmy Industrial Corporation, Taiwan) prior the following centrifugation step.

After a third centrifugation, which was carried out at 15000 g at 4 ^{o}C for 1 hour, the cream layer was suspended in a solution of 2% SDS and kept under gently agitation at room temperature for 48 hours before being centrifuged for the last time, at 120000 g at 20 ^{o}C for 1 hour. The aqueous phase with SPs associated with the emulsion droplets, indicated as PA (Proteins/peptides Adsorbed), was concentrated using Centriprep® Centrifugal Filter Units with a cut-off membrane of 3 kDa (4302, Milipore B.V., Amsterdam, the Netherlands) before further analysis, while the cream layer was discarded. Protein determination was performed, after each step, according to the BCA method and/or with RCDC protein method from Bio-Rad. Besides the concentration step, no further treatment was performed on the samples prior SELDI-TOF-MS. Extensive dialysis of sample PA followed by filtration with a Centricon 30 filter (30 kDa cut-off, Millipore USA) was performed, instead, prior to RP-HPLC analysis. Samples concentration after recovery was about 70 μg mL^{-1}. For comparison, only the aqueous phase after the first centrifugation step (PNA, Proteins/peptides Non-Adsorbed) was analyzed.

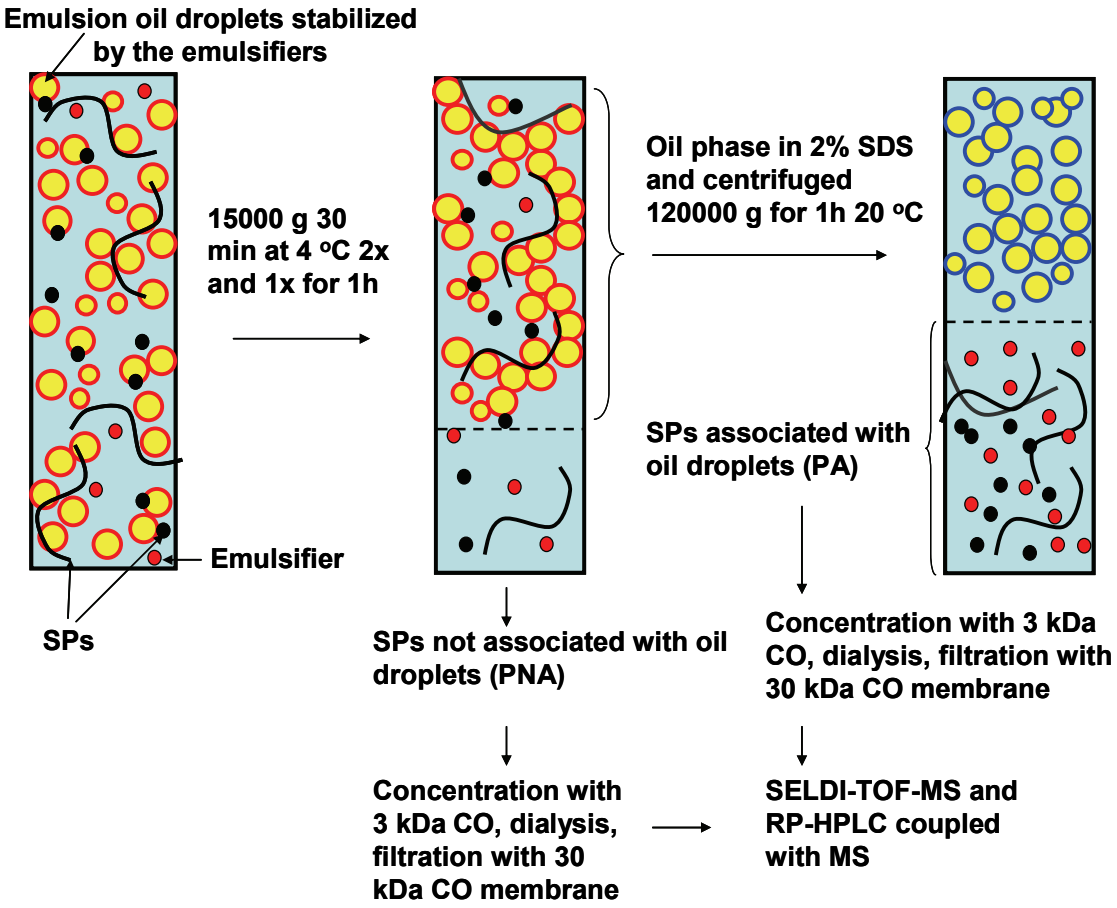

Figure 1 – Schematic representation of the experimental procedure for sample preparation and analysis with SELDI-TOF-MS and RP-HPLC coupled to MS.

SELDI-TOF-MS

SELDI-TOF MS analysis was carried out according to Schipper et al [43], with some minor modifications. Normal phase (NP20, C57-30043, Bio-Rad Laboratories, USA) and weak cation exchange (CM 10, C57-30075, Bio-Rad Laboratories, USA) chip surfaces were used. Samples were diluted to a concentration of 1 mg mL^{-1} with Milli Q water or binding buffer (BB) 0.1 mM ammonium acetate pH 4.5, containing 0.1% Triton-X100, prior being applied on NP20 and CM10, respectively. Before sample loading, NP20 was premoistened with Milli Q water while CM10 were washed 3 times (5 minutes each) with BB. 1 µL of diluted sample was applied directly onto NP20, while onto CM10 99 µL of BB was also added to the chips and incubated for 1 hour. NP20 sample spots were rinsed with 4 µl Milli Q water, while CM10 was washed once with BB, twice with BB without Triton-X100 and once

more with Milli Q water. After air-drying, 0.8 µl SPA saturated solution (SPA in 50% ACN/0.5% TFA) was added twice to the spots of both NP20 and CM10, and air-dried prior to reading on a Protein Chip Reader IIC instrument (Bio-Rad Laboratories, USA). Laser intensities of 160 and 165 were used for spectra acquisition in the *m/z* range 1-10000 and 5000-20000, respectively.

RP-HPLC

HPLC analyses were performed using an Ultimate 3000 (LcPackings). 20 µL of each sample (about 1.4 µg of SPs) was injected onto a C18 trapping column (Zorbax 300SB-C18, 5 µm particle size, 5 x 0.3 mm, Agilent Technologies, Santa Clara, CA, USA) using an autosampler. The sample was washed over the trapping column for 3 minutes with 95% buffer A (water, 0.1% TFA), 5% buffer B (ACN, 0.1% TFA) at a flow rate of 30 µl/min. Flow was then reversed over the trapping column, and the sample was eluted onto a 150 mm × 75 µm Zorbax 300SB capillary analytical C18 column with 3.5 µm particle size (Agilent Technologies, Santa Clara, CA, USA) at a flow rate of 0.3 µl/min. A linear gradient of 5% to 55% buffer B was run over a period of 35 minutes. The column was then washed with a 3-minutes gradient from 55% to 90% buffer B, followed by a 5-minutes hold at 90% buffer B. The column was re-equilibrated in 5% buffer B prior to future analyses. The separation was monitored at 214 nm using a UV detector (Dionex/LC Packings, Sunnyvale, CA) equipped with a 3 nL flow cell. The proteins and peptides eluting off the monolithic capillary column were deposited onto 384-well MALDI plates at 20 seconds intervals for each spot using Probot (Dionex/LC Packings) adding 270 *n*L of α-CHCA matrix solution, which was prepared by diluting saturated α-CHCA with 70% ACN/0.3% TFA with the addition of 15fmol of Glu-Fib as internal standard.

RP-HPLC Separation and On-MALDI Plate Enzymatic Digestion

LC separation of the SPs (1.4 µg) was performed as above-described with minor modifications. The proteins and peptides eluting off the monolithic capillary column were directly deposited onto the MALDI plates at 20 seconds intervals for each spot (100 nL/fraction), starting 5 minutes after the beginning of the separation process, mixed with a continuous flow of trypsin solution at 800 nL/min (0.1 µg mL^{-1} prepared in 50 mM NH_4HCO_3/ACN 10%) resulting on the deposition of a final volume of 370 nL per spot. In

total, for every separation run, 156 fractions were collected. Following sample collection, the plate was kept for 10 minutes at 37ºC temperature in a humid chamber (more than 90% of humidity) for digestion. Then, the plate was placed again in the Probot for matrix deposition under a continuous flow rate of 270 nL of α-CHCA matrix solution (2mg mL^{-1} in 70% acetonitrile/0.3% TFA and internal standard Glu-Fib at 15ftmol for MALDI-TOF/TOF MS analysis).

Mass spectrometry and database search

Two different mass spectrometry analysis were performed for the same LC-run: concerning the fraction of SPs < 5 kDa and on-plate digestion, peptide mass spectra were obtained with ca. 1500 laser shots on a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive ion reflector mode in the *m/z* range between 800 and 5000. For each sample spot, a data dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, trypsin autolysis, or acrylamide peaks, for subsequent MS/MS data acquisition. Trypsin autolysis peaks were used for internal calibration of the mass spectra, allowing a routine mass accuracy of better than 20 ppm.

Spectra were processed and analysed by the Global Protein Server Workstation (Applied Biosystems, Foster City, CA, USA), which uses internal Mascot (Matrix Science Ltd, U.K.) software for searching the peptide mass fingerprints and MS/MS data. Searches were performed against the Swiss-Prot non-redundant protein database with a tolerance of 200 ppm and 0.3 Da on the *m/z* from MS and MS/MS spectra, respectively.

Screening of SPs with M_r > 5 kDa was obtained in the linear mode with ca. 1200 laser shots on a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the *m/z* range between 800 and 20000.

Results

As a first approach to study the association of SPs to emulsion droplets, we performed SELDI-TOF-MS analysis using two different arrays, i.e NP20 and CM10. An overview of the $[MH]^+$ m/z peaks detected in the PA fraction is provided in Table 1.

Table 1 – Summary of the SPs associated with emulsion droplets of lysozyme and β-lg stabilized emulsions after mixing with saliva as determined by SELDI-TOF-MS $[MH]^+$ *m/z* in the range 2000-20000 with NP20 and CM10 protein chips. Identified components by MALDI-TOF-MS after tryptic digestion and data bank research are also reported. When identification was not possible, a tentative identity is proposed based on literature.

m/z	Lys	β-lg	Salivary components	SELDI	MALDI	Reference
2012.9	-	+	QGGNKPQGPPPPPGKPGGPPP from b PRP 1 [42-62], P-E [12-32]; b PRP 2 [29-49]		Yes	*
2028.7	-	+	GPPPPPGKPQGPPPQGGNKPQ from b PRP 1 [49-69], b PRP 2 [36-56]		Yes	*
2029.0	-	+	GPPPQGGNQPQGPPPPPGKPQ from b PRP 1 [99-119], P-E [8-28], b PRP 2 [25-45]		Yes	*
2087.9	-	+	GPPPPAGGNPQQPQAPPAGQPQ from b PRP 1 [345-366], b PRP 2 [355-376]		Yes	*
2162.2	-	+	HRP-5b	Yes		[74, 75]
2185.1	-	+	PGKPQGPPPQDKSRSPQS from b PRP 1 [257-275] with 3 phoshorylations		Yes	*
2342.6	-	+	HHGYKRKFHEKKHHSHRGY	Yes		[74, 75]
2367.7	-	+	PPGPAGPAGERGEQGPAGSPGFQGL from Collagen alpha 1(I) chain [621-645] with 1 phoshorylation		Yes	*
2367.7	-	+	PPPGKPQGPPPQGGDQSQGPPPPPG b PRP 4 [35-59]		Yes	*
2377.2	-	+	GPPPQGGNQPQGPPPPPGKPQGPPP from b PRP 1 [99-123], P-E [8-32], b PRP 2 [25-49]		Yes	*
2499.1	-	+	HRP-5a	Yes		[74]
2622.4	+	-	Histatin 1 precursor [37-57]	Yes	Yes #	*
2626.6	-	+	HRP-6	Yes		[74, 75]
2690.6	+	-	Unknown	Yes		
2880.6	+	-	Unknown	Yes		
3017.6	+	-	Collagen alpha 6(IV) chain precursor [237-265] with 1 oxidation and 2 phoshorylations	Yes	Yes #	*
3036.6	-	+	Histatin 5	Yes		[3, 12, 74-76]
3160.0	+	-	Unknown	Yes		
3255.8	-	+	Unknown	Yes		
3347.9	+	-	Unknown	Yes		
3371.9	+	-	Unknown	Yes		

Table 1 Continued

3448.0	+	+	α-defensin 1	Yes		[31]
3651.3	+	-	Unknown	Yes		
3782.8	-	+	Unknown	Yes		
3931.7	-	+	Collagen alpha 1(XVII) chain precursor [1107-1140] with 1 oxidation and 6 phoshorylations	Yes	Yes [#]	*
4081.8	+	-	Unknown	Yes		
4129.3	+	-	Statherin	Yes		[5]
4258.4	-	+	Unknown	Yes		
4377.7	+	+	P-C	Yes	Yes [#]	*
4550.4	-	+	Unknown	Yes		
4928.3	-	+	Histatin 1	Yes	Yes [#]	*
5006.6	-	+	Histatin 1 with 1 sulphation		Yes	*
5082.1	-	+	Histatin 1 with 2 sulphations		Yes	*
5117.7	+	-	Unknown	Yes		
5140.3	+	-	Unknown	Yes		
5203.6	-	+	Unknown	Yes		
5218.7	+	-	Unknown	Yes		
5253.4	+	-	Statherin isoforms [a]	Yes		[77]
5261.2	+	+	Cyclo-statherin Q-37		Yes	*
5271.9	+	-	Unknown	Yes		
5365.3	+	+	Unknown	Yes	Yes [#]	
5377.6	-	+	Statherin	Yes	Yes [#]	*
5387.4	+	-	Unknown	Yes	Yes [#]	
5409.3	+	-	Unknown	Yes		
5514.9	-	+	Unknown	Yes		
5560.0	-	+	Unknown	Yes		
5568.3	+	-	Unknown	Yes		
5578.8	+	-	Unknown	Yes		
5590.5	+	+	P-H		Yes	*
5672.4	-	+	Granulin-5	Yes	Yes [#]	[77]
5742.1	-	+	Unknown	Yes		
5782.5	+	-	Unknown	Yes		
5796.3	-	+	P-B		Yes	[16]
5844.8	+	+	P-F		Yes	*
5944.6	+	+	P-J		Yes	*

6006.4	-	+	Unknown	Yes		
6024.0	+	+	P-E		Yes	*
6112.9	-	+	P-E with 1 phoshorylation		Yes	*
6289.1	+	+	Unknown	Yes		
6510.7	+	-	Unknown	Yes		
6641.2	-	+	Unknown	Yes	Yes [#]	
6868.3	-	+	Unknown	Yes	Yes [#]	
6951.5	+	+	P-D		Yes	*
6999.0	-	+	Unknown	Yes		
7078.3	+	+	Unknown	Yes	Yes [#]	
7147.4	+	-	Unknown	Yes	Yes [#]	
7165.6	-	+	Neutrophil peptide 3 precursor	Yes		[29]
7245.9	+	-	Unknown	Yes		
7420.7	+	-	Small proline-rich protein 2E	Yes		[78]
7609.3	+	+	II-2		Yes	*
7714.7	-	+	Unknown	Yes		
7729.9	+	-	Unknown	Yes		
7737.1	+	-	Unknown	Yes		
8056.0	-	+	Unknown	Yes		
8107.7	-	+	Neutrophil activating peptide	Yes	Yes [#]	[45]
8266.2	-	+	Homodomain-only protein	Yes		[78]
8503.4	-	+	Unknown	Yes		
8757.7	-	+	Unknown	Yes		
8906.5	-	+	Unknown	Yes		
9147.3	-	+	Unknown	Yes		
9253.5	-	+	Unknown	Yes		
9509.5	-	+	IB 1		Yes	*
9678.4	+	-	Unknown	Yes		
9721.0	-	+	Unknown	Yes		
9756.5	+	+	Unknown	Yes		
9919.7	-	+	Gene c-abl (fragment) or Acyl-CoA-binding protein	Yes	Yes [#]	[29, 78]
10381.3	-	+	Unknown	Yes		
10392.8	-	+	S-100 protein P	Yes		[29]
10600.8	-	+	Unknown	Yes		
10761.5	-	+	Unknown	Yes		
10861.5	-	+	Unknown	Yes		

Table 1 Continued

10954.4	+	+	Unknown	Yes		
11009.5	-	+	a PRP 3		Yes	*
11031.6	+	-	Unknown	Yes		
11075.7	-	+	a PRP 3 with 1 phoshorylation		Yes	*
11109.8	+	-	Unknown	Yes	Yes [#]	
11163.9	-	+	a PRP 3 with 2 phoshorylations		Yes	*
11316.5	+	-	S100 calcium-binding protein A7	Yes		[78]
11453.9	-	+	Unknown	Yes		
11465.0	-	+	Unknown	Yes		
11546.5	+	-	Unknown	Yes		
11667.2	-	+	S100 calcium-binding protein A14 or B PRP 1	Yes		[77, 78]
11673.7	-	+	Unknown	Yes		
11836.5	-	+	Unknown	Yes		
12553.6	+	-	Unknown	Yes		
13409.0	-	+	Cystatin S with 2 phoshorylations		Yes	*
13594.9	+	-	Unknown	Yes		
13615.9	-	+	Unknown	Yes		
13667.5	-	+	Histidin triad nucleotide-binding protein 1	Yes		[78]
14207.8	-	+	Unknown	Yes		
14260.0	+	-	Chicken egg-white lysozyme	Yes		
14610.8	-	+	CCP1 14600	Yes		[79]
14697.3	-	+	Human lysozyme with 2 phoshorylations		Yes	
15431.7	+	-	a PRP 1, PIF-s	Yes		[10]
15443.1	+	-	Unknown	Yes		[29]
15522.3	-	+	a PRP 1 with 2 phoshorylations		Yes	*
15650.3	-	+	Unknown	Yes		
18258.0	-	+	β-lactoglobulin	Yes		
19158.4	-	+	Unknown	Yes		

+ and - indicate the presence or absence of the peak in the sample, respectively; a: acidic; b: basic; [ ] indicates the position in the protein precursor amino acidic sequence; [a] Statherin isoform corresponds to statherin sequence with a cleavage of one aspartic acid residue on N-terminal; * *This study; [#] Difference between m/z values observed with SELDI-TOF-MS and MALDI-TOF-MS < 5 Da.*

SELDI-TOF-MS analysis of PA and PNA obtained from lysozyme stabilized emulsions after mixing with saliva

Figure 2 shows the SELDI profile in the $[MH]^+$ *m/z* range from 2000 to 5000 (a-d) and 5000-20000 (e, f) for the lysozyme emulsion/saliva mixture. The intensity scale is optimized to offer a good visualization of the peaks. With respect to fraction PA, a comparable number of peaks was detected on both chips in the $[MH]^+$ *m/z* range from 2000 to 5000, although several peaks were only found on one array. For example, peaks at $[MH]^+$ *m/z* of 2690.6, 3651.3, 4550.4 have been observed on the NP20 array (Figure 2a), while peaks at 2880.6, 3017.6, 3448.0 and 4377.7 were detected only with CM10 (Figure 2c). In the PNA fraction, a larger number of peaks was detected using the NP20 Proteinchip (i.e. 12 peaks on NP20 vs. 6 on CM10) in the *m/z* range 2000-5000. All the peaks detected on CM10, with the exception of *m/z* 4772.1, were also found on NP20. Comparison between PA and PNA fractions in *m/z* 2000-5000, showed that only one $[MH]^+$ *m/z*, i.e. 4377.7 (peptide P-C, identified by LC-MS/MS approach), was found in both samples.

Figure 2e and 2f illustrate typical SELDI-TOF-MS spectra obtained in the $[MH]^+$ *m/z* range 5000-20000 with NP20. This protein chip offered better results than CM10 in terms of number of detected peaks. 26 different peaks have been detected in fraction PA. Four major peaks were observed at $[MH]^+$ *m/z* 5365.3, 11109.8, 14260, 15431.7, plus several of minor intensities as, for example, at *m/z* 5117.7, 5578.8, and 9756.5 (Figure 2e).

In the PNA fraction, 28 peaks were observed, of which 3 at $[MH]^+$ *m/z* 5782.5, 7136.9 and 14223.9 with high intensities (Figure 2f). Four peaks, i.e. 5365.3±4.3, 5578.8±0.4, 5782.5±0.7 and the large peak at 14200, have been seen in both PA and PNA. However the intensity of the peaks at *m/z* 5365.3 and 5782.5 varied respectively 4- and 10 fold, indicating that the former is principally present in PA and the latter in PNA. The peak at 14200 is assigned to the chicken lysozyme molecule used to prepare the emulsions, which is also present in the aqueous phase of the emulsion with a concentration of 7.6 mg mL^{-1}. The large peak at *m/z* 7136.9 is probably the double charge of the lysozyme molecule.

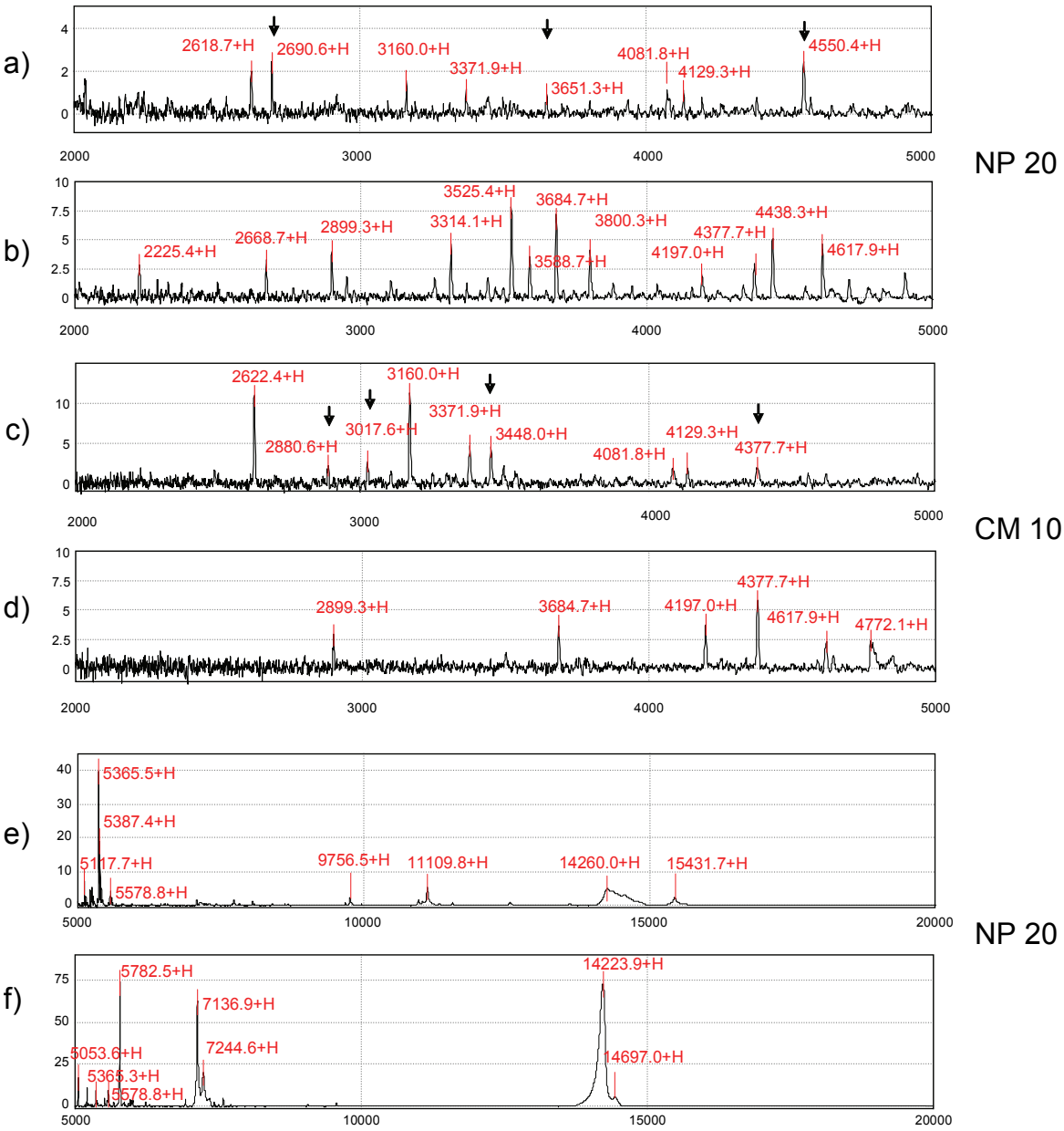

Figure 2 – SELDI-TOF-MS profiles of PA (a, c and e) and PNA (b, d and f) fractions of emulsion droplets of lysozyme stabilized emulsions after mixing with saliva in the *m/z* ranges 2000-5000 (a-d) and 5000-20000 (e, f) using NP 20 and CM 10 arrays. Arrows indicate the positions of peaks detected only in the specified chip.

SELDI-TOF-MS analysis of PA and PNA obtained from β-lactoglobulin stabilized emulsions after mixing with saliva

Analogously to the results described above, Figure 3 illustrates the PA and PNA SELDI-TOF-MS profiles obtained from β-lg stabilized emulsion/saliva mixtures in the two $[MH]^+$ *m/z* ranges.

Between *m/z* 2000 and 5000, PA sample showed comparable number of peaks on NP20 and CM10 (Figure 3a and 3c) with only 2 peaks, i.e. $[MH]^+$ *m/z* of 3036.6 and 4928.3 (histatin 1, identified by LC-MS/MS approach), present on both arrays. In the PNA fraction (Figure 3b and 3d), the amount of peaks detected with NP20 and CM10 was substantially lower compared to the number of peaks observed in PNA from lysozyme emulsion/saliva mixture. Moreover, when PA and PNA of β-lg stabilized emulsion/saliva mixtures were analyzed in search of similarities, no peaks in common were discovered in the considered *m/z* interval.

Figure 3e and 3f report the SELDI-TOF-MS spectra obtained with NP20 in $[MH]^+$ *m/z* 5000-20000. 26 and 29 peaks have been detected in the PA and PNA fractions, respectively. Unfortunately, as seen in Figure 3e and 3f, not all the detected peaks are easily visible. 4 peaks have been found in both PA and PNA, i.e. $[MH]^+$ *m/z* averaged 5365.0±1.7, 6006.4 ±1.0, 7714.7±5.3, 9253.5±1.8, with *m/z* 5365 which was also found in the lysozyme emulsion/saliva mixture.

In line with the findings from the lysozyme stabilized emulsion/saliva mixture, SPs are associating with β-lg stabilized emulsion droplets as well (see Table 1). Furthermore, since only 4 SPs were present in both PA and PNA fractions in *m/z* range 5000-20000, a specific mechanism is likely responsible for the interaction of SPs with the surface of β-lg emulsion droplets.

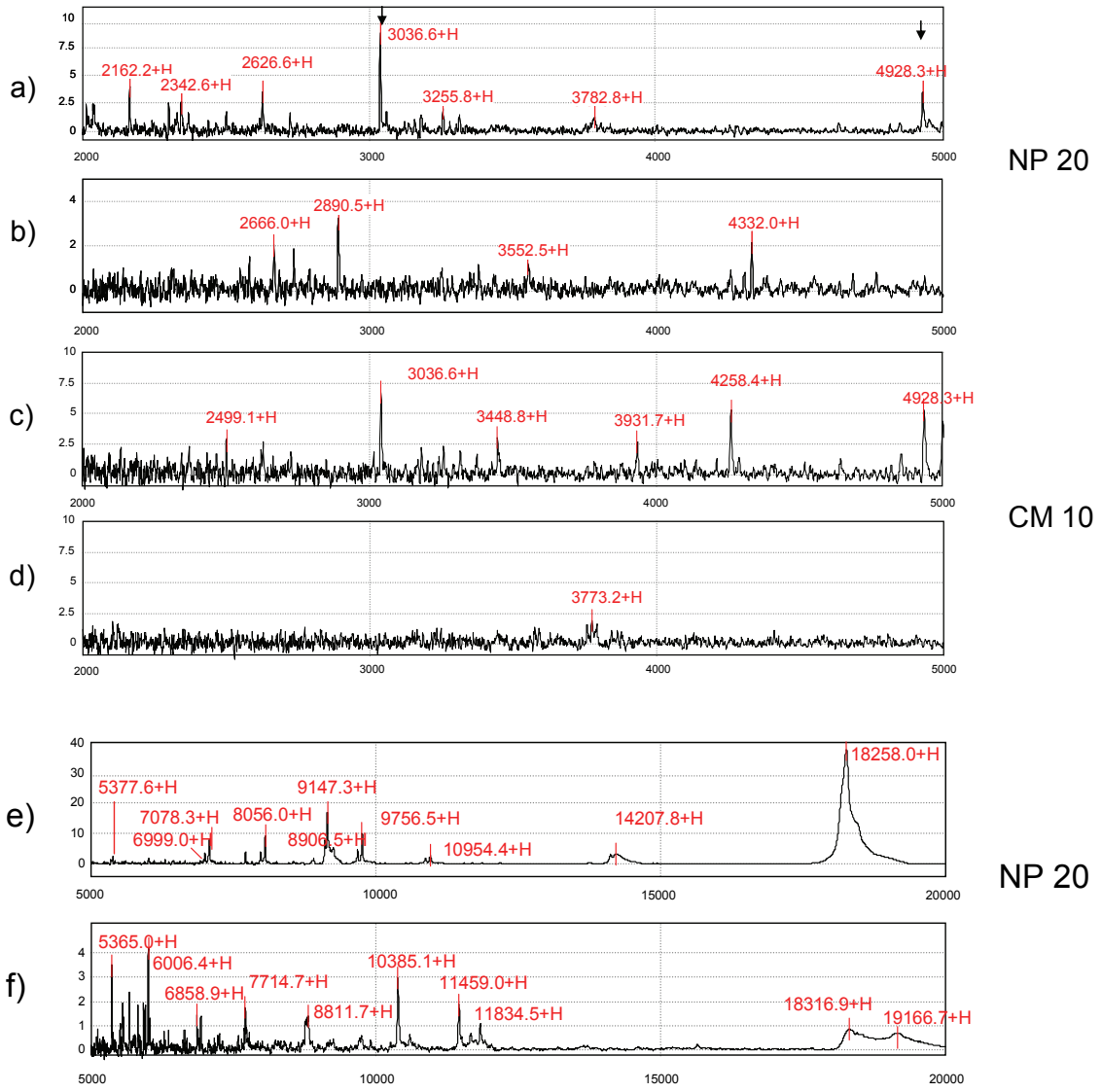

Figure 3 – SELDI-TOF-MS profiles of PA (a, c and e) and PNA (b, d and f) of β-lg stabilized emulsions after mixing with saliva in the *m/z* ranges 2000-5000 (a-d) and 5000-20000 (e and f) using NP 20 and CM 10 arrays. Arrows indicate the position of the peaks found in PA which have been detected with both chips.

LC-MS analysis of PA and PNA obtained from lysozyme stabilized emulsion/saliva mixtures

Figure 4 illustrates typical RP-HPLC chromatograms of PA and PNA fractions of lysozyme emulsion/saliva mixtures. The LC pattern of whole unstimulated saliva is only provided as reference and will not be further discussed. PA and PNA exhibited unique chromatographic profiles with respectively 7 peaks (retention time 40-48 min) and 44 peaks (retention time 14-48 min).

A total of 14 peptides have been identified in the PA fraction in the $[MH]^+$ *m/z* range 800-5000. Eleven peptides between *m/z* 800 and 2000 are separately reported in Table 2. As shown, 8 peptides, mostly containing the sequence HEKHHSH, derived from the histatin 3 precursor while sequences YLYEIAR ($[MH]^+$ *m/z* 927.51), KVPQVSTPTLVEVSR ($[MH]^+$ *m/z* 1639.93) and PFIAIHAESKL ($[MH]^+$ *m/z* 1225.71) originated from serum albumin and pancreatic α-amylase precursor, respectively. All peptides have basic pIs (pI> 7.2) with the exception of YLYEIAR, which displays a calculated isoelectric point of 6. Moreover, they are characterized by a hydrophilic motif and/or the presence of basic amino acids i.e. lysine, arginine, histidine and hydrophilic tyrosine as terminal residues (Table 2). In addition to the above mentioned SPs, $[MH]^+$ *m/z* 2622.4 and 3017.6 have been assigned to fragment 37-57 of histatins 1 precursor and fragment 237-265 of collagen alpha 6(IV) chain precursor, respectively. $[MH]^+$ *m/z* 4377.7 corresponds to the peptide P-C (Table1).

Figure 5a shows, as an example, a typical MALDI-TOF-MS spectrum of the fraction collected at elution time of 43.6 minutes in sample PA. Numerous ions were easily resolved as well as the identified peptides KRHHGYK ($[MH]^+$ *m/z* 925.52), SHAKRHHGYK ($[MH]^+$ *m/z* 1220.66) and HEKHHSHRGY ($[MH]^+$ *m/z* 1287.60) from histatin 3. Figure 5b illustrates the MALDI-TOF/TOF MS/MS spectra of peptide SHAKRHHGYK. The majority of the detected fragments belonged to the N-terminal end "b" series and only y_4 and y_6 were obtained for the "y" series (C-terminal end). An "a" fragment for the dipeptide HA as well as other internal fragments, e.g. HH or HG, were found. In addition, we have reported the two fragments AKRHHGYK and HAKRHHGY which putatively correspond to the observed *m/z* 1036.09 and 1092.58.

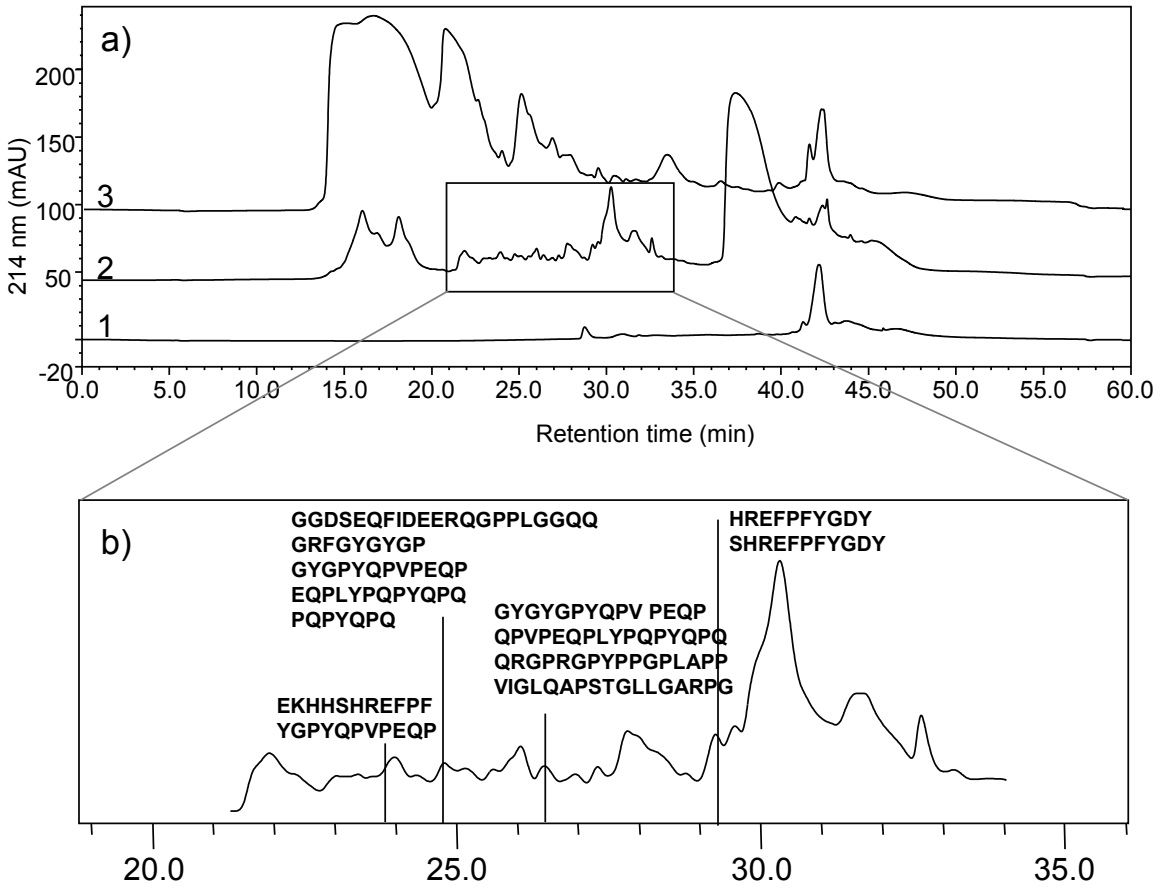

Figure 4 – (a) RP-HPLC profiles of PA (1) and PNA (2) of lysozyme stabilized emulsions mixed with saliva. As reference, saliva is shown in profile 3. (b) Enlargement of the 21-34 min elution range of PNA meant to illustrate some peaks and elution time of some peptides identified after MS.

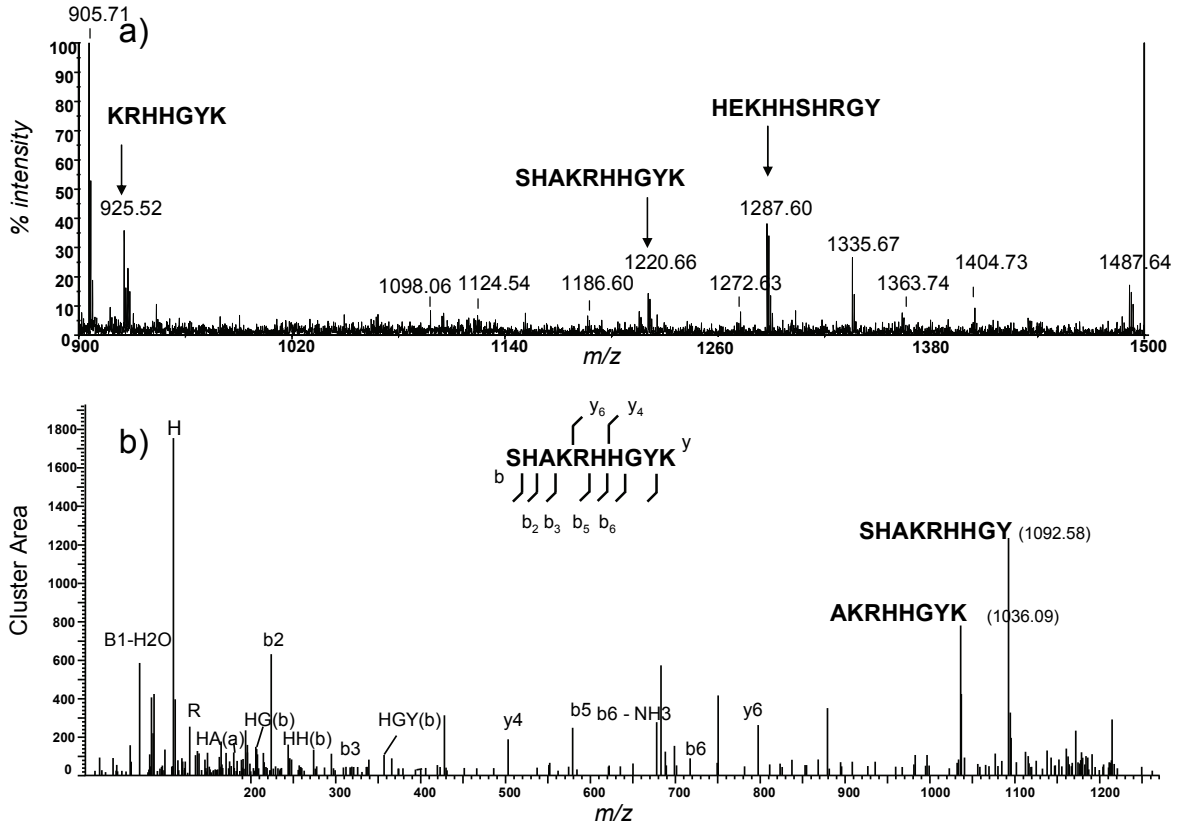

Figure 5 – (a) MALDI-TOF-MS spectra obtained with reflector mode in the [MH]+ *m/z* range between 900 and 1500 of the fraction PA eluted at retention time of 43.6 min. As an example, the identified peptides at *m/z* 925.52, 1220.66, 1287.60 are shown. (b) MALDI-TOF/TOF MS/MS analysis of the histatin 3 peptide SHAKRHHGYK ($[MH]^+$ *m/z* 1220.66). Product b-ions and y-ions are indicated with the subscript which denotes the number of amino acid residue retained by the production. Putative internal sequences AKRHHGYK and SHAKRHHGY and the corresponding *m/z* value are reported.

LC-MS analysis of the PNA fraction of the lysozyme emulsion/saliva mixtures resulted in the identification of 169 different peptides in the M_r range 800-5000 Da. Enlargement of the region 21 to 34 minutes shows the position and some identified sequences (Figure 4b). The complete list, as well as retention time, observed mass, pI, and precursor protein is reported in the Appendix A. Peptides originated from protein precursors as basic salivary proline-rich protein 3 (b PRP 3) precursor, b PRP 4 allele S precursor, statherin, histatin 1,

Wiskott-Aldrich syndrome protein family member 2, CG1 protein, bromodomain-containing protein, splicing factor and polymeric-immunoglobulin receptor. The isoelectric points, calculated using the Expasy ProtParam Tool without taking in account PTM, show that 96 peptides have pI < 6.5, while 73 possess a basic pI. Phosphorylation, oxidation and pyroglutamination were found in 26 peptides. With regard to amino acid composition, the presence of hydrophobic stretches alternated to hydrophilic single amino acids, or short clusters of hydrophilic amino acids, seems to be the most common feature. Furthermore, we found 24 peptides derived from histatins 1, of which 3 are very similar to some of the peptides from histatins 3 in the PA fraction. Interestingly, these peptides (i.e. HEKHHSHREFPF $[MH]^+$ *m/z* 1587.73, FHEKHHSHREFPF $[MH]^+$ *m/z* 1784.89, EKHHSHREFPF $[MH]^+$ *m/z* 1450.75) differ from those in PA (e.g. HEKHHSHRGY), mainly for the presence at the C-terminal end of the motif EFPF instead of GY. It seems therefore that the substitution of the polar aromatic tyrosine with the non-polar aromatic phenylalanine, which lacks the OH group on the benzene ring, could be important in preventing the interaction of the peptides with the lysozyme molecules at the emulsion droplet interfaces.

Figure 6a and 6b illustrate as example two MALDI-TOF-MS spectra for PA and PNA samples, respectively conducted to evaluate the presence of proteins with M_r > 5 kDa. In general, in the PA sample, only a few peaks above *m/z* 5000 were identified. Seven proteins have been identified in the PA sample with LC-MS in the $[MH]^+$ *m/z* range 5000-20000 (Table 1). Just to name a few, $[MH]^+$ *m/z* 5261.2 belongs to cyclo-statherin Q-37, $[MH]^+$ *m/z* 5590.5 and 6024 correspond to peptides P-H and P-E (Figure 6a), respectively. Other peaks at higher *m/z*, such as 14141, 15734, 16904 and 17094 (not shown), had very low MS intensities and could not be identified.

In case of PNA, the MALDI-TOF-MS spectrum in the $[MH]^+$ *m/z* range 5000-20000, as displayed in Figure 6b, strongly resembled the SELDI-TOF-MS profile in Figure 2f. A major peak of *m/z* 14293 corresponds to the chicken egg-white lysozyme used to prepare the emulsion. In the same $[MH]^+$ *m/z* range, peptides P-H (*m/z* 5590.5), non-phosphorylated II-2 (*m/z* 7525.9) and IB1 (*m/z* 9509.5) were identified as well.

.

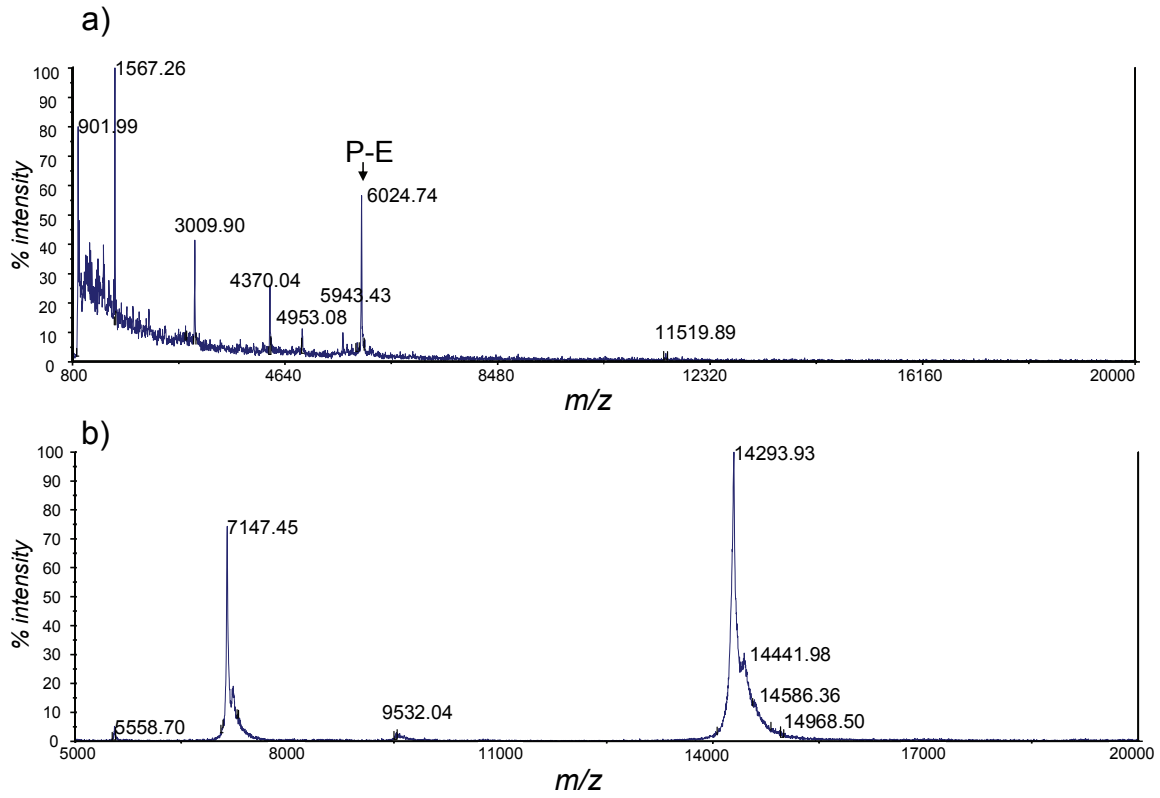

Figure 6 – MALDI-TOF-MS spectra obtained with linear mode of PA (a, *m/z* range 800-20000) and PNA (b, *m/z* range 5000-20000) from lysozyme stabilized emulsion/saliva mixture. As example, the identified proline-rich peptide P-E is shown. The peak *m/z* 7147 is the double charged species of the *m/z* 14293.

LC-MS analysis of PA and PNA obtained from β-lactoglobulin stabilized emulsion/saliva mixtures.

Figure 7a shows the RP-HPLC chromatograms of PA and PNA from β-lg stabilized emulsions after mixing with saliva. The samples have distinct patterns with 17 and 43 peaks resolved, respectively.

A total of 166 different peptides from 800 to 5000 Da have been identified, of which 48 were associated with the emulsions oil droplets and 118 were instead found in the PNA fraction. Table 2 reports a summary of the peptide sequence, retention time, PTM, pI, as well as start and end position of the sequence in the precursor proteins for the peptides identified associated with β-lg stabilized emulsion droplets below *m/z* 2000, while *m/z* in the range 2000-20000 are reported in Table 1. Pyroglutamination, phosphorylation and oxidation have been detected in 13 peptides (Tables 1 and 2). The foremost protein

precursors were the basic salivary proline-rich proteins 1 (b PRP 1) precursor, b PRP 2 precursor, b PRP 4 allele M precursor, proline-rich peptide P-E, salivary acidic proline-rich phosphoprotein 1/2 precursor. Chondroitin sulfate glucuronyltransferase, collagen alpha 2 (VIII) and collagen alpha 1(I) chain have been seen as precursors, as well. The identified sequences, with a pI 5.96-10.18, are constituted mainly of stretches of 3-6 non-polar amino acids, in particular proline and glycine as shown in the motif PPPGKPQ or QGPPPPG, which are frequently repeated or alternated combined together to form longer peptides. Only the PGKPQGPPPQGDKSRSPQS sequence ($[MH]^+$ *m/z* 2185.12) was identified to contain the hydrophilic underlined cluster of 5 aa combined with the common motif PGKPQ or QGPPP. No aromatic amino acids were found, with the exception of phenylalanine in the PPGPAGPAGERGEQGPAGSPSGFQGL sequence ($[MH]^+$ *m/z* 2367.72).

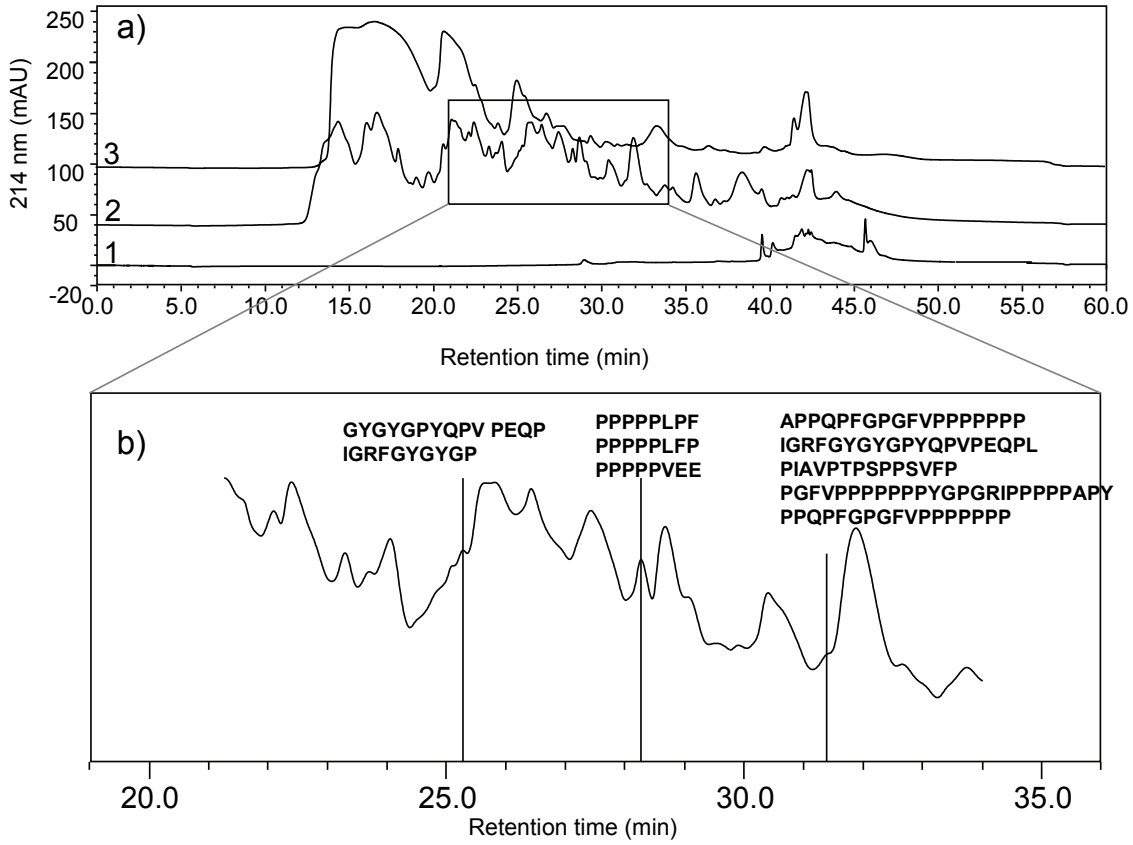

Figure 7 – (a) RP-HPLC profiles of PA (1) and PNA (2) of β-lg stabilized emulsions mixed with saliva. As reference saliva is shown in profile 3. (b) Enlargement of the 21-34 min elution range of PNA meant to illustrate some of the peptides identified after MS.

Table 2 – Identified peptides after RP-HPLC coupled with MALDI-MS-MS in the PA extracted after sample procedure from a lysozyme and β-lg stabilized emulsion after mixing with saliva.

Retention time	Sequence	PTM	pI*	Position	Observed Mass (Da)	Calculated Mass (Da)	Error (Da)	Precursor
PA sample from lysozyme stabilized emulsion upon mixing with saliva								
36.6	YLYEIAR		6.0	162-168	927.51	927.49	0.02	Serum albumin (P02768)
38.3	KVPQVSTPTLVEVSR		8.7	438-452	1639.94	1639.93	0.01	Serum albumin (P02768)
41.6	PFIAIHAESKL		7.2	501-511	1225.71	1225.69	0.02	Pancreatic alpha-amylase (P04746)
43.3	DSHAKRHHGYK		9.7	20-30	1335.67	1335.66	0.01	Histatin 3 (P15516)
43.6	HEKHHSHRGY		8.6	34-43	1287.60	1287.60	<0.01	Histatin 3 (P15516)
	KRHHGYK		10.2	24-30	925.52	925.51	0.01	Histatin 3 (P15516)
	SHAKRHHGYK		10.2	21-30	1220.66	1220.63	0.03	Histatin 3 (P15516)
44.0	FHEKHHSHRGY		8.6	33-43	1434.67	1434.67	<0.01	Histatin 3 (P15516)
	RKFHEKHHSH		9.9	31-40	1342.69	1342.68	0.01	Histatin 3 (P15516)
44.3	KFHEKHHSHRGY		9.7	32-43	1562.77	1562.77	<0.01	Histatin 3 (P15516)
44.6	RKFHEKHHSHRGY		10.2	31-43	1718.89	1718.87	0.02	Histatin 3 (P15516)
PA sample from β-lg stabilized emulsion upon mixing with saliva								
27.7	QGPPPPGKPQGPPPP	#	8.75	224-238	1430.74	1430.74	<0.01	b PRP 4 allele M (P10161)
	QGPPPPPGKPQGPPP	#	8.75	18-32; 35-49; 48-62	1430.74	1430.74	<0.01	P-E (P02811); b PRP 2 (P02812); b PRP 1 (P04280)
	QPQGPPPPPGKPQGPPP	#	8.75	16-32; 33-49; 107-123	1655.87	1655.85	0.02	P-E (P02811); b PRP 2 (P02812); b PRP 1 (P04280)
	QQGPPPPPPGKPQGPPP	#	8.75	135-151	1655.87	1655.85	0.02	a proline-rich phosphoprotein 1/2 (P02810)
34.3	GRPQGPPQQGGHQQ		9.76	123-136	1471.69	1471.72	-0.03	a proline-rich phosphoprotein 1/2 (P02810)

Table 2 Continued

42.7	GPPPQGGRPQGPPQGQSPQ		9.75	148-166	1866.59	1866.92	-0.33	a proline-rich phosphoprotein 1/2 (P02810)
	NQPQGPPPPPGKPQGPPP		8.75	15-32; 32-39; 106-123	1786.57	1786.92	-0.35	P-E (P02811); b PRP 2 (P02812); b PRP 1 (P04280)
	QGGNKPQGPPPPPGKPQ	#	10	42-58	1663.57	1663.86	-0.29	b PRP 1 (P04280)
	QGGNQPQGPPPPPGKPQ	#	8.75	12-28; 29-45	1663.57	1663.82	-0.25	P-E (P02811); b PRP 2 (P02812)
	SPPGKPQGPPPQGGNQPQ		8.47	1-18; 18-35; 92-109	1767.57	1767.88	-0.31	P-E (P02811); b PRP 2 (P02812); b PRP 1 (P04280)
43.0	GPPPQEGNKSRX		Nd	161-172	1333.48	1333.59	-0.11	b PRP 4 (P81489)
	GPPPPGKPQGPPPP		8.75	225-238	1319.50	1319.71	-0.21	b PRP 4 allele M (P10161);
	AGQPQGPPRPPQ		9.79	362-373	1229.46	1229.64	-0.17	b PRP 1 (P04280)
				372-383	1229.46	1229.64	-0.17	b PRP 2 (P02812)
	GPPPPPGKPQGPPP		8.75	19-32 36-49 49-62	1319.50	1319.71	-0.21	P-E (P02811) b PRP 2 (P02812) b PRP 1 (P04280)
	GPPPPPPGKPQ		8.75	137-147	1068.44	1068.58	-0.15	a proline-rich phosphoprotein 1/2 (P02810)
	GPPPQEGNKSRS	*[12]	8.75	193-204	1333.48	1333.59	-0.11	b PRP 4 allele M (P10161)
	GPPPQGGNKPQ		8.75	46-56; 59-69	1076.40	1076.55	-0.14	b PRP 2 (P02812); b PRP 1 (P04280)
	GPPPQGGNQPQ		5.52	8-18	1076.40	1076.51	-0.11	P-E (P02811)
	GPPPQGGNRPQ		9.75	29-39	1104.38	1104.55	-0.17	P-E (P02811)
	GPPQQGGNRPQ		9.75	242-252	1135.39	1135.56	-0.17	b PRP 1 (P04280)
	PGPAGEKGSPGA	*[9]	6.43	928-939	1104.38	1104.47	-0.09	Collagen alpha 1(I) chain (P02452)
	PGRPQGGNQPQ		10.18	21-31	1135.39	1135.56	-0.17	b PRP 4 (P81489)
	PPGPPGAGPDPPSP	*[13]	3.8	636-649	1319.50	1319.57	-0.06	Chondroitin sulfate glucuronyltransferase (Q9P2E5)
	PPGPPGPPGAPGA		5.96	523-535	1068.44	1068.55	-0.11	Collagen alpha 2 (VIII) chain (P25067)
	SPPGKPQGPPPQGGN		8.47	1-15; 18-32; 92-106	1414.48	1414.71	-0.22	P-E (P02811); b PRP 2 (P02812); b PRP 1 (P04280)

43.0	SPPGKPQGPPPQGGNQP		8.47	1-17; 18-34; 92-108	1639.54	1639.82	-0.28	P-E (P02811); b PRP 2 (P02812) b PRP 1 (P04280)
43.3	GPPPPGKPQ		8.75	40-48; 57-65; 70-78; 225-233	874.37	874.48	-0.11	P-E (P02811); b PRP 2 (P02812); b PRP 1 (P04280); b PRP 4 allele M (P10161)
	GPPPPGKPQGPPP		8.75	40-52; 57-69; 70-82; 225-237	1222.49	1222.66	-0.17	P-E (P02811); b PRP 2 (P02812) ; b PRP 1 (P04280); b PRP 4 allele M (P10161)
	GPPPPPGKPQ		8.75	19-28; 36-45; 49-58	971.40	971.53	-0.13	P-E (P02811); b PRP 2 (P02812); b PRP 1 (P04280)
	GPPPQGGRPQ		9.75	148-157	990.38	990.51	-0.13	a proline-rich phosphoprotein 1/2 (P02810)
	GQPQGPPRPPQ		9.75	363-373; 373-383	1158.44	1158.60	-0.16	b PRP 2 (P02812); b PRP 1 (P04280)
	PGPPGAPGPQ		5.96	190-199	874.37	874.44	-0.07	Collagen alpha 1(I) chain (P02452)
	PGPPGPPGAPGA		5.96	524-535	971.40	971.49	-0.10	Collagen alpha 2 (VIII) chain (P25067)
	PPGPPGAGPDPPS	*[13]	3.8	636-648	1222.49	1222.51	-0.03	Chondroitin sulfate glucuronyltransferase (Q9P2E5)
43.7	PPPPGKPQ		9.18	17-24; 21-28; 34-41	817.36	817.46	-0.10	b PRP 4 allele M (P10161); P-E (P02811); b PRP 4 (P81489)
	PPPPGKPQ		9.18	38-45; 51-58; 140-147	817.36	817.46	-0.10	b PRP 2 (P02812); b PRP 1 (P04280); a proline-rich phosphoprotein 1/2 (P02810)
	SPPGKPQGPPP		8.47	1-11; 18-28; 92-102	1058.43	1058.56	-0.14	P-E (P02811); b PRP 2 (P02812); b PRP 1 (P04280)

X: unspecified amino acid; #: pyroglutamination at Glutamine (Q) at N-terminal end of the sequence;: phosphorylation occurs at Serine (S); [ ] indicates the amino acid residues of the peptide sequence where PTM is occurring; Nd: not determined; a: acidic; b: basic; ( ) indicates the Swiss-Prot data base accession number.*

The identities of SPs non-associated with β-lg stabilized emulsions are reported in Appendix A. As an example, Figure 7b shows an enlargement of the RP-HPLC region with retention time 21 to 34 minutes of the PNA fraction. Peptide precursors were PRPs, statherin precursor, Wiskott-Aldrich syndrome protein family member 2 and 4, Abl-interactor 2, early growth response protein 2, fosB, splicing factor and acetylcholinesterase collagenic tail peptide. 61 peptides had an acid isoelectric point (pI< 6.7) while 56 had a pI in the range 8-12.3. Although peptides were rich in proline clusters, unique structural features could not be clearly recognized. Larger variations in terms of type and positions of amino acids, as well as length of repeated motifs or of the total sequence were observed when compared with the PA fraction. Furthermore, cysteines and methionines, not detected in PA, were present in 11 PNA sequences, within the motifs ACC, CTM or PPPPPPPMI. Moreover, only the following 3 peptides GPPPPPGKPQ, GPPPPPPGKPQ and GPPPQGGNKPQ were found in both PA and PNA fractions analyzed from β-lg emulsion/saliva mixtures.

Analogously to lysozyme, Figure 8 illustrates the MALDI-TOF-MS spectrum obtained in the high molecular range (*m/z* 5000-20000) for the fraction associated with β-lg stabilized emulsion droplets.

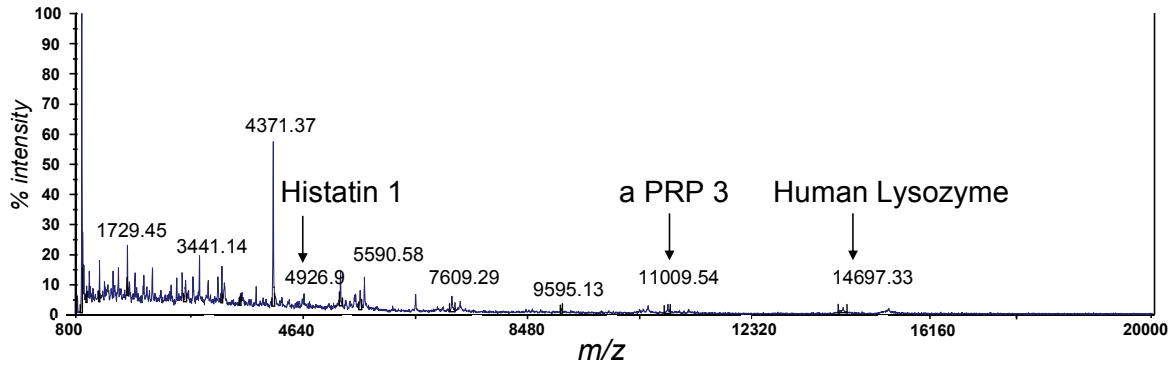

Figure 8 – MALDI-TOF-MS spectra obtained with linear mode in the *m/z* range 800-20000 of PA from β-lg stabilized emulsion/saliva mixture. The identified histatin 1, acidic (a) PRP 3 and human lysozyme are indicated.

Several proteins such as histatin, statherin and proline-rich proteins, as well as their PTMs isoforms, could be identified in this sample. For example, *m/z* 5006.6 and 5082.1 were identified as fragment 20-57 of the histatins 1 precursor (peak *m/z* 4926.9) having 1 sulphation and 2 sulphations, respectively. Cyclo-statherin Q-37 ($[MH]^+$ *m/z* 5262,3), statherin ($[MH]^+$ *m/z* 5374,8) identified as well. Basic proline-rich peptide P-E and the same peptide with 1 phosphorylation were assigned to $[MH]^+$ *m/z* 6024.3 and 6112.9, respectively. Furthermore, other proline-rich proteins were also found, such as the acidic PRP 1 (2 phosphorylations), acidic PRP 3 (non-phosphorylated) and acidic PRP 3 (1 phosphorylation) at 15522.3, 11009.5 and 11075.7, respectively. Cystatins S with 2 phosphorylation ($[MH]^+$ *m/z* 13409.7) and human lysozyme ($[MH]^+$ *m/z* 14697.3) were also identified.

With regard to the PNA sample, instead, peaks in the *m/z* above 5000 were only observed in a few MS spectra. No spectra of this sample are presented since they were very similar to those observed in Figure 6b with two major peaks at 7000 and 14000. In the $[MH]^+$ *m/z* range 5000-20000, peptide P-H (*m/z* 5590.5), and non-phosphorylated II-2 (*m/z* 7525.9) which were also present in PNA from lysozyme emulsion/saliva mixtures were identified. Moreover, basic proline-rich peptide P-E with 1 phosphorylation, as in PA was assigned to $[MH]^+$ *m/z* 6024.3.

Discussion

This paper describes the application of SELDI-TOF-MS and LC-MS to study the interaction between salivary components (in the range between 800 and 20000 Da) and oil-water interfaces which have been stabilized by chicken egg-white lysozyme or bovine β-lactoglobulin. Combining these two techniques offers several advantages. For example, compared to LC-MS experiments, SELDI-TOF-MS provides a rapid response and a clear overview of the differences and/or similarities between samples within the same emulsion/saliva mixture or between mixtures, with modest effort in data managing. Moreover little sample preparation, with no need for the extensive dialysis necessary to remove the SDS, was required. On the other hand, LC-MS and data bank research provided us with the identification of salivary proteins and peptides in the emulsion/saliva mixtures, as well as other useful features, such as the peptide sequence and the presence of PTMs. Furthermore, LC-MS enabled the detection of small peptides in the *m/z* range 800-2000

which were not observed with SELDI-TOF-MS experiments. This combined information might increase our understanding of the interaction mechanism of salivary proteins and peptides with the oil-droplets surface.

More than 200 protein and peptides were detected in each mixture in total, considering both SELDI-TOF-MS results and the LC-MS identified compounds. Our findings suggest that salivary components distinctly separated between the aqueous phase of the emulsion, indicated as PNA, and the oil phase constituted of the emulsion droplets (PA samples). Only few components were found to be in common in both PA and PNA fractions. For example, peptide P-C and P-H were the only ones observed in both fractions of lysozyme emulsion/saliva mixtures in the *m/z* range 800-5000 and 5000-20000, respectively. Similar results have been seen in the β-lg stabilized emulsion where 5 SPs, i.e. peptide P-H, P-E and the small 3 peptides GPPPPPGKPQ (*m/z* 971.5), GPPPPPPGKPQ (*m/z* 1068.5) and GPPPQGGNKPQ (*m/z* 1076.5) were present in both fractions in the considered M_r range.

To unravel the mechanism of the interaction of salivary proteins with lysozyme and β-lg stabilized emulsion droplets, several aspects must be considered. Firstly, it is important to realize that protein adsorption at the oil-water interface, which occurs predominantly via hydrophobic segments, often results in changes of the molecular conformation of the emulsifying protein until the most favorable is reached [80-82]. Information about exposure of aromatic amino acids, percentage of secondary structure (e.g. α-helix), surface hydrophobicity upon adsorption of lysozyme and β-lg to air-water interfaces is available in literature [83-86]. However, detailed information, for example, of which amino acids of the proteins at the oil-water interfaces are exposed to the aqueous is not available yet. Globular proteins, such as lysozyme and β-lg adsorb mostly as relatively compact globules, with a low degree of unfolding [85, 87]. Analogously to the air-water interfaces [83, 85], it is likely that at the oil-water interfaces these proteins expose the same amino acids as in their native state in solution. Secondly, due to the different pIs, lysozyme and β-lg, at physiological pH, carry a net positive and negative charge, respectively, inducing a different overall net surface charge on the droplets. Therefore, one might simply expect an electrostatically driven interaction mechanism of oppositely charged salivary molecules with the emulsion droplets to occur. Nevertheless, the exposure of polar amino acids, or the presence of a particular protein sequence could favor adsorption of salivary compounds to the surface, not only via electrostatics but also via hydrogen bonds, or a sequence specific interaction, as well. In addition, a last point of attention concerning the structure and

protein organization of saliva must be mentioned. Since it has been calculated that 4-20% of whole salivary proteins is present in form of micellar aggregates [13, 88-92], it is plausible that some of the SPs found in the PA fraction belong to adsorbed complexes rather than being single molecules interacting with the droplet surface. The presence of crosslinks or complexes between several salivary proteins, could allow SPs that do not have affinity to the emulsion droplets to be included into the oil droplets phase. Since at this stage of the research, we are not able to distinguish how SPs are interacting with the emulsion droplets, i.e. directly or as part of adsorbed complex, we use both the terms adsorbed or associated as synonyms.

Bearing these considerations in mind, our work suggests that properties of the oil droplets interfaces are important for determining which SPs are associated to the emulsions oil-phase. A total number of 60 and 119 different components have been detected in the fractions associated with droplets of lysozyme and β-lg stabilized emulsions, respectively. Taking into account the amino acid composition, hydrophilicity and hydrophobicity of the sequences rather than only the net charge carried at physiological pH, the results provide indications that interaction of SPs in the *m/z* range 800-2000 with emulsion droplets surfaces is driven by a surface specific mechanism. For example, presence of GY or EFPF at the C-terminal end of the HEKHHSHR determined, without variation in the positive net charge, if this peptide was associated or not with the lysozyme stabilized emulsion droplets. It might also be interesting to note that lysozyme molecules possess sequences which could interact with the detected peptides. The sequence KFESNFNTQATNRNTDGSTDY, from the position 33 to 53, is particularly rich in polar side chain residues and contains two aspartic acids, one arginine and one lysine. Although in the native state only the sequence QATNRNTD is exposed as part of a loop between two β-sheets, these 8 aa could be potentially sufficient for determining an interaction. Moreover, the hydrophilic sequence from aa 112 to 129 at the C-terminal end of the lysozyme molecules was found to be exposed towards the aqueous solution at air-water interfaces [85] and might play a role as well.

In case of SPs associated with β-lg stabilized emulsions, the presence of long stretches of non-polar residues contained in both net positively and negatively charged peptides point to a role of surface specific interaction with the droplets as well. Although no clear evidence for electrostatics could be pointed out, due to the complexity of the system and the amount of SPs involved, it also could not be completely ruled out. The presence of PTM such as

pyroglutamination, phosphorylation and sulphation, which were found mainly in PA from β-lg emulsion/saliva mixtures, could be another indication for a specific role of the protein surface layer of the emulsions.

Furthermore, when comparing the so-far detected/identified salivary species, 14 SPs, e.g. basic proline-rich peptide P-E, were found in PA fractions of both emulsions (Table 1). This last finding might also suggest that adsorption of saliva components to emulsion droplets could involve a multi-step process. Specifically driven adsorption of small peptides (M_r < 2 kDa) onto emulsion droplets could favor further interactions of the droplets with other salivary proteins with a mechanism similar to the multi-layer deposition on emulsions droplets [93, 94]. Although beyond the scope of this chapter, it would be of interest to study the different adsorption kinetics of the here-indicated salivary molecules which would also provide information on a possible multi-step mechanism.

The identification of salivary components associated with emulsion droplets can also be very helpful in understanding the sensory perception of food emulsions. In fact, in line with the mechanism of astringency induced by tannins, we showed that saliva-induced flocculation of lysozyme stabilized emulsions was due to complex formation between SPs and the droplets producing subsequently a clear astringent sensation of this emulsion (Chapters 3 and 7) [59, 71]. In this study, we have identified several SPs involved in droplet flocculation of lysozyme stabilized emulsion and therefore likely in astringency. Both small peptides derived from histatin 3 (M_r < 2 kDa) and a large number of other not completely identified salivary proteins in the M_r 2-20 kDa, have been found to be associated with these emulsion droplets. Furthermore, we have also shown that interaction between SPs (M_r 1-20 kDa) and the β-lactoglobulin emulsion droplets surface is also occurring and typical astringency-related proteins, such as PRP and histatins, were found associated with these emulsions. Even though emulsions stabilized by whey protein, which is composed mainly of β-lg, have been reported to be perceived as creamy, a 10% (w/v) solution of whey protein has also been reported as astringent [68, 69]. However, this protein solution is 40-fold more concentrated compared with the emulsifier concentration in the studied emulsion/saliva mixtures.

Our results indicate that different SPs are involved in adsorption with emulsion droplets and might play a role in the sensory perception of the studied emulsions, as well. To conclude, this work, which showed that protein-protein interaction between SPs and food emulsion droplets occurs, might provide a new point of view for investigating the cause of

the sensorial experience of food emulsions in the mouth by studying at a molecular level the interaction with salivary components.

Conclusions

In this chapter we combined two proteomics techniques, i.e. SELDI-TOF-MS and LC-MS, to study which SPs associated with emulsion droplets. In this way, the advantage of having rapid results and general overview of the data are joined with the identification and sequence characterization provided by LC-MS.

Results show that a substantial number of SPs in the M_r range 800-20000 Da are interacting with emulsion droplets stabilized by lysozyme and β-lg. To our knowledge, this is the first time that salivary components have been reported to adsorb onto the oil-water interface of a β-lg stabilized emulsion.

Eleven peptides (< 2 kDa), mostly containing a hydrophilic sequence derived from the histatin 3 precursor, serum albumin and pancreatic α-amylase precursor, were found in PA of the lysozyme stabilized emulsion after mixing with unstimulated saliva. In contrast, 37 peptides, containing non-polar proline stretches, which derived mostly from different salivary PRPs, were identified in PA from β-lg stabilized emulsions. In the M_r range above 2 kDa, among others SPs, different statherin isoforms have been observed to be associated with lysozyme emulsions droplets, while histatins, PRPs, cystatin S associated with β-lg stabilized emulsions. Furthermore, only a few SPs were found in common in the PA and PNA fractions in each emulsion/saliva mixtures, as well as in the two PA fractions from both emulsions, e.g. peptide P-E. Our results suggest that adsorption/association of salivary protein and peptides onto the droplets surface is related to the type of proteins at the emulsion oil-water interfaces. Interaction with the droplets seems to be driven by the presence of specific sequences structural surfaces features rather than being only an electrostatically driven interaction mechanism.

Acknowledgements

We would like to thank Raymond Schipper, George van Aken and Willem Norde for critically reading of the manuscript.

References

1. Nieuw Amerongen, A.v., Bolscher, J., and Veerman, E.C.I., *Salivary proteins: Protective and diagnostic value in cariology?* Caries Research., 2004. **38**: p. 247.
2. Humphrey, S.P. and Williamson, R.T., *A review of saliva: Normal composition, flow and function.* Journal of Prosthetic Dentistry, 2001. **85**: p. 162.
3. Troxler, R.F., Offner, G.D., Xu, T., Vanderspek, J.C., and Oppenheim, F.G., *Structural relationship between human salivary histatins.* Journal of Dental Research, 1990. **69**: p. 2.
4. De Smet, K. and Contreras, R., *Human antimicrobial peptides: Defensins, cathelicidins and histatins.* Biotechnology Letters, 2005. **27**: p. 1337.
5. Inzitari, R., Cabras, T., Rossetti, D.V., Fanali, C., Vitali, A., Pellegrini, M., Paludetti, G., Manni, A., Giardina, B., Messana, I., and Castagnola, M., *Detection in human saliva of different statherin and p-b fragments and derivatives.* Proteomics, 2006. **6**: p. 6370.
6. Isemura, S., Saitoh, E., Sanada, K., and Minakata, K., *Identification of full-sized forms of salivary (s-type) cystatins (cystatin SN, cystatin SA, cystatin S, and two phosphorylated forms of cystatin S) in human whole saliva and determination of phosphorylation sites of cystatin S.* Journal of Biochemistry, 1991. **110**: p. 648.
7. Lamkin, M.S., Jensen, J.L., Setayesh, M.R., Troxler, R.F., and Oppenheim, F.G., *Salivary cystatin SA-III, a potential precursor of the acquired enamel pellicle, is phosphorylated at both its amino- and carboxyl-terminal regions.* Archives of Biochemistry and Biophysics, 1991. **288**: p. 664.
8. Drzymala, L., Castle, A., Cheung, J.C., and Bennick, A., *Cellular phosphorylation of an acidic proline-rich protein, PRP1, a secreted salivary phosphoprotein.* Biochemistry, 2000. **39**: p. 2023.
9. Cabras, T., Fanali, C., Monteiro, J.A., Amado, F., Inzitari, R., Desiderio, C., Scarano, E., Giardina, B., Castagnola, M., and Messana, I., *Tyrosine polysulfation of human salivary histatin 1. A post-translational modification specific of the submandibular gland.* Journal of Proteome Research, 2007. **6**: p. 2472.
10. Inzitari, R., Cabras, T., Onnis, G., Olmi, C., Mastinu, A., Sanna, M.T., Pellegrini, M.G., Castagnola, M., and Messana, I., *Different isoforms and post-translational modifications of human salivary acidic proline-rich proteins.* Proteomics, 2005. **5**: p. 805.
11. Perinpanayangam, H.E.R., Wuyckhuyze, B.C.v., Ji, Z.S., and Tabak, L.A., *Characterization of low-molecular-weight peptides in human parotid saliva.* Journal of Dental Research, 1995. **71**: p. 345.
12. Castagnola, M., Inzitari, R., Rossetti, D.V., Olmi, C., Cabras, T., Piras, V., Nicolussi, P., Sanna, M.T., Pellegrini, M., Giardina, B., and Messana, I., *A cascade of 24 histatins (histatin 3 fragments) in human saliva: Suggestions for a pre-secretory sequential cleavage pathway.* Journal of Biological Chemistry, 2004. **279**: p. 41436.
13. Helmerhorst, E.J. and Oppenheim, F.G., *Saliva: A dynamic proteome.* Journal of Dental Research, 2007. **86**: p. 680.
14. Cai, K. and Bennick, A., *Processing of acid proline-rich proprotein by human salivary gland convertase.* Archives of Oral Biology, 2004. **49**: p. 871.
15. Jensen, J.L., Lamkin, M.S., Troxler, R.F., and Oppenheim, F.G., *Multiple forms of statherin in human salivary secretions.* Archives of Oral Biology, 1991. **36**: p. 529.
16. Messana, I., Cabras, T., Fadda, M.B., Inzitari, R., Zuppi, C., Olmi, C., Giardina, B., Castagnola, M., Lupi, A., and Cordaro, M., *Characterization of the human salivary basic proline-rich protein complex by a proteomic approach.* Journal of Proteome Research, 2004. **3**: p. 792.
17. Distler, W. and Kroncke, A., *Biologically-active, low-molecular-weight peptides in human saliva.* Journal of Dental Research, 1987. **66**: p. 603.
18. Hay, D.I. and Oppenheim, F.G., *The isolation from human parotis saliva of a further group of proline-rich proteins.* Archives of Oral Biology, 1974. **19**: p. 627.
19. Hay, D.I., *Fractionation of human parotid salivary proteins and the isolation of an histidine-rich acidic peptide which shows high affinity for hydroxyapatite surfaces.* Archives of Oral Biology, 1975. **20**: p. 553.
20. Hay, D.I., Ahern, J.M., Schluckebier, S.K., and Schlesinger, D.H., *Human salivary acidic proline-rich protein polymorphisms and biosynthesis studied by high-performance liquid-chromatography.* Journal of Dental Research, 1994. **73**: p. 1717.
21. Omichi, K. and Ikenaka, T., *Separation of human salivary [alpha]-amylase isozymes by high-performance liquid chromatography with a continuous monitor system of the activity.* Analytical Biochemistry, 1988. **168**: p. 332.
22. Siqueira, W.L., Helmerhorst, E.J., Zhang, W., Salih, E., and Oppenheim, F.G. (2007) *Acquired enamel pellicle and its potential role in oral diagnostics*. Annals of the New York Academy of Sciences, **1098**: p. 504.

23. Peluso, G., De Santis, M., Inzitari, R., Fanali, C., Cabras, T., Messana, I., Castagnola, M., and Ferraccioli, G.F., *Proteomic study of salivary peptides and proteins in patients with Sjogren's syndrome before and after pilocarpine treatment.* Arthritis and Rheumatism, 2007. **56**: p. 2216.
24. Inzitari, R., Vento, G., Capoluongo, E., Boccacci, S., Fanali, C., Cabras, T., Romagnoli, C., Giardina, B., Messana, I., and Castagnola, M., *Proteomic analysis of salivary acidic proline-rich proteins in human preterm and at-term newborns.* Journal of Proteome Research, 2007. **6**: p. 1371.
25. Vitorino, R., Lobo, M.J.C., Ferre-Correira, A.J., Dubin, J.R., Tomer, K.B., Domingues, P.M., and Amado, F.M.L., *Identification of human whole saliva protein components using proteomics.* Proteomics, 2004. **4**: p. 1109.
26. Yao, Y., Berg, E.A., Costello, C.E., Troxler, R.F., and Oppenheim, F.G., *Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches.* The Journal of Biological Chemistry, 2003. **278**: p. 5300.
27. Huang, C.-M., *Comparative proteomic analysis of human whole saliva.* Archives of Oral Biology, 2004. **49**: p. 951.
28. Ghafouri, B., Tagesson, C., and Lindahl, M., *Mapping of proteins in human saliva using two-dimensional gel electrophoresis and peptide mass fingerprinting.* Proteomics, 2003. **3**: p. 1003.
29. Hu, S., Li, Y., Wong, D.T., Xie, Y., Ramachandran, P., Loo, J.A., and Loo, R.R.O., *Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry.* Proteomics, 2005. **5**: p. 1714.
30. Cabras, T., Inzitari, R., Fanali, C., Scarano, E., Patamia, M., Sanna, M.T., Pisano, E., Giardina, B., Castagnola, M., and Messana, I., *HPLC-MS characterization of cyclo-statherin Q-37, a specific cyclization product of human salivary statherin generated by transglutaminase.* Journal of Separation Science, 2006. **29**: p. 2600.
31. Pisano, E., Cabras, T., Montaldo, C., Piras, V., Inzitari, R., Olmi, C., Castagnola, M., and Messana, I., *Peptides of human gingival crevicular fluid determined by HPLC-ESI-MS.* European Journal of Oral sciences, 2005. **113**: p. 462.
32. Messana, I., Loffredo, F., Inzitari, R., Cabras, T., Giardina, B., Onnis, G., Piludu, M., and Castagnola, M., *The coupling of RP-HPLC and ESI-MS in the study of small peptides and proteins secreted in vitro by human salivary glands that are soluble in acidic solution.* European Journal of Morphology, 2003. **41**: p. 103.
33. Hutchens, T.W. and Yip, T.T., *New desorption strategies for the mass spectrometric analysis of macromolecules.* Rapid Communications in Mass Spectrometry, 1993. **7**: p. 576.
34. Streckfus, C.F., Bigler, L.R., and Zwick, M., *The use of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry to detect putative breast cancer markers in saliva: A feasibility study.* Journal of Oral Pathology and Medicine, 2006. **35**: p. 292.
35. Zhang, H., Wu, G., Tu, H., and Huang, F., *Discovery of serum biomarkers in astrocytoma by SELDI-TOF-MS and proteinchip technology.* Journal of Neuro-Oncology, 2007. **84**: p. 315.
36. Scarlett, C.J., Samra, J.S., Xue, A., Baxter, R.C., and Smith, R.C., *Classification of pancreatic cystic lesions using SELDI-TOF mass spectrometry.* ANZ Journal of Surgery, 2007. **77**: p. 648.
37. Wang, S.X., Zhao, X.F., Wei, M.T., Wu, Y., Zeng, J.W., Liang, X.C., Yi, J.H., He, B., and Tu, Z.G., *Screening of serum biomarkers and establishment of a decision tree in silica-exposed populations by surface-enhanced laser desorption ionization time-of-fly mass spectrometry.* Journal of Occupational and Environmental Medicine, 2007. **49**: p. 764.
38. Kanmura, S., Uto, H., Kusumoto, K., Ishida, Y., Hasuike, S., Nagata, K., Hayashi, K., Ido, A., Stuver, S.O., and Tsubouchi, H., *Early diagnostic potential for hepatocellular carcinoma using the SELDI proteinchip system.* Hepatology, 2007. **45**: p. 948.
39. Hinshelwood, J., Spencer, D.I.R., Edwards, Y.J.K., and Perkins, S.J., *Identification of the C3B binding site in a recombinant VWF-A domain of complement factor b by surface-enhanced laser desorption-ionisation affinity mass spectrometry and homology modelling: Implications for the activity of factor b.* Journal of Molecular Biology, 1999. **294**: p. 587.
40. Rawel, H.M., Rohn, S., Kroll, J., and Schweigert, F.J., *Surface enhanced laser desorptions ionization-time of flight-mass spectrometry analysis in complex food and biological systems.* Molecular Nutrition and Food Research, 2005. **49**: p. 1104.
41. Rawel, H.M., Ranters, H., Rohn, S., and Kroll, J., *Assessment of the reactivity of selected isoflavones against proteins in comparison to quercetin.* Journal of Agricultural and Food Chemistry, 2004. **52**: p. 5263.
42. Schipper, R., Loof, A., De Groot, J., Harthoorn, L., Van Heerde, W., and Dransfield, E., *Salivary protein/peptide profiling with SELDI-TOF-MS.* in *Annals of the New York Academy of Sciences*. 2007.**1098**: p. 498.

43. Schipper, R., Loof, A., de Groot, J., Harthoorn, L., Dransfield, E., and van Heerde, W., *SELDI-TOF-MS of saliva: Methodology and pre-treatment effects.* Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 2007. **847**: p. 45.
44. Ryu, O.H., Atkinson, J.C., Hoehn, G.T., Illei, G.G., and Hart, T.C., *Identification of parotid salivary biomarkers in Sjogren's syndrome by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional difference gel electrophoresis.* Rheumatology, 2006. **45**: p. 1077.
45. Harthoorn, L.F., Schipper, R.G., Loof, A., Van Heerde, W.L., Cordewener, J.H.G., America, A.H.P., Vereijken, P., and Dransfield, E., *Identification of saliva biomarkers associated with food consumption and body weight in humans.* Submitted for publication.
46. de Roos, K.B., *Effect of texture and microstructure on flavour retention and release.* International Dairy Journal, 2003. **13**: p. 593.
47. Engelen, L., de Wijk, R.A., Prinz, J.F., and Bosman, F., *The relation between saliva flow after different stimulations and the perception of flavor and texture attributes in custard desserts.* Physiology & Behavior, 2003. **78**: p. 165.
48. Engelen, L., de Wijk, R.A., Prinz, J.F., Janssen, A.M., van den Bilt, A., Weenen, H., and Bosman, F., *A comparison of the effects of added saliva, alpha-amylase and water on texture perception in semisolids.* Physiology & Behavior, 2003. **78**: p. 805.
49. Malone, M.E., Appelqvist, I.A.M., and Norton, I.T., *Oral behaviour of food hydrocolloids and emulsions. Part 1. Lubrication and deposition considerations.* Food Hydrocolloids, 2003. **17**: p. 763.
50. Malone, M.E., Appelqvist, I.A.M., and Norton, I.T., *Oral behaviour of food hydrocolloids and emulsions. Part 2. Taste and aroma release.* Food Hydrocolloids, 2003. **17**: p. 775.
51. Vingerhoeds, M.H., Blijdenstein, T.B.J., Zoet, F., and van Aken, G.A., *Emulsion flocculation induced by saliva and mucin.* Food Hydrocolloids, 2005. **19**: p. 915.
52. Silletti, E., Vingerhoeds, M.H., Norde, W., and van Aken, G.A., *The role of electrostatics in saliva-induced emulsion flocculation.* Food Hydrocolloids, 2007. **21**: p. 596.
53. Canfield, R.E., *The amino acid sequence of egg white lysozyme.* Journal of Biological Chemistry, 1963. **238** p. 2698.
54. Jolles, J., Jauregui, A.J., Bernier, I., and Jolles, P., *La structure chimique du lysozyme de blanc dóeuf de poule: Etude detailee.* Biochimica et Biophysica Acta., 1963. **78**: p. 668.
55. McKenzie, H.A., *Milk protein chemistry and molecular biology*, ed. M. HA. Vol. 2. 1971, New York: Academic press. 257.
56. Hambling, S.G., McAlpine, A.S., and Sawyer, L., *Advance dairy chemistry -1: Proteins*, E.A. Science, Editor. 1992: Barking, UK. p. 141.
57. Monaco, H.L., Zanotti, G., Spadon, P., Bolognesi, M., Sawyer, L., and Eliopoulos, E.E., *Crystal structure of the trigonal form of bovine beta-lactoglobulin and of its complex with retinol at 2.5 a resolution.* Journal of Molecular Biology, 1987. **197**: p. 695.
58. Godovac-Zimmermann, J., *The structural motif of beta-lactoglobulin and retinol-binding protein: A basic framework for binding and transport of small hydrophobic molecules?* Trends in biochemical sciences, 1988. **13**: p. 64.
59. Vingerhoeds, M.H., Silletti, E., de Groot, J., Schipper, R.G., and van Aken, G.A., *Relating the effect of saliva-induced emulsion flocculation on rheological properties and adherence to the tongue surface with sensory perception.* Submitted for publication, 2007.
60. Yan, Q. and Bennick, A., *Identification of histatins as tannin-binding proteins in human saliva.* Biochemical Journal, 1995. **311**: p. 341.
61. Naczk, M., Amarowicz, R., Zadernowski, R., and Shahidi, F., *Protein precipitating capacity of condensed tannins of beach pea, canola hulls, evening primrose and faba bean.* Food Chemistry, 2001. **73**: p. 467.
62. Oh, H.I. and Hoff, J.E., *pH dependence of complex formation between condensed tannins and proteins.* Journal of Food Science, 1987. **52**: p. 1267.
63. Naurato, N., Wong, P., Lu, Y., Wroblewski, K., and Bennick, A., *Interaction of tannin with human salivary histatins.* Journal of Agricultural and Food Chemistry, 1999. **47**: p. 2229.
64. Lu, Y. and Bennick, A., *Interaction of tannin with human salivary proline-rich proteins.* Archives of Oral Biology, 1998. **43**: p. 717.
65. Yan, Q. and Bennick, A., *Identification of histatins as tannin-binding proteins in human saliva.* Biochemical Journal, 1995. **311**: p. 341.
66. Luck, G., Liao, H., Murray, N.J., Grimmer, H.R., Warminski, E.E., Williamson, M.P., Lilley, T.H., and Haslam, E., *Polyphenols, astringency and proline-rich proteins.* Phytochemistry, 1994. **37**: p. 357.

67. Gambuti, A., Rinaldi, A., Pessina, R., and Moio, L., *Evaluation of aglianico grape skin and seed polyphenol astringency by SDS-PAGE electrophoresis of salivary proteins after the binding reaction.* Food Chemistry, 2006. **97**: p. 614.
68. Russell, T.A., Drake, M.A., and Gerard, P.D., *Sensory properties of whey and soy proteins.* Journal of Food Science, 2006. **71**: p. S447.
69. Carunchia Whetstine, M.E., Croissant, A.E., and Drake, M.A., *Characterization of dried whey protein concentrate and isolate flavor.* Journal of Dairy Science, 2005. **88**: p. 3826.
70. Sano, H., Egashira, T., Kinekawa, Y., and Kitabatake, N., *Astringency of bovine milk whey protein.* Journal of Dairy Science, 2005. **88**: p. 2312.
71. Silletti, E., Vingerhoeds, M.H., Norde, W., and van Aken, G.A., *Complex formation in mixtures of lysozyme-stabilized emulsions and human saliva.* Journal of Colloid and Interface Science, 2007. **313**: p. 485.
72. de Jongh, H.H.J., Groneveld, T., and de Groot, J., *Mild isolation procedure discloses new protein structural properties of beta-lactoglobulin.* Journal of Dairy Science, 2001. **84**: p. 562.
73. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C., *Measurement of protein using bicinchoninic acid.* Analytical Biochemistry, 1985. **150** p. 76.
74. Hardt, M., Thomas, L.R., Newport, G., Agabian, N., Prakobphol, A., Hall, S.C., Witkowska, H.E., Fisher, S.J., and Dixon, S.E., *Toward defining the human parotid gland salivary proteome and peptidome: Identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry.* Biochemistry, 2005. **44**: p. 2885.
75. Xu, L., Lal, K., Santarpia, R.P., and Pollock, J.J., *Salivary proteolysis of histadine-rich polypeptides and the antifungal activity of peptide degradation products.* Archives of Oral Biology, 1993. **38**: p. 277.
76. Vitorino, R., Lobo, M.J.C., Duarte, J.R., Ferrer-Correia, A.J., Domingues, P.M., and Amado, F.M.L., *The role of salivary peptides in dental caries.* Biomedical Chromatography, 2005. **19**: p. 214.
77. Vitorino, R., Lobo, M.J.C., Duarte, J.A.R., Ferrer-Correia, A.J., Domingues, P.M., and Amado, F.M.L., *Analysis of salivary peptides using HPLC-electrospray mass spectrometry.* Biomedical Chromatography, 2004. **18**: p. 570.
78. Guo, T., Rudnick, P.A., Wang, W., Lee, C.S., DeVoe, D.L., and Balgley, B.M., *Characterization of the human salivary proteome by capillary isoelectric focusing/nanoreversed-phase liquid chromatography with ESI-tandem MS.* Journal of Proteome Research, 2006. **5**: p. 1469.
79. Al-Hashimi, I., Dickinson, D.P., and Levine, M.J., *Purification, molecular-cloning, and sequencing of salivary cystatin SA-I.* Journal of Biological Chemistry, 1988. **263**: p. 9381.
80. Walstra, P. and de Roos, A.L., *Proteins at air-water and oil-water interfaces - static and dynamic aspects.* Food Reviews International, 1993. **9**: p. 503.
81. Dickinson, E., *Protein-stabilized emulsions.* Journal of Food Engineering, 1994. **22**: p. 59.
82. Caessens, P.W.J.R., de Jongh, H.H.J., Norde, W., and Gruppen, H., *The adsorption-induced secondary structure of β-casein and of distinct parts of its sequence in relation to foam and emulsion properties.* Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology, 1999. **1430**: p. 73.
83. Lechevalier, V., Croguennec, T., Pezennec, S., Guerin-Dubiard, C., Pasco, M., and Nau, F., *Ovalbumin, ovotrasferrin, lysozyme: Three model proteins for structural modifications at the air-water interface.* Journal of Agricultural and Food Chemistry, 2003. **51**: p. 6354.
84. de Jongh, H.H.J., Kosters, H.A., Kudryashova, E., Meinders, M.B., Trofimova, D., and Wierenga, P.A., *Protein adsorption at air-water interfaces: A combination of details.* Biopolymers, 2004. **74**: p. 131.
85. Lu, J.R., Su, T.J., Thomas, R.K., Penfold, J., and Webster, J., *Structural conformation of lysozyme layers at the air-water interfaces studied by neutron reflection.* Journal of the Chemical Society Faraday Transactions, 1998. **94**: p. 3279.
86. Martin, A.H., Meinders, M.B.J., Bos, M.A., Cohen Stuart, M.A., and van Vliet, T., *Conformational changes of proteins at the air/water interface studied by IRRAS.* Langmuir, 2002. **19**: p. 2922.
87. de Roos, A.L. and Walstra, P., *Loss of enzyme activity due to adsorption onto emulsion droplets.* Colloids and Surfaces B: Biointerfaces, 1996. **6**: p. 201.
88. Iontcheva, I., Oppenheim, F.G., and Troxler, R.F., *Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins.* Journal of Dental Research, 1997. **76**: p. 734.
89. Soares, R.V., Lin, T., Siqueira, C.C., Bruno, L.S., Li, X., Oppenheim, F.G., Offner, G.D., and Troxler, R.F., *Salivary micelles: Identification of complexes containing MG2, sIgA, lactoferrin, amylase, glycolsylated proline-rich protein and lysozyme.* Archives of Oral Biology, 2004. **49**: p. 337.

90. Soares, R.V., Siqueira, C.C., Bruno, L.S., Oppenheim, F.G., Offner, G.D., and Troxler, R.F., *MG2 and lactoferrin form a heterotypic complex in salivary secretions.* Journal of Dental Research, 2003. **82**: p. 471.
91. Yao, Y., Lamkin, M.S., and Oppenheim, E.G., *Pellicle precursor proteins: Acidic proline-rich proteins, statherin, and histatins, and their crosslinking reaction by oral transglutaminase.* Journal of Dental Research, 1999. **78**: p. 1696.
92. Yao, Y., Lamkin, M.S., and Oppenheim, F.G., *Pellicle precursor protein crosslinking: Characterization of an adduct between acidic proline-rich protein (PRP-1) and statherin generated by transglutaminase.* Journal of Dental Research, 2000. **79**: p. 930.
93. Ogawa, S., Decker, E.A., and McClements, D.J., *Production and characterization of o/w emulsions containing cationic droplets stabilized by lecithin-chitosan membranes.* Journal of Agricultural and Food Chemistry, 2003. **51**: p. 2806.
94. Ogawa, S., Decker, E.A., and McClements, D.J., *Production and characterization of o/w emulsions containing droplets stabilized by lecithin-chitosan-pectin mutilayered membranes.* Journal of Agricultural and Food Chemistry, 2004. **52**: p. 3595.

Appendix A - Identified peptide after RP-HPLC coupled with MALDI-MS-MS in the PNA extracted after sample procedure from a lysozyme and β-lg stabilized emulsion after mixing with saliva.

PNA from lysozyme emulsion/saliva mixture								
Retention time	**Sequence**	**PTM**	**pI***	**Position**	**Observed Mass (Da)**	**Calculated Mass (Da)**	**Error (Da)**	**Precursor**
14.7	GPPPPPQGGRPHRPPQGQPPQ		12	289-309	2179.30	2179.13	0.18	b PRP 3 (Q04118)
	GRPQGPPQQGGHQQGPPPPP PGKPQ		11	123-147	2521.49	2521.28	0.21	a proline-rich phosphoprotein 1/2 (P02810)
	PPQGGNQSQGPPPHPGKPEG PPPQEGNKSRSA	*[8]	9.01	145-176	3292.00	3291.53	0.47	b PRP 4 allele S (P10163)
15.0	GPPPPPPGKPQGPPPQGGRPQ		11	137-157	2040.22	2040.08	0.15	a proline-rich phosphoprotein 1/2 (P02810)
	SPPGKPQGPPQQEGNKPQGP PPPGKPQ		9.7	178-204	2726.59	2726.40		b PRP 4 allele S (P10163)
15.3	PPPGKPQGPPPQGGRPQGPP QGQSPQ		11.01	141-166	2568.50	2568.31	0.14	a proline-rich phosphoprotein 1/2 (P02810)
15.7	GPPPPPQGGRPPRPAQGQQPPQ		12	226-247	2241.31	2241.16	0.15	b PRP 4 allele S (P10163)
16.0	PGPPPPPFTGADGQPAIPPP LSDTTKPK	*[9,25]	6.38	400-427	2937.62	2937.38	0.24	Wiskott-Aldrich syndrome protein family member 2 (Q9Y6W5)
17.7	GPPPPPPGKPQGPPPQGGRP QGPPQGQSPQ		11	137-166	2916.63	2916.49	0.14	a proline-rich phosphoprotein 1/2 (P02810)
19.3	PPPGGNPQQPLPPPAGKPQ		9.18	270-288	1874.05	1873.99	0.06	b PRP 3 (Q04118)
20.0	QRGPRGPYPP	#	10.84	23-32	1107.61	1107.57	0.04	b PRP 3 (P02814); PRP 5 (Q99954)
20.7	GPPPPGGNPQQPLPPPAGKP QGPPPPPQ		8.75	268-295	2698.56	2698.41	0.15	b PRP 3 (Q04118)
	GPPPPGGNPQQPLPPPAGKP QGPPPPPQG		8.75	268-296	2755.57	2755.43	0.14	b PRP 3 (Q04118)
	HHSHREFP		7.03	37-44	1046.53	1046.49	0.04	Histatin 1(P15515)
	PPPGGNPQQPLPPPAGKPQG PPPPPQ		9.18	270-295	2544.47	2544.34	0.13	b PRP 3 (Q04118)

Appendix A Continued

20.7	QRGPRGPYPPGP	#	10.84	23-34	1261.69	1261.64	0.05	b PRP 3 (P02814); PRP 5 (Q99954)
	VPPPPPPPYGPGR		8.72	46-58	1327.77	1327.72	0.05	b PRP 3 (P02814)
21.0	PQYQQY		5.95	55-60	826.40	826.37	0.03	Statherin (P02808)
	YQPQYQQY		5.52	53-60	1117.53	1117.50	0.04	Statherin (P02808)
21.3	GPGRIPPPPPAP		9.75	55-66	1152.69	1152.65	0.03	b PRP 3 (P02814)
	LFAEEKAVADTRDQADGSR		4.44	604-622	2079.09	2079.01	0.08	Polymeric-immunoglobulin receptor (P01833)
21.7	FHEKHHSHREFP		7.1	33-44	1587.73	1587.76	-0.03	Histatin 1(P15515)
	FIDEERQGPPLGGQQ		4.14	41-55	1670.80	1670.81	-0.01	a proline-rich phosphoprotein 1/2 (P02810)
	FIDEERQGPPLGGQQSQPS		4.14	41-59	2069.96	2069.99	-0.03	a proline-rich phosphoprotein 1/2 (P02810)
	LFAEEKAVADTR		4.68	604-615	1349.69	1349.71	-0.02	Polymeric-immunoglobulin receptor (P01833)
	PYQPQYQQY		5.94	52-60	1214.53	1214.55	-0.02	Statherin (P02808)
	YPQPYQPQYQQ		5.52	49-59	1439.64	1439.66	-0.02	Statherin (P02808)
22.3	GRIPPPPPAPYGPG		8.75	57-70	1372.80	1372.74	0.06	b PRP 3 (P02814)
	LYPQPYQPQ		5.52	48-56	1133.61	1133.56	0.05	Statherin (P02808)
23.3	GPGRIPPPPPAPY		8.75	55-67	1315.67	1315.72	-0.05	b PRP 3 (P02814)
	GPGRIPPPPPAPYGPG		8.75	55-70	1526.76	1526.81	-0.05	b PRP 3 (P02814)
	GRFGYGY		8.59	31-37	819.34	819.38	-0.04	Statherin (P02808)
	HEKHHSHREFPF		7.1	34-45	1587.71	1587.76	-0.05	Histatin 1(P15515)
	PTGGAPPPPPPPPPGP		5.96	386-402	1526.76	1526.80	-0.04	Wiskott-Aldrich syndrome protein family member 2 (Q9Y6W5)
	YGPYQPVPE		4.6	37-45	1049.45	1049.49	-0.04	Statherin (P02808)
23.7	EKHHSHREFPF		7.12	35-45	1450.76	1450.70	0.04	Histatin 1(P15515)
	YGPYQPVPEQP		4	37-47	1274.66	1274.61	0.05	Statherin (P02808)
24.0	IPPPPPAPY		5.52	59-67	948.55	948.52	0.03	b PRP 3 (P02814)
	KHHSHREFPF		8.77	36-45	1321.70	1321.65	0.05	Histatin 1(P15515)
	LPPPPPPFS		5.52	280-288	948.55	948.52	0.03	CG1 protein (Q13495)
	QPLYPQPYQPQ		5.52	46-56	1358.73	1358.67	0.05	Statherin (P02808)
	SSEEKFLRRIG	*[1]	8.46	21-31	1401.74	1401.69	0.05	Statherin (P02808)

24.0	YPQPYQPQY		5.52	49-57	1183.59	1183.54	0.05	Statherin (P02808)
24.3	EQPLYPQPYQPQ		4	45-56	1487.78	1487.72	0.06	Statherin (P02808)
	FHEKHHSHREFPF		7.1	33-45	1734.89	1734.82	0.07	Histatin 1(P15515)
	GGDSEQFIDE ERQGPPLGGQQ		3.83	35-55	2244.12	2244.02	0.11	a proline-rich phosphoprotein 1/2 (P02810)
	GRFGYGYGP		8.59	31-39	973.49	973.45	0.03	Statherin (P02808)
	GYGPYQPVPEQP		4	36-47	1331.62	1331.63	0.06	Statherin (P02808)
	PQPYQPQ		5.95	50-56	857.44	857.42	0.03	Statherin (P02808)
24.7	FVPPPPPPPYGPGR		8.75	45-58	1474.85	1474.78	0.06	b PRP 3 (P02814)
	GYGYGPYQP		5.52	34-42	1001.47	1001.44	0.04	Statherin (P02808)
	HHSHREFPF		7.03	37-45	1193.61	1193.56	0.05	Histatin 1(P15515)
	RGPYPPGPL		8.75	27-35	953.56	953.52	0.04	b PRP 3 (P02814); PRP 5 (Q99954)
	RIPPPPPAPY		8.75	58-67	1104.66	1104.62	0.04	b PRP 3 (P02814)
25.0	FYGDYGSNY		3.8	45-53	1085.46	1085.42	0.04	Histatin 1(P15515)
	QPLYPQPYQPQYQQ		5.52	46-59	1777.94	1777.85	0.09	Statherin (P02808)
	YPQPYQPQYQQY		5.52	49-60	1602.80	1602.72	0.08	Statherin (P02808)
	YQPVPEQPL		4	40-48	1070.59	1070.55	0.04	Statherin (P02808)
25.3	SLFLISGKPEGRRPQGGNQPQ		10.83	28-48	2266.31	2266.21	0.10	b PRP 4 allele S (P10163)
25.7	HSHREFPF		6.92	38-45	1056.54	1056.50	0.04	Histatin 1(P15515)
	QRGPRGPYPPGPL	#	10.84	23-35	1374.80	1374.73	0.07	b PRP 3 (P02814); PRP 5 (Q99954)
	RGPYPPGPLA PPQP		8.75	27-40	1443.83	1443.77	0.06	b PRP 3 (P02814)
	VPEQPLYPQPYQPQ		4	43-56	1683.92	1683.84	0.08	Statherin (P02808)
26.0	EQPLYPQPY		4	45-53	1134.59	1134.55	0.04	Statherin (P02808)
	EQPLYPQPYQPQYQ		4	45-58	1778.91	1778.84	0.07	Statherin (P02808)
	IGRFGYGY		8.59	30-37	932.50	932.46	0.04	Statherin (P02808)
	QRGPRGPYPPGPLAPPQP	#	10.84	23-40	1865.06	1864.98	0.08	b PRP 3 (P02814)
	SLFLISGKPEGRRPQ		10.83	28-42	1684.96	1684.95	0.01	b PRP 4 allele S (P10163)
26.3	AAPDEKVLDSGFREIENK		4.51	580-597	2018.10	2018.02	0.08	Polymeric-immunoglobulin receptor (P01833)
	GFVPPPPPPPYGPGR		8.75	44-58	1531.86	1531.81	0.06	b PRP 3 (P02814)
	GYGYGPYQPVPE		4.6	34-45	1326.66	1326.60	0.06	Statherin (P02808)
	GYGYGPYQPVPEQP		4	34-47	1551.78	1551.71	0.07	Statherin (P02808)
	QPVPEQPLYPQPYQPQ		4	41-56	1909.04	1908.95	0.09	Statherin (P02808)

Appendix A Continued

26.3	QRGPRGPYPPGPLAPP	#	10.84	23-38	1639.94	1639.87	0.07	PRP 5 (Q99954); b PRP 3 (P02814)
	VIGLQAPSTGLLGARPG		9.72	839-855	1606.83	1606.93	-0.10	Splicing factor, arginine/serine-rich 15 (O95104)
	VPEQPLYPQPYQPQYQQ		4	43-59	2103.11	2103.02	0.09	Statherin (P02808)
26.7	GPGFVPPPPPPYGPGR		8.75	42-58	1685.95	1685.88	0.07	b PRP 3 (P02814)
	HREFPF		6.75	40-45	832.44	832.41	0.03	Histatin 1(P15515)
	HREFPFYGD		5.45	40-48	1167.56	1167.52	0.04	Histatin 1(P15515)
	HSHREFPFYGDYGSN		5.99	38-52	1812.85	1812.77	0.07	Histatin 1(P15515)
	LAPPQPFGPG		5.52	35-44	980.55	980.52	0.03	b PRP 3 (P02814)
	SHREFPF		6.47	39-45	919.48	919.44	0.04	Histatin 1(P15515)
27.0	QFIDEERQGPPLGGQQSQPS	#	4.14	40-59	1225.71	1225.69	0.02	a proline-rich phosphoprotein 1/2 (P02810)
	VPEQPLYPQP YQPQYQ		4	43-58	1975.04	1974.96	0.08	Statherin (P02808)
27.3	GYGYGPYQPVP		5.52	34-44	1197.59	1197.56	0.03	Statherin (P02808)
	SHREFPFYG		6.47	39-47	1139.56	1139.53	0.04	Histatin 1(P15515)
	SHREFPFYGDYGSN		5.3	39-52	1675.77	1675.71	0.06	Histatin 1(P15515)
27.7	GRFGYGYGPYQPVPEQP		6	31-47	1911.90	1911.90	0.00	Statherin (P02808)
	HREFPFYGDYGSN		5.32	40-52	1588.68	1588.68	0.00	Histatin 1(P15515)
	PPPPPQPQ		5.96	976-983	857.41	857.45	-0.04	Bromodomain-containing protein 4 (O60885)
	QFIDEERQGPPLGGQQ	#	4.14	40-55	1781.84	1781.85	0.01	a proline-rich phosphoprotein 1/2 (P02810)
	QPVPEQPLYPQPY		4	41-53	1555.77	1555.78	-0.01	Statherin (P02808)
	YQPVPEQPLYPQPYQPQ		4	40-56	2072.03	2072.01	0.02	Statherin (P02808)
28.0	GPYQPVPEQPLYPQPYQPQ		4	38-56	2226.18	2226.09	0.10	Statherin (P02808)
	RFGYGYGPYQPVPEQP		6	32-47	1854.95	1854.88	0.06	Statherin (P02808)
	YGPYQPVPEQ PL		4	37-48	1387.74	1387.69	0.05	Statherin (P02808)
	YQPVPEQPLYPQPYQPQYQQ		4	40-59	2491.32	2491.19	0.13	Statherin (P02808)
28.3	GPLAPPQPFGPG		5.52	33-44	1134.63	1134.59	0.03	b PRP 3 (P02814)
	GYGPYQPVPEQPL		4	36-48	1444.76	1444.71	0.05	Statherin (P02808)
	HREFPFY		6.75	40-46	995.50	995.47	0.03	Histatin 1(P15515)
	PPPLSPPYGP GRIPPSPPPP	*[5,8,16]	9.18	64-83	2257.30	2256.99	0.31	PRP 5 (Q99954)

28.3	SHREFPFY		6.47	39-46	1082.55	1082.51	0.04	Histatin 1(P15515)
	VPPPPPPPYGPGRIPPPPPAPY		8.56	46-67	2257.30	2257.22	0.08	b PRP 3 (P02814)
28.7	GIFPPPPPQP		5.52	70-79	1046.54	1046.57	-0.03	b PRP 3 (P02814)
	GPGIFPPPPPQP		5.52	68-79	1200.62	1200.64	-0.02	b PRP 3 (P02814)
	PPPPPPFL		5.96	718-725	861.54	861.49	0.05	Splicing factor, arginine/serine-rich 15 (O95104)
	PPPPPPMI	O [7]	5.96	323-330	861.54	861.45	0.08	Wiskott-Aldrich syndrome protein family member 2 (Q9Y6W5)
	REFPFYGDYG SN		4.37	41-52	1451.60	1451.62	-0.03	Histatin 1(P15515)
29.0	GPGFVPPPPPPPY		5.52	42-54	1318.67	1318.68	-0.02	b PRP 3 (P02814)
	GPGFVPPPPPPPYGPG		5.52	42-57	1529.76	1529.78	-0.01	b PRP 3 (P02814)
	HREFPFYGDYGSNY		5.32	40-53	1751.73	1751.74	-0.01	Histatin 1(P15515)
	IGRFGYGYGPYQPVPEQP		6	30-47	2024.97	2024.99	-0.01	Statherin (P02808)
	PPLPLPPPPPPFSP	*[13]	5.96	276-289	1529.76	1529.78	-0.02	CG1 protein (Q13495)
	SHREFPFYGDYGSNY		5.3	39-53	1838.76	1838.78	-0.02	Histatin 1(P15515)
	YQPVPEQPLYPQPY		4	40-53	1718.84	1718.84	0.00	Statherin (P02808)
29.3	FGYGYGPYQPVPEQP		4	33-47	1698.84	1698.78	0.06	Statherin (P02808)
	HREFPFYGDY		5.32	40-49	1330.63	1330.59	0.04	Histatin 1(P15515)
	SHREFPFYGDY		5.3	39-49	1417.66	1417.62	0.05	Histatin 1(P15515)
	YGPYQPVPEQPLYPQP		4	37-52	1872.99	1872.92	0.08	Statherin (P02808)
	YGPYQPVPEQPLYPQPYQPQ		4	37-56	2389.27	2389.15	0.12	Statherin (P02808)
29.7	FGPGFVPPPPPPPYGPGR		8.75	41-58	1832.95	1832.95	0.01	b PRP 3 (P02814)
	GYGPYQPVPEQPLYPQP		4	36-52	1929.96	1929.94	0.02	Statherin (P02808)
	GYGPYQPVPEQPLYPQPYQPQ		4	36-56	2446.20	2446.17	0.03	Statherin (P02808)
	GYGYGPYQPVPEQPL		4	34-48	1664.80	1664.80	0.01	Statherin (P02808)
	RGPYPPGPLAPPQPFGPG		8.75	27-44	1801.94	1801.94	0.01	b PRP 3 (P02814)
	YGPGIFPPPPPQP		5.52	67-79	1363.70	1363.70	0.00	b PRP 3 (P02814)
30.0	GPRGPYPPGPLAPPQPFGPG		8.75	25-44	1956.06	1956.01	0.05	b PRP 3 (P02814)
	QPFGPGFVPPPPPPPYGPGR		8.75	39-58	2058.12	2058.06	0.06	b PRP 3 (P02814)
	QRGPRGPYPPGPLAPPQPFGPG	#	10.84	23-44	2223.22	2223.15	0.07	b PRP 3 (P02814)
30.3	REFPFYGDYGSNY		4.37	41-53	1614.72	1614.69	0.04	Histatin 1(P15515)
31.0	APPQPFGPGFVPPPPPPPYGPGR		8.79	36-58	2323.26	2323.20	0.05	b PRP 3 (P02814)

Appendix A Continued

	GPGFVPPPPPPPYGPGRIPP PPPAPY		8.59	42-67	2615.45	2615.38	0.07	b PRP 3 (P02814)
	GPGRIPPPPPAPYGPGIFPPPPPQP		8.75	55-79	2497.40	2497.34	0.06	b PRP 3 (P02814)
31.0	GRIPPPPPAPYGPGIFPPPP PQP		8.75	57-79	2343.32	2343.26	0.05	b PRP 3 (P02814)
	PPPPAPYGPGIFPPPPPQP		5.95	61-79	1920.05	1920.01	0.05	b PRP 3 (P02814)
	QRGPRGPYPPGPLAPPQPF	#	10.84	23-41	2012.10	2012.05	0.05	b PRP 3 (P02814)
	RGPYPPGPLAPPQPF		8.75	27-41	1590.88	1590.84	0.04	b PRP 3 (P02814)
	RIPPPPPAPYGPGIFPPPPPQP		8.75	58-79	2286.30	2286.24	0.05	b PRP 3 (P02814)
31.3	GPYPPGPLAPPQPFGPG		5.52	28-44	1645.88	1645.84	0.04	b PRP 3 (P02814)
	YPPGPLAPPQPFGPG		5.52	30-44	1491.80	1491.76	0.04	b PRP 3 (P02814)
31.7	GPGRIPPPPPAPYGPGIFP		8.75	55-73	1884.06	1884.02	0.04	b PRP 3 (P02814)
	PAPVPQQPPPPPQQPPPPPPPQ		5.96	750-771	2285.27	2285.21	0.06	Bromodomain-containing protein 4 (O60885)
32.0	IPPPPPAPYGPGIFPPPPPQP		6	59-79	2130.21	2130.14	0.07	b PRP 3 (P02814)
	LAPPQPFGPGFVPPPPPPPYGPGR		8.75	35-58	2436.35	2436.29	0.06	b PRP 3 (P02814)
	RIPPPPPAPYGPGIFP		8.75	58-73	1672.96	1672.92	0.04	b PRP 3 (P02814)
	YPPGPLAPPQPF		5.52	30-41	1280.69	1280.67	0.01	b PRP 3 (P02814)
32.3	GPYPPGPLAPPQPF		5.52	28-41	1434.77	1434.74	0.03	b PRP 3 (P02814)
32.7	FGPGFVPPPPPPPY		5.52	41-54	1465.73	1465.75	-0.02	b PRP 3 (P02814)
	GPLAPPQPFGPGFVPPPPPP PYGPGR		8.75	33-58	2590.32	2590.36	-0.04	b PRP 3 (P02814)
	QPPPLPLPPPPPPFSPQSLMVSC	O [20]; *[22]; P[23]	5.52	274-296	2590.32	2590.25	0.07	CG1 protein (Q13495)
	VIGLQAPSTGLLGARPGLI		9.72	839-857	1832.91	1833.10	-0.18	Splicing factor, arginine/serine-rich 15 (O95104)
33.0	APPQPFGPGFVPPPPPPPYG PG		5.57	36-57	2167.12	2167.10	0.02	b PRP 3 (P02814)
	QPFGPGFVPPPPPPPYGPGR	#	8.75	39-58	2041.04	2041.03	0.01	b PRP 3 (P02814)
33.3	APPQPFGPGFVPPPPPPPY		5.57	36-54	1956.03	1956.01	0.02	b PRP 3 (P02814)
	QLSLPRFPS	#	9.75	101-109	1027.56	1027.56	0.00	PRP 4 (Q16378)
	QRGPRGPYPPGPLAPPQPFGPGF	#	10.84	23-45	2370.23	2370.21	0.01	b PRP 3 (P02814)
33.7	GPLAPPQPFGPGF		6.52	33-45	1281.64	1281.66	-0.02	b PRP 3 (P02814)

33.7	GPRGPYPPGPLAPPQPFGPGF		8.75	25-45	2103.06	2103.08	-0.02	b PRP 3 (P02814)
34.0	QLSLPRFPSVS	#	9.75	101-111	1200.63	1200.64	-0.01	PRP 4 (Q16378)
34.7	PPPPPPPY		5.95	47-54	861.44	861.45	-0.01	b PRP 3 (P02814)
35.3	GPYPPGPLAPPQPFGPGF		5.52	28-45	1792.91	1792.91	0.00	b PRP 3 (P02814)
	QLSLPRFP	#	9.75	101-108	940.52	940.53	0.00	PRP 4 (Q16378)
	QLSLPRFPSVSLQEAS	#	6	101-116	1741.91	1741.91	0.00	PRP 4 (Q16378)
35.7	PPGPLAPPQPFGPGF		5.96	31-45	1475.77	1475.77	0.00	b PRP 3 (P02814)
36.0	QLSLPRFPSV	#	9.75	101-110	1315.71	1315.72	0.00	PRP 4 (Q16378)
	QLSLPRFPSVSLQ	#	9.75	101-113	1454.79	1454.80	-0.01	PRP 4 (Q16378)
	VPPPPPPPY		5.49	46-54	960.60	960.52	0.08	b PRP 3 (P02814)
45.3	LLILLSVA	*[6]	5.52	2-9	927.51	927.49	0.02	a proline-rich phosphoprotein 1/2(P02810); b PRP 3 (Q04118); b PRP 4 allele S (P10163)
49.0	QQQPPPPPPPQ	#	5.52	971-981	1193.58	1193.60	-0.02	Bromodomain-containing protein 4 (O60885)

PNA from β-lg emulsion/saliva mixture

Retention time	Sequence	PTM	pI*	Position	Observed Mass (Da)	Calculated Mass (Da)	Error (Da)	Precursor
16.0	GPPQQGGHPPPPQGRPQGPP QQGGHPRPP		12	93-121	2937.56	2937.47	0.08	a proline-rich phosphoprotein 1/2 (P02810)
19.3	GPPQQEGNNPQGPPPPAGGN PQQPQAPPAGQPQGPPRPPQ		6	344-383	3964.10	3963.92	0.18	b PRP 1 (P04280)
19.7	GPPPPAGGNPQQPQAPPAGQ PQGPPRPP		9.75	355-382	2689.49	2689.36	0.13	b PRP 1 (P04280)
	GPPPPAGGNPQQPQAPPAGQ PQGPPRPPQG		9.75	355-384	2874.59	2874.44	0.15	b PRP 1 (P04280)
	GPPPPGGNPQQPLPPPAGKP Q		8.75	268-288	2028.16	2028.07	0.09	b PRP 3 (Q04118)
	GPPQQGGHPRPP		9.76	110-121	1224.69	1224.62	0.07	a proline-rich phosphoprotein1/2 (P02810)
	PPPPGGNPQQPLPPPAGKPQ		9.18	269-288	1971.13	1971.04	0.08	b PRP 3 (Q04118)
21.3	GPGRIPPPPPAP		9.75	55-66	1152.70	1152.65	0.05	b PRP 3 (P02814)
	GRIPPPPPAP		9.75	57-66	998.63	998.58	0.05	b PRP 3 (P02814)

Appendix A Continued

	LRRIGRF		12.3	27-33	917.62	917.58	0.05	Statherin (P02808)
	PGRIPPPPPAP		10.18	56-66	1095.69	1095.63	0.06	b PRP 3 (P02814)
21.3	PTLPPPPLS	* [9]	5.96	376-384; 504-512	998.63	998.50	0.13	Wiskott-Aldrich syndrome protein family member 2 (Q9Y6W5); Wiskott-Aldrich syndrome protein family member 4 (Q8IV90)
	YPQPYQPQYQQ		5.52	49-59	1439.70	1439.66	0.04	Statherin (P02808)
22.0	FIDEERQGPPLGGQQ		4.14	41-55	1670.85	1670.81	0.04	A proline-rich phosphoprotein 1/2 (P02810)
	GRIPPPPPAPY		8.75	57-67	1161.67	1161.64	0.03	b PRP 3 (P02814)
	QPTGGAPPPPPPPP		5.52	385-398	1306.71	1306.68	0.03	Wiskott-Aldrich syndrome protein family member 2 (Q9Y6W5)
	RIPPPPPAPY		8.75	58-67	1104.65	1104.62	0.03	b PRP 3 (P02814)
	YPTLPPPPLS	*[10]	5.52	375-384; 503-512	1161.67	1161.56	0.11	Wiskott-Aldrich syndrome protein family member 2 (Q9Y6W5) and member 4 (Q8IV90)
22.3	GPGRIPPPPPAPYGPG		8.75	55-70	1526.85	1526.81	0.04	b PRP 3 (P02814)
	PPVPPPPPY		5.95	814-822	960.53	960.52	0.02	Large proline-rich protein BAT2 (P48634)
	PTGGAPPPPPPPPPGP		5.96	386-402	1526.85	1526.80	0.05	Wiskott-Aldrich syndrome protein family member 2 (Q9Y6W5)
	VPPPPPPPY		5.49	46-54	960.53	960.52	0.02	b PRP 3 (P02814)
22.7	GPGRIPPPPPAPY		8.75	55-67	1315.75	1315.72	0.04	b PRP 3 (P02814)
	GPGRIPPSPPP	* [8]	9.75	72-82	1151.74	1151.56	0.17	PRP 5 (Q99954)
	PGRIPPPPPAPY		9.18	56-67	1258.76	1258.69	0.06	b PRP 3 (P02814)
23.3	GPGRIPPPPPAPYGP		8.75	55-69	1469.76	1469.79	-0.03	b PRP 3 (P02814)
	IPPPPPAPY		5.52	59-67	948.49	948.52	-0.03	b PRP 3 (P02814)
23.7	QNLNEDVSQEESPSLIAGNP QGPSPQGGNKPQGPPPPPGKPQ	#; * [8]	4.41	17-58	4332.07	4332.02	0.05	b PRP 1 (P04280)
	RGPYPPGPL		8.75	27-35	953.53	953.52	0.01	b PRP 3 (P02814)

24.7	QNLNEDVSQEESPSLIAGNP QGPSPQGGNKPQ	#; * [12]	4	17-48	3379.48	3379.51	-0.03	b PRP 1 (P04280)
	QRGPRGPYPPGPL	#	10.84	23-35	1374.72	1374.73	0.00	b PRP 3 (P02814)
24.7	RGPYPPGPLAPPQP		8.75	27-40	1443.76	1443.77	-0.02	b PRP 3 (P02814)
25.0	GPYQPVPEQPL		4	38-48	1224.58	1224.63	-0.05	Statherin (P02808)
	QRGPRGPYPPGPLAPPQP	#	10.84	23-40	1864.94	1864.98	-0.04	b PRP 3 (P02814)
25.3	GFVPPPPPPPYGPGR		8.75	44-58	1531.77	1531.81	-0.04	b PRP 3 (P02814)
25.7	GYGYGPYQPVPEQP		4	34-47	1551.66	1551.71	-0.06	Statherin (P02808)
	IGRFGYGYGP		8.59	30-39	1086.54	1086.54	0.01	Statherin (P02808)
26.0	AVRPPPAPATRVL		12	2007-2019	1344.66	1344.81	-0.15	Large proline-rich protein BAT2 (P48634)
26.3	GPGFVPPPPPPPYGPGR		8.75	42-58	1685.87	1685.88	-0.01	b PRP 3 (P02814)
	SSVVSPSHPPPAP	* [1,5,7]	6.46	292-304; 420-432	1498.74	1498.54	0.20	Wiskott-Aldrich syndrome protein family member 2 (Q9Y6W5) and member 4 (Q8IV90)
26.7	GRFGYGYGPYQPVPEQP		6	31-47	1911.90	1911.90	-0.01	Statherin (P02808)
27.7	GIFPPPPPQP		5.52	70-79	1046.55	1046.57	-0.02	b PRP 3 (P02814)
	GPGIFPPPPPQP		5.52	68-79	1200.63	1200.64	-0.01	b PRP 3 (P02814)
28.3	PPPPPLFP		5.96	56-63	861.46	861.49	-0.03	Acetylcholinesterase collagenic tail peptide (Q9Y215)
	PPPPPLPF		5.96	259-266	861.46	861.49	-0.03	Protein fosB (P53539)
	PPPPPVEE		4.24	403-410	861.46	861.44	0.03	Abl-interactor 2 (Q9NYB9)
28.7	GPGFVPPPPPPPY		5.52	42-54	1318.67	1318.68	-0.01	b PRP 3 (P02814)
29.3	GPRGPYPPGPLAPPQPFGPG		8.75	25-44	1956.04	1956.01	0.03	b PRP 3 (P02814)
	GYGYGPYQPVPEQPL		4	34-48	1664.79	1664.80	-0.01	Statherin (P02808)
	QRGPRGPYPPGPLAPPQPFGPG	#	10.84	23-44	2223.17	2223.15	0.02	b PRP 3 (P02814)
	RGPYPPGPLAPPQPFGPG		8.75	27-44	1801.96	1801.94	0.02	b PRP 3 (P02814)
29.7	APPQPFGPGF		5.57	36-45	1014.52	1014.50	0.02	b PRP 3 (P02814)
	FVPPPPPPPYGPGRIPPPPPAPY		8.59	45-67	2404.33	2404.29	0.05	b PRP 3 (P02814)
	PPQAPFGFPG		5.96	315-324	1014.52	1014.50	0.02	Splicing factor, arginine/serine-rich 15 (O95104)
	RGPYPPGPLAPPQPF		8.75	27-41	1590.87	1590.84	0.03	b PRP 3 (P02814)

Appendix A Continued

30.0	GPGRIPPPPPAPYGPGIFPPPPPQP		8.75	55-79	2497.39	2497.34	0.05	b PRP 3 (P02814)
	GPRGPYPPGPLAPPQPF		8.75	25-41	1744.95	1744.92	0.03	b PRP 3 (P02814)
	QRGPRGPYPPGPLAPPQPF	#	10.84	23-41	2012.09	2012.05	0.04	b PRP 3 (P02814)
	RFGYGYGPYQPVPEQPL		6	32-48	1968.03	1967.96	0.06	Statherin (P02808)
30.7	GFVPPPPPPPYGPGRIPPPP PAPY		8.59	44-67	2461.35	2461.31	0.04	b PRP 3 (P02814)
31.3	APPQPFGPGFVPPPPPPP		5.57	36-53	1792.96	1792.94	0.02	b PRP 3 (P02814)
	IGRFGYGYGPYQPVPEQPL		6	30-48	2138.12	2138.07	0.05	Statherin (P02808)
	PGFVPPPPPPPYGPGRIPPPPPAPY		9	43-67	2558.43	2558.36	0.07	b PRP 3 (P02814)
	PIAVPTPSPPSVFP	* [6,8,11]	5.96	270-283	1645.85	1645.67	0.18	Abl-interactor 2 (Q9NYB9)
	PPQPFGPGFVPPPPPPP		5.96	37-53	1721.92	1721.91	0.02	b PRP 3 (P02814)
31.7	LAPPQPFGPGFVPPPPPPPY GPGR		8.75	35-58	2436.27	2436.29	-0.01	b PRP 3 (P02814)
	YPPGPLAPPQPF		5.52	30-41	1280.64	1280.67	-0.03	b PRP 3 (P02814)
32.3	ACCLLTPPPPLFP		5.55	50-63	1465.76	1465.76	0.00	Acetylcholinesterase collagenic tail peptide (Q9Y215)
	FGPGFVPPPPPPPY		5.52	41-54	1465.76	1465.75	0.01	b PRP 3 (P02814)
	GPYPPGPLAPPQPF		5.52	28-41	1434.76	1434.74	0.02	b PRP 3 (P02814)
32.7	QPFGPGFVPPPPPPPY		5.52	39-54	1690.88	1690.86	0.02	b PRP 3 (P02814)
33.0	ACCLLTPPPPLFPP		5.55	50-64	1562.83	1562.81	0.02	Acetylcholinesterase collagenic tail peptide (Q9Y215)
	PFGPGFVPPPPPPPY		5.95	40-54	1562.83	1562.80	0.02	b PRP 3 (P02814)
	PQPFGPGFVPPPPPPPY		5.95	38-54	1787.94	1787.92	0.02	b PRP 3 (P02814)
	QLSLPRFPS	#	9.75	101-109	1027.59	1027.56	0.04	PRP 4 (Q16378)
33.3	DGFSWLLPPPPPPPLPF	* [4]	3.8	250-266	1954.11	1953.96	0.15	Protein fosB (P53539)
	PPPPPAPYGPGIFP	* [8]	5.95	60-73	1483.83	1483.70	0.12	b PRP 3 (P02814)
33.7	APPQPFGPGFVPPPPPPPY		5.57	36-54	1956.06	1956.01	0.05	b PRP 3 (P02814)
	APPQPFGPGFVPPPPPPPYG P		5.57	36-56	2110.14	2110.08	0.06	b PRP 3 (P02814)
	GHKACCLLTPPPPLFPP		8.07	47-64	1885.03	1884.99	0.04	Acetylcholinesterase collagenic tail peptide (Q9Y215)
	GHKACCLLTPPPPLFPP	P [6]	8.07	47-64	1956.06	1956.02	0.03	Acetylcholinesterase collagenic tail peptide (Q9Y215)

33.7	PPPPPPFL		5.96	718-725	861.49	861.49	0.00	Splicing factor, arginine/serine-rich 15 (O95104)
	PPPPPPMI	O [7]	5.96	323-330; 451-458	861.49	861.45	0.03	Wiskott-Aldrich syndrome protein family member 2 (Q9Y6W5)and member 4 (Q8IV90)
	PPPPPPPY		5.95	47-54; 167-174	861.49	861.45	0.04	b PRP 3 (P02814); early growth response protein 2 (P11161)
	PPQPFGPGFVPPPPPPPY		5.95	37-54	1885.03	1884.97	0.06	b PRP 3 (P02814)
	RGPYPPGPLAPPQPFGPGFV PPPPPPP		8.75	27-53	2727.56	2727.44	0.11	b PRP 3 (P02814)
34.0	LYPPPSFLYSPAFCP	*[2,9,10]	5.52	1906-1920	1938.98	1938.72	0.25	Large proline-rich protein BAT2 (P48634)
	PPQPFGPGFVPPPPPPPYGP		5.95	37-56	2039.11	2039.04	0.06	b PRP 3 (P02814)
	RGPYPPGPLAPPQPFGPGFVPPPPP		8.75	27-51	2533.44	2533.34	0.10	b PRP 3 (P02814)
	YSQNPVSDTPPPPPPVEE		3.91	393-410	1950.07	1949.91	0.16	Abl-interactor 2 (Q9NYB9)
34.3	QLSLPRFPSVS	#	9.75	101-111	1213.69	1213.66	0.04	PRP 4 (Q16378)
35.0	LAPPQPFGPGFVPPPPPPPY		5.52	35-54	2069.13	2069.09	0.04	b PRP 3 (P02814)
35.3	QLSLPRFP	#	9.75	101-108	940.55	940.53	0.03	PRP 4 (Q16378)
	QLSLPRFPSVSLQEASS	#	6	101-117	1829.01	1828.94	0.06	PRP 4 (Q16378)
	RGPYPPGPLAPPQPFGPGFV PPPPPPPY		8.59	27-54	2890.63	2890.51	0.12	b PRP 3 (P02814)
	RGPYPPGPLAPPQPFGPGFV PPPPPPPYGP		8.59	27-56	3044.72	3044.58	0.14	b PRP 3 (P02814)
35.7	GPLAPPQPFGPGFVPPPPPPPY		5.52	33-54	2223.22	2223.16	0.06	b PRP 3 (P02814)
	GVCTMSQTQPDLDHLYSPPP PPPPY	* [4]; P[3]	4.2	150-174	2888.60	2888.27	0.33	Early growth response protein 2 (P11161)
	PAPPLGSPPSSKPGFAPPPA PPPPPPPMI	* [11]; O [28]	9.18	430-458	2888.60	2888.45	0.15	Wiskott-Aldrich syndrome protein family member 4 (Q8IV90)
	QLSLPRFPSVSLQEAS	#	6	101-116	1741.95	1741.91	0.04	PRP 4 (Q16378)
36.0	PPGPLAPPQPFGPGFVPPPPPPPY		5.95	31-54	2417.35	2417.27	0.09	b PRP 3 (P02814)
36.3	QLSLPRFPSV	#	9.75	101-110	1126.65	1126.63	0.03	PRP 4 (Q16378)
	QLSLPRFPSVSLQ	#	9.75	101-113	1454.84	1454.80	0.04	PRP 4 (Q16378)

Appendix A Continued

36.7	PASNQPKRPPAAP		11.01	117-129	1330.71	1330.72	-0.01	Large proline-rich protein BAT2 (P48634)
37.3	DQKKRGGHKACCLLTPPPPPLFP	* [15]	9.39	41-63	2580.44	2580.30	0.15	Acetylcholinesterase collagenic tail peptide (Q9Y215)
	YPPGPLAPPQPFGPGFVPPPPPPPY		5.52	30-54	2580.44	2580.33	0.11	b PRP 3 (P02814)
40.3	GPGRIPPSPPPPY		8.75	72-84	1331.68	1331.71	-0.03	PRP 5 (Q99954)
41.0	DVAGTEMSQSDSGVDLSGDS QVSSGPCSQ	O [7]; * [23,24]	3.28	1625-1653	3006.55	3006.09	0.46	Large proline-rich protein BAT2 (P48634)
42.7	ATGPLGVCTMSQTQPDLDHL YSPPPPPPPY SGC	O [10]; * [2,9,11]; P [33]	4.2	145-177	3751.05	3750.50	0.55	Early growth response protein 2 (P11161)
44.0	GPPPPPPGKPQ		8.75	137-147	1068.56	1068.58	-0.03	a proline-rich phosphoprotein 1/2 (P02810)
	PGPPPPITPPV		5.96	741-751	1068.56	1068.61	-0.05	Splicing factor, arginine/serine-rich 15 (O95104)
45.7	PPPPPPPPAADYPTLPPPP	* [14]	3.8	364-382	1994.97	1994.97	0.00	Wiskott-Aldrich syndrome protein family member 2 (Q9Y6W5)
46.3	GPPPPITPPV		5.52	742-751	971.51	971.56	-0.05	Splicing factor, arginine/serine-rich 15 (O95104)
	GPPPPPGKPQ		8.75	49-58	971.51	971.53	-0.02	b PRP 1 (P04280)
49.7	GPPQQGGHQQ		6.74	127-136	1033.50	1033.48	0.02	a proline-rich phosphoprotein 1/2 (P02810)
	PGKPQGPPQQ		9.18	237-246	1033.50	1033.54	-0.04	b PRP 1 (P04280)
51.0	GPPPHPGKPQ		8.76	238-247	1011.52	1011.54	-0.02	b PRP 3 (Q04118)
53.0	GPPPQGGNKPQ		8.75	59-69	1076.53	1076.55	-0.02	b PRP 1 (P04280)

#: pyroglutamination at Glutamine (Q) at N-terminal end of the sequence;: phosphorylation at Serine (S), Threonine (T) and Tyrosine (Y);O: oxidation at M; P: propionamide at C; [ ] indicates the amino acid residue of the peptide sequence where PTM is occurring; a: acidic; b: basic; ( ) indicates the Swiss-Prot data base accession number.*

Chapter 6

Characterization of salivary proteins interacting with lysozyme and β-lactoglobulin stabilized emulsions

In memory of Nol

Alla memoria di Nol

E. Silletti, R.M.P. Vitorino, R.G. Schipper, F.M.L. Amado and M.H. Vingerhoeds

To be submitted

Abstract

In this chapter, we investigated the interaction of emulsions, stabilized by lysozyme and β-lactoglobulin (β-lg), with salivary proteins and peptides (SPs) with a molecular mass (M_r) above about 10 kDa using different techniques, i.e. infrared spectroscopy, Western blotting, PAS staining and SDS-PAGE coupled to MS.

Results show that the high M_r mucin MUC5B was strongly bound to lysozyme stabilized emulsions, whereas β-lg stabilized emulsion droplets associated with mucin MUC7 and, moderately, MUC5B. Furthermore, we observed that salivary proteins in the range M_r 10-100 kDa also associated differently with emulsion droplets. A large majority of SPs was found to interact with lysozyme stabilized emulsion droplets while in case of β-lg stabilized emulsions, the SPs distribute more evenly between the fraction associated and non-associated with the droplets. A clear example is α-amylase (M_r ~ 55 kDa) which predominantly associates with lysozyme stabilized emulsion droplets, but not with β-lg emulsion droplets. Our findings indicate that adsorption/association of salivary protein components onto the droplets surface is related to the type of emulsifying proteins at the oil-water interfaces.

Introduction

Saliva has multifunctional roles in speech, lubrication, digestion of food and maintaining oral and general health [1-3]. Among other proteins, saliva is constituted of glycoproteins, α-amylase, immunoglobulins, lactoferrin and a wide range of peptides such as cystatins, statherin, histatins and proline-rich proteins [2, 4-6]. The molecular mass (M_r) of salivary proteins and peptides varies from a few kDa for small peptides to more than 200 kDa for the large secreted glycoproteins, i.e. mucins [7]. Two types of secretory mucins are present in human saliva: the polymeric MUC5B (M_r 1000-40000 kDa), which consists of about 15% protein and 78% carbohydrate, and the monomeric MUC7 (M_r 200-300 kDa) comprised of about 30% protein and 68% carbohydrate [8-11]. Fucose, galactose, mannose, sialic acid, N-acetyl-glucosamine and N-acetyl-galactosamine constitute the typical monosaccharides present in salivary mucins [12-14]. Different species of MUC5B varying in the carbohydrate moiety and length of polysaccharide chains have been detected [14-19]. Besides the difference in M_r, MUC7 differs from MUC5B in the number and type of residues on the carbohydrate chain. Two different isoforms of MUC7 varying in sialic acid and fucose content have been reported [12].

Several separation techniques have been used to analyze saliva and to evaluate its protein composition (see [20] for a recent review). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been widely employed to study the protein composition, typically in the range M_r 10-120 kDa, of whole saliva [21], specific gland saliva [22-24] and salivary complexes [25, 26]. Moreover, SDS-PAGE has proven a valuable technique for characterization of salivary proteins interacting with the tooth surface, thereby forming the acquired enamel pellicle [21, 23, 27, 28] and those adsorbed at air-water interfaces [29]. When SDS-PAGE is used to analyze mucin containing samples, these molecules are found on the top of the gel, since even in a 4% running gel mucins migrate only a small distance into the gel. Detection of mucins (and other glycoproteins) is typically performed with period acid Schiff (PAS) staining or by using specific monoclonal antibodies [16, 18, 23, 25, 30, 31].

Another technique which is emerging as a rapid tool to analyze, for example, mucous samples, is Fourier Transform Infrared spectroscopy (FTIR) [32-34]. Recently, a methodology using Attenuated Total Reflection infrared spectroscopy (ATR-FTIR) for the analysis of swabs taken from distinct parts of the oral cavity after oral processing of

dressings and emulsions was reported (Chapter 7) [35, 36]. The interpretation of infrared spectra, for the analysis of the sample composition, involves the correlation of absorption bands in the obtained infrared spectrum with the known absorption frequencies for different types of molecular bonds.

The increasing interest of food scientists to understand the influence of saliva on sensory perception has raised the issue of how saliva interacts with food products and its ingredients. Biochemical techniques, e.g., SDS-PAGE, have been used to investigate, for example, the interaction between polyphenols and salivary proteins in order to understand the molecular origin of astringency [37, 38].

Recently, we used SELDI-TOF-MS and liquid chromatography coupled to mass spectrometry (LC-MS) to study the interaction between salivary proteins/peptides (SPs) and emulsion droplets (Chapter 5) [39]. A large number of SPs in the molecular mass range below 20 kDa were found to be associated with emulsion droplets stabilized by lysozyme and β-lactoglobulin (β-lg). The differences in the salivary proteins found at the oil-water interface of the two investigated emulsions were attributed to a selective interaction between SPs and the droplet surface (Chapter 5) [39].

Knowledge of the interaction between emulsion droplets and salivary proteins is important to understand the oral behavior of emulsions in relation to sensory perception. Positively charged emulsions, e.g., stabilized by lysozyme (M_r of about 14 kDa and a pI ~ 10.5), and negatively charged emulsions, i.e. stabilized by β-lg (M_r ~ 18 kDa and a pI ~ 4.9) showed, as a consequence of flocculation, different rheological behavior upon mixing with saliva (Chapters 2 and 4) [40, 41] and differences in sensory perception (Chapter 7) [35]. Lysozyme stabilized emulsions were characterized by attributes such as dryness, roughness and astringency, while emulsions stabilized by isolated whey protein, of which β-lg is the main component, were perceived as creamy and fatty (Chapter 7) [35, 42]. Astringent sensation characterizes, for example, the perception of red wine, grapefruit and tea. It is attributed to the formation of insoluble complexes between salivary histatins and proline-rich proteins with tannins [43, 44]. In case of lysozyme stabilized emulsion/saliva mixtures it was observed that emulsion droplets where imbedded in a proteinaceous matrix which led to the formation of flocs (Chapter 3) [45].

This chapter describes the results of a study aiming to investigate the interaction between emulsion droplets stabilized by lysozyme and β-lg, respectively, and salivary proteins with

M_r above 10 kDa by using different techniques, i.e. infrared spectroscopy, Western blotting and SDS-PAGE coupled to mass spectrometry (1D-MS).

Materials and methods

Materials

Freeze-dried β-lactoglobulin was provided by TI Food and Nutrition (TIFN, Wageningen, The Netherlands) and was purified as described previously [46]. The powder contains 93.6 % w/w proteins (N x 6.38). Lysozyme from chicken egg white, purchased from Belovo (L6876, batch 016K1189), was desalted on a Sephadex G10 column (flow rate was 40 ml min^{-1} and pressure 0.4 mPa, eluted with water de-gassed with He). The eluent was freeze dried and stored at –20 ^{o}C until further use. Bovine serum albumin (A-7030-100g, batch 109H1072) and casein (C8654-500g batch 026K0156) were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands) and used without further purification. Sunflower oil (Reddy, Vandemoortele, The Netherlands) was purchased from a local retailer; BCA^{TM} Protein Assay Kit was from Pierce Biotechnology Inc. (Rockford, IL, USA), RCDC Protein Assay from Bio-Rad (500-0121, Bio-Rad Laboratories, USA) and sodium azide was obtained from Merck (Shuchardt, Germany). Protease inhibitors (Proteases inhibitor Cocktail P8340-5mL, 096K4130) were purchased from Sigma (Sigma, St Louis, MI, USA). Primary antibody against MUC5B (F2; batch 5G2) and MUC7 (CpMG2; batch $2A_4$) were kindly donated by Prof. dr. E.C.I. Veerman from the Vrije Universiteit (Amsterdam). Polyclonal Goat Anti-Rabbit Immunoglobulins/AP (D0487, DakoCytomation Denmark A/S, Glostrup, Denmark) and Rabbit anti-Mouse IgG (H+L) conjugated with alkaline phosphatase (81-6722, Zymed Laboratories, CA94080, USA) were used as secondary antibodies for staining MUC5B and MUC7, respectively. Fuchin Sulfite reagent (S5133) was purchased from Sigma (St Louis, MI, USA), trypsin from Promega (Madison, WI, USA), and α-Cyano-4-hydroxycinnamic acid and calibrant mixture for the 4700 Proteomics Analyser were purchased from Applied Biosystems (Foster City, CA, USA).

Saliva, emulsions and samples preparation

Whole human unstimulated saliva was collected according to the procedure described in chapter 2 [40]. After collection and centrifugation, saliva supernatant, indicated as saliva, was frozen in liquid nitrogen, stored at –80 ^{o}C and used within six weeks. Salivary protein content, determined according to the BCA method of Pierce [47] using bovine serum albumin as standard, varied from 1.1 mg mL^{-1} to 1.4 mg mL^{-1}.

β-Lg and lysozyme stabilized emulsions, pH 6.7, contained 40 % w/w and 20 % w/w sunflower oil, respectively, 1 % w/w protein, 10 mM NaCl and were prepared according to the procedure in chapter 2 [40]. Sodium azide (0.02 % w/w) was added to the emulsions to prevent microbial growth.

Emulsion/saliva mixtures were prepared by mixing the required amount of emulsions with saliva, which was removed from –80 ^{o}C and thawed at room temperature shortly before each experiment. The final SP concentration in the mixtures was 0.6 mg mL^{-1}. The emulsion oil content was 2.5 % w/w in infrared spectroscopy experiments, and 10 % w/w in SDS-PAGE and Western blotting experiments. If necessary the volume was adjusted by addition of a 10 mM NaCl solution.

Protease inhibitors were added to the mixtures according to Schipper et al. [48]. Sample fractionation (Figure 1) was performed according to the protocol reported in chapter 5 with some minor changes [39]. Briefly, the mixtures were centrifuged twice for 30 minutes and once for 1 hour at 15000 g and 4 ^{o}C using a Beckman LB-60 Ultracentrifuge (rotor SW 41, Beckman Coulter, Mijdrecht, The Netherlands). After each centrifugation step, the aqueous phases containing proteins not associated with the emulsion droplets, were collected and stored at -80 ^{o}C until further use. The emulsion oil droplets, present in the so-called cream layers at the top of the tube, were removed and gently suspended in 10 mM NaCl by using a Vortex Mix VM-300 (Gemmy Industrial Corporation, Taiwan) prior to the following centrifugation step. After the third centrifugation, the cream layer was suspended in a solution of 2% SDS and kept under gentle agitation at room temperature for 48 hours before being centrifuged for the last time, at 120000 g at 20 ^{o}C for 1 hour. The aqueous phase with salivary proteins initially associated with the emulsion droplets is indicated in the text with PA (Protein-Associated). Before further analysis, samples were concentrated using Centriprep® Centrifugal Filter Units with a cut-off membrane of 3 kDa (4302, Milipore B.V., Amsterdam, the Netherlands). Protein content determination and SDS-PAGE were performed as control before and after sample concentration with all samples

after each centrifugation step. For comparison with PA, we only show the aqueous phase containing SPs not associated with emulsion droplets after the first centrifugation (PNA; Protein Non-Associated), since samples after second and third centrifugation only contained the emulsifying proteins.

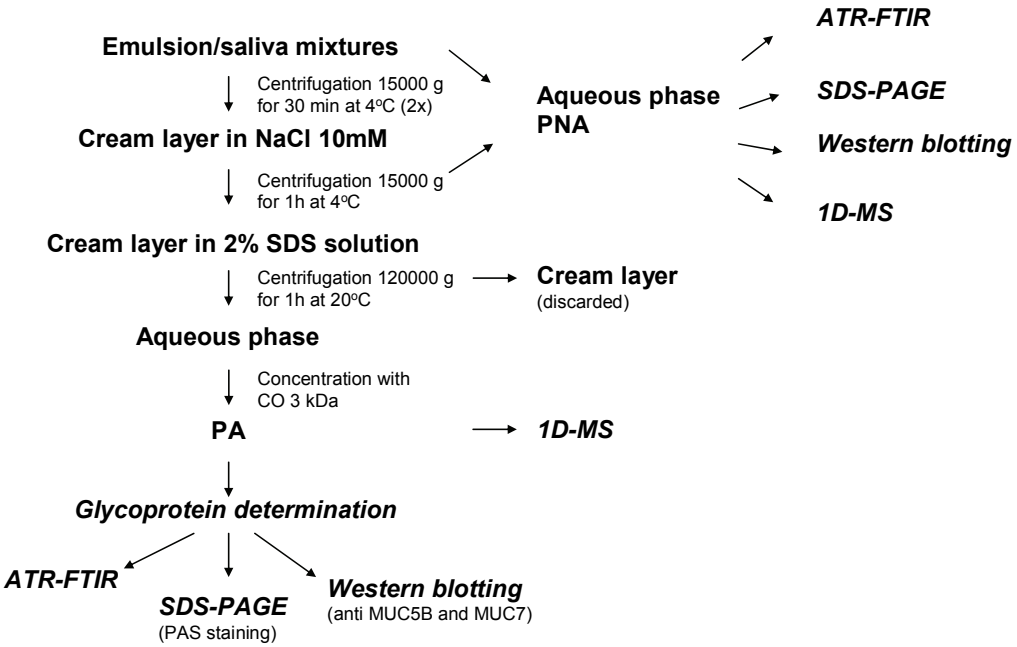

Figure 1 – Experimental procedure for sample preparation and analysis of salivary protein associated (PA) or non-associated (PNA) with the emulsion oil phase of lysozyme and β-lg stabilized emulsions.

Attenuated Total Reflectance Fourier Transform InfraRed spectroscopy (ATR-FTIR)

ATR-FTIR spectra were recorded on a Bio-Rad FTS 6000 equipped with a DTGS detector. 20 μL of the sample was transferred onto a germanium crystal (1 x 8 cm) and air dried before analysis. After 10 minutes of sample equilibration inside the sample chamber, 50 spectra were accumulated in the region of 4000-800 cm^{-1}, with a spectral resolution of 2 cm^{-1} prior to zero-filling and Fourier transformation, using a speed of 5 kHz and a filter of 1.2 kHz and subsequently averaged.

A mixture of fucose (357.9 μg mL^{-1}), N-acetyl-galactosamine (495 μg mL^{-1}), N-acetyl-glucosamine (586.7 μg mL^{-1}), galactose (485.9 μg mL^{-1}), glucose (441.5 μg mL^{-1}) and mannose (478.8 μg mL^{-1}) was analyzed as a reference for carbohydrates. As second

standard, fetuin (1.91 mg mL^{-1}) was used. The influence of SDS used in the sample preparation procedure was taken into account by separately analyzing the SDS solution.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were mixed 1:1 with a buffer containing 0.62 M TrisHCl pH 6.8, 12.5% SDS, 50% glycerol, 0.25% bromophenolblue and 2.5% β-mercaptoethanol. Subsequently, they were incubated for 5 minutes at 100 ^{o}C and 15 μL, corresponding to the amount of 30 μg proteins, were applied to a 4–20 % gradient gel (25244, Pierce, Rockford IL, USA). Electrophoresis was carried out at 120 V for 45 minutes with a MINI-PROTEAN II (Bio-Rad Laboratories, Ivry sur Seine, France). HEPES solution from Pierce (28398, Pierce, Rockford IL, USA) containing 0.1M Tris, 0.1 M HEPES, 3 mM SDS pH 8 was used as running buffer. The gels were fixed in 40% methanol, 10% acetic acid for 1 hour and glycoproteins were visualized with the Schiff's Fuchin Sulfite reagent (S5133, Sigma, St Louis, MI, USA) according to the method of Kapitany [49]. Precision Plus Protein Standard All Blue (161-0373, Bio-Rad, USA) was used as a standard.

Before performing SDS electrophoresis, the samples have been treated with 2D-Clean Up Kit (84-6484-51, Amersham-Biosciences, UK) according to the manufacturer instructions and suspended in rehydration buffer containing 8 M urea, 2 M thiourea, 1% CHAPS and 13 mM DTT. Samples were then diluted 1:5 in the above mentioned sample buffer and 15 μL, corresponding to 50 μg protein, was loaded on 15% running gel. Electrophoresis was run on a Hoefer Mini VE (Amersham-Biosciences 80-6418-77 SN: 20086062, UK) at 200 V for 1 hour and 30 minutes. Gels were stained overnight with Coomassie staining. The proteins in each band were quantified using Quantitation software (Quantity One, Bio-Rad, USA) and the amount was indicated by the measured volume i.e. optical density (OD)*mm^2. The volume of each band (V_{band}) was normalized with the volume (V_{st}) of the band of phophorylase b from rabbit muscle, at 97 kDa contained in the marker (Marker Protein Mixture, 17044601, Lot. 214172, Amersham-Biosciences, UK). The average of 8 repeated gel electrophoresis experiments was taken.

Western blotting

Western blotting was performed to determine mucins using antibodies directed against MUC5B (mAbF2) [30] and MUC7 (CpMG2) [16]. After SDS gel electrophoresis, each

sample was transferred to a polyvinylidene difluoride membrane (162-0177, Bio-Rad Hercules, CA, USA) and washed briefly with Tris Buffered Saline (TBS; 20 mM Tris, 150 mM NaCl pH 7.6). Membranes were incubated at room temperature for 1 hour with a blocking solution containing 3% bovine serum albumin and 1% casein in TBS, before being washed 3 times for 10 minutes each with TTBS buffer (0.02 M Tris, 0.15 M NaCl pH 7.5, 0.1% Tween-20). Incubation with a primary antibody anti MUC5B and MUC7 (dilution 1:1000 in TBS) was performed overnight at 4 ^{o}C under gentle agitation. Monoclonal anti-MUC5B recognizes the SO^{3}-3Galβ1-3GlcNAc moiety of the sulfo-Lewis^{a} antigen [30] while the antibody anti-MUC7 targets the peptide sequence NLLNRIIDDMVEQ, which occupies the amino acids position from 365 to 377 at the C-terminal end [16]. After three washing steps with TTBS of 10 minutes, the membranes were incubated with secondary antibodies (dilution of 1:2000 in TBS) for 2 hours at room temperature. Polyclonal Goat Anti-Rabbit Immunoglobulins/AP (Dako, Denmark) and Rabbit anti-Mouse IgG (H+L) conjugated with alkaline phosphatase (Zymed, USA) were used against the primary antibodies anti-MUC5B and anti-MUC7, respectively. Membranes were washed and color development was obtained by addition of BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitrobluetetrazolium) according to the manufacturer's instructions (Sigma, USA).

Tryptic digestion, mass spectrometry and database search

The protein bands were excised with a pipette tip from the gel and transferred to an eppendorf tube. The gel pieces were washed three times with 25 mM ammonium bicarbornate/50 % acetonitrile (ACN), once with ACN and dried in a SpeedVac (Thermo Savant). 25 μL of 10 μg mL^{-1} sequence grade modified porcine trypsin in 25 mM ammonium bicarbonate was added to the dried gel pieces and the samples were incubated overnight at 37 ºC. Extraction of tryptic peptides was performed by addition of 10% of formic acid (FA)/50% ACN, three times, before being lyophilised in a SpeedVac (Thermo Savant, Farmingdale, NY, USA).

Tryptic peptides were resuspended in 10 μL of a 50% ACN/0.1% FA solution. The samples were mixed (1:1) with a matrix consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid prepared in 50% ACN/0.1% FA. Aliquots of samples (1 μL) were spotted onto the MALDI sample target plate.

Peptide mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive ion reflector mode. Spectra were obtained in the mass range between 800 and 5000 Da with ca. 1500 laser shots. For each sample spot, a data dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, trypsin autolysis, or acrylamide peaks, for subsequent MS/MS data acquisition. Trypsin autolysis peaks were used for internal calibration of the mass spectra, allowing a routine mass accuracy of better than 20 ppm.

Spectra were processed and analysed by the Global Protein Server Workstation (Applied Biosystems, Foster City, CA, USA), which uses internal Mascot (Matrix Science Ltd, U.K.) software for searching the peptide mass fingerprints and MS/MS data. Searches were performed against the Swiss-Prot non-redundant protein database with a tolerance of 200 ppm and 0.3 Da on the *m/z* from MS and MS/MS spectra, respectively.

Results

ATR-FTIR

Figure 2 shows the ATR-FTIR spectra obtained for saliva (spectrum a) and the fractions PA and PNA (spectra b and c) obtained from lysozyme stabilized emulsion/saliva mixtures. As references, the spectra of SDS solution and monosaccharides mixture are shown in spectra d and e, respectively. The spectrum of PA contains a number of peaks which were also observed in the reference spectra, while the PNA spectrum shows great similarities with the spectrum of saliva. Several peaks belonging to different functional groups were identified. The peak at 2926 cm^{-1}, visible in spectra b-d, is assigned to the CH_2, CH_3 and OH of lipid and carbon backbone structure, while the peak at 2058 cm^{-1} (spectra a and c) corresponds to the diazoketones found in saliva [32, 33]. The 1700-1500 cm^{-1} mid infrared region exhibited the characteristic absorption band associated to the peptide bond (CO-NH) of proteins and peptides. The peaks at 1658 cm^{-1} and 1544 cm^{-1} correspond respectively to the stretching vibration of C=O group (amide I) and to the bending vibration of the N-H (amide II). Amide I was detected only in PA, whereas both peaks of amide I and amide II were found in saliva as well as in the PNA fraction. The 1300-900 cm^{-1} range (enlarged in Figure 2, bottom) is the typical infrared absorption interval of phosphate-carrying groups and carbohydrates. The 1086 cm^{-1} peak is usually assigned to PO_2^- and the peak at 1080

cm^{-1} corresponds to the C-O and C=S bonds of sugar. Although not clearly visible due to the broadness of the band, both these two peaks were found in saliva, PNA and the monosaccharides mixture used as reference (spectra a, c and e).

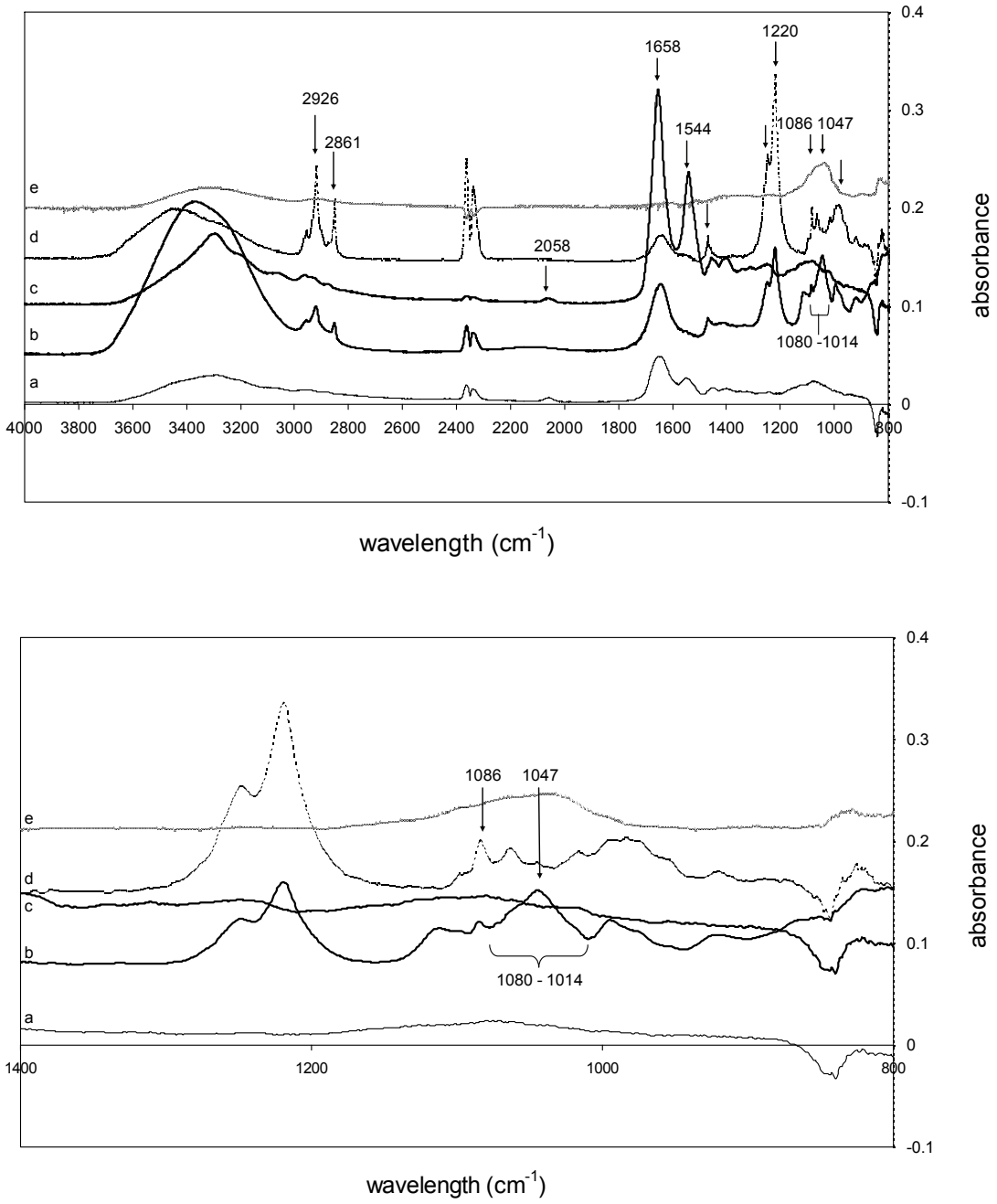

Figure 2 – Infrared spectra obtained in the 4000-800 cm^{-1} range (top) and 1400-800 cm^{-1} (bottom) for whole saliva (a), PA and PNA from lysozyme emulsion upon mixing with saliva (b and c respectively), SDS (d) and reference solution containing fucose, N-acetyl-galactosamine, N-acetyl-glucosamine, galactose, glucose and mannose (e).

The peak at 1086 cm^{-1} is found in both the PA and SDS solution spectra (b and d) as well. In line with the FTIR spectra of the monosaccharides solution, a large infrared band from 1080-1014 cm^{-1}, with a maximum at 1047 cm^{-1} is found in the PA fraction (spectrum b), which indicates the presence of glycoprotein in this sample. Similar results were obtained with the PA fraction from β-lg emulsion/saliva mixture (not shown).

Western blotting for mucin identification

The presence of mucins in PA and PNA fractions of lysozyme and β-lg stabilized emulsions upon mixing with saliva was analyzed with SDS-PAGE and Western blotting (Figure 3). PAS staining was used to detect the glycoproteins (Figure 3a). Mucins were further identified with Western blotting using anti-MUC5B and anti-MUC7 antibodies (Figure 3b and 3c, respectively).

Two PAS-positive bands were visible in the PA fraction (Figure 3a, lane 2) obtained from lysozyme emulsion/saliva mixtures. The first band appeared on top of the stacking gel and the second band was observed between 150 and 250 kDa. The positions of these bands are in line with literature [12, 16, 18, 23, 50, 51] and correspond to the high-M_r mucin MUC5B (the first band) and to MUC7 (the second band). No bands were visible with the PAS colouring in the PNA fraction of lysozyme emulsion/saliva mixtures (Figure 3a, lane 3). Western blotting performed on the lysozyme emulsion/saliva mixture revealed that both MUC5B and MUC7 were present in the PA fraction (lane 2, Figures 3b and 3c). Moreover, MUC7, which was not visible with the PAS staining, was detected in the PNA fraction (Figure 3c, lane 3).

PAS staining of β-lg stabilized emulsions mixed with saliva showed two bands corresponding respectively to MUC5B and MUC7 in the PA fraction and one band, i.e. MUC5B, in the PNA fraction (Figure 3a, lanes 4 and 5). This finding was further confirmed by the Western blotting analysis (Figures 3b and 3c).

These results, in line with the ATR-FTIR measurements, confirm the presence of glycoprotein, namely mucins, associated with the emulsion oil droplets. Moreover, they revealed that MUC5B and MUC7 interact differently with emulsions stabilized by lysozyme and β-lg.

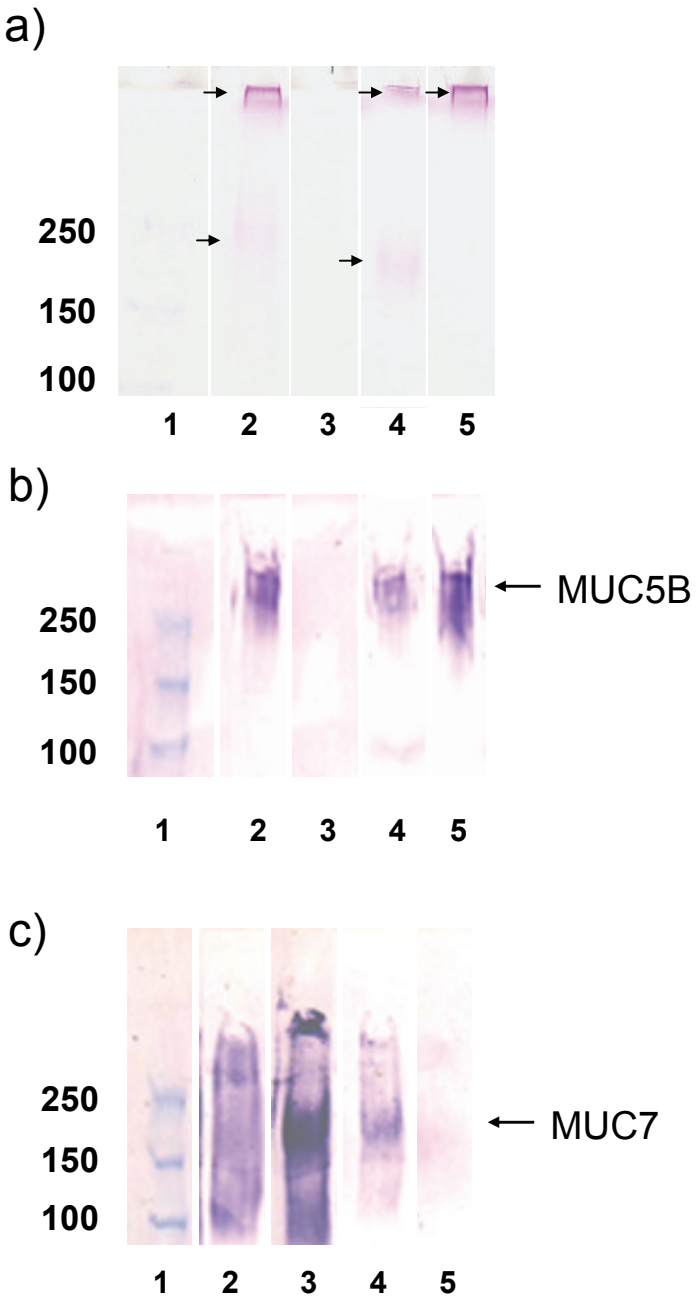

Figure 3 – SDS-PAGE on a 4-20 % gradient gel after PAS staining (a) and Western blotting performed with monoclonal anti-MUC5B (b) and anti-MUC7 antibodies (c). Lane 1 shows Stain All Marker, lanes 2-5 show the different fractions, i.e. PA (lanes 2 and 4) and PNA (lanes 3 and 5) obtained from lysozyme emulsion/saliva mixtures (lanes 2-3) and β-lg emulsion/saliva mixtures (lanes 4-5). Arrows indicate the position of the bands.

1D-MS

Figure 4 shows the SDS-PAGE results of the PA and PNA fractions obtained from both lysozyme and β-lg stabilized emulsions upon mixing with saliva. Table 1 summarizes the identity of the proteins, M_r, isoelectric points (pIs) and the normalized volume of each band. Unfortunately, only a few proteins could be identified with this approach. Possibly, protein identification was compromised by the low SPs concentrations and the extremely low yields of the peptide extraction procedure which is commonly not more than 1% [52].

As observed in case of lysozyme emulsions mixed with saliva, the majority of the SPs was present in the PA fraction, as clearly visible in lane 3. Polymeric immunoglobulin receptor precursor (M_r 83261.7 Da), serum albumin precursor (M_r 69321.5 Da) and different isoforms of α-amylase, i.e. salivary α-amylase precursor (M_r 57730.9 Da), pancreatic α-amylase precursor (M_r 57670 Da) and α-amylase 2B precursor (M_r 57673 Da) were identified. The large band at 14 kDa is chicken egg-white lysozyme used to stabilize the emulsions (Chapter 5) [39]. Cystatin S precursor (M_r 16204 Da) was identified as well. The PNA fraction of lysozyme emulsions mixed with saliva showed only a few bands with low intensity, with M_r of about 55 kDa and below 14 kDa (Figure 4a, lane 3). As in the PA sample, the intense band at 14 kDa is chicken egg-white lysozyme, which is present in the aqueous phase of the emulsion at a concentration of 7.6 mg mL^{-1} (Chapter 5) [39].

In case of β-lg emulsion/saliva mixtures, SPs are more equally distributed between the PA and PNA fractions (Figure 4a lanes 5 and 6, respectively). To better evaluate the differences between these two samples, Figure 4b reports the optical density patterns of both fractions. The arrows are meant to illustrate the major differences in optical density profiles between the PA and PNA fractions while the asterisks indicate β-lg that is present in both fractions.

Two peaks in the M_r range > 66 kDa were only clearly visible in the PA fraction. Polymeric immunoglobulin receptor precursor (M_r 83261.7 Da) was identified in the peak with the highest M_r. Moreover PA showed enrichment in SPs of about 40-45 kDa which is attributed to the actin cytoplasmic 1 and 2 (M_r 41765.8 Da and M_r 41709.7 Da, respectively), and Ig α-2 chain C region and Ig α-1 chain C region (M_r 36485.1 Da and 37630.6 Da, respectively). Carbonic anhydrase IV precursor (M_r 35342.6 Da) was also identified while the basic proline-rich proteins at about 43 kDa [22] were not observed.

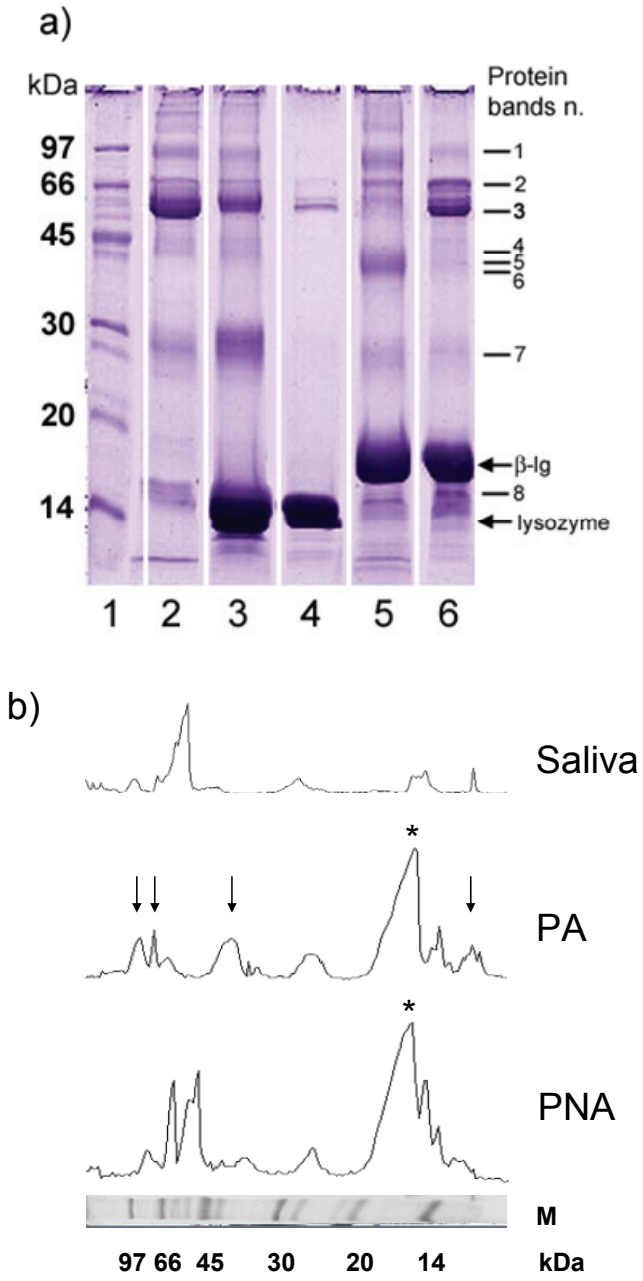

Figure 4 – (a) SDS-PAGE of a 15 % gel loaded with 50 μg of proteins and stained with Coomassie: marker (lane1), saliva (lane 2), PA and PNA obtained from lysozyme emulsion/saliva mixtures (lane 3-4) and β-lg emulsion/saliva mixtures (lane 5-6). Bands were excised and subjected to in-gel digestion and MS analysis. Numbers on the right of the gel refer to the excised bands for which proteins have been identified and reported Table 1. (b) OD analysis performed with density tracer tool of Quantity One software of the PA and PNA fractions of β-lg emulsion/saliva mixtures. The saliva profile is reported for comparison and the marker (M) is reported to clarify position of the observed peaks. Arrows indicate the position of major changes in the profiles of the PA fraction compared to the PNA fraction. * indicates the β-lg band.

A large peak with M_r < 14 kDa (Figure 4b), probably containing proline-rich proteins, such as the previously detected acidic PRP 3 (Chapter 5) [39], was observed in this fraction.
The band containing salivary α-amylase at about 55 kDa is almost exclusively present in the PNA sample as well as cystatin S precursor (M_r 16204 Da) and cystatin SN precursor (M_r 16351.3 Da) which were identified in the PNA but not in the PA fraction.
Comparison of the PA fractions of the two emulsion/saliva mixtures points to dissimilarities in terms of positions and intensities of the bands (Figure 4a and Table 1). For example, α-amylase (band 3) is mostly associated with emulsion droplets in the lysozyme stabilized emulsion/saliva mixture, but not in that of β-lg emulsions. Oppositely, bands 5 and 6 were predominantly detected in the PA fraction of β-lg emulsion/saliva mixtures. Furthermore, an intense and broad band at about 30 kDa was detected in PA from lysozyme emulsions while a less intense band (band 7) was seen in case of the β-lg emulsion/saliva mixture. In line with the results obtained from the PAS staining and Western blotting, the emulsifiers used to stabilize emulsions affect the interaction of emulsion droplets with SPs in the M_r range < 100 kDa as well.

Discussion

This chapter describes the characterization of the salivary components (M_r > about 10 kDa) interacting with oil-water interfaces, which have been stabilized by egg-white chicken lysozyme or bovine β-lg. Different techniques were used to investigate the presence of high molecular mass glycoproteins, namely mucins, as well as protein molecules in the M_r range 10-100 kDa.
ATR-FTIR and PAS staining, used as rapid screening techniques, showed some first indications of the presence of salivary glycoproteins in the fractions containing SPs associated with emulsion droplets (Figures 2 and 3a). Salivary mucins were subsequently identified with monoclonal antibodies. In our study, both MUC5B and MUC7 were associated with PA fractions of the investigated emulsions. Interestingly, MUC7 was also detected in the PNA fraction from the lysozyme emulsion/saliva mixtures, whereas for the β-lg emulsion/saliva mixture MUC5B was present in the PNA fraction (Figures 3b and 3c).
The global analysis of salivary proteins between 10 and 120 kDa is nowadays generally performed using two-dimensional gel electrophoresis (2DE) [53-55]. In our case, the presence of mucins in PA fractions interferes with the isoelectric focusing in the first

Table 1 – Identified proteins in fractions associated with emulsion droplets (PA) and not associated with emulsion droplets (PNA) of lysozyme (Lys) and β-lg stabilized emulsions upon mixing with saliva (sal).

Band no.	Identified Protein	Swiss-Prot number	M_r (Da)	pI	Lys emul/sal mix *		β-lg emul/sal mix *	
					PA	PNA	PA	PNA
1	Polymeric Ig receptor precursor	P01833	83261.7	5.58	0.83 (0.04)	0.12 (0.01)	0.84 (0.10)	0.64 (0.06)
2	Myotubularin	Q13496	69887.6	8.38	0.65 (0.09)	0.31 (0.02)	0.61 (0.02)	0.83 (0.21)
	Serum albumin precursor	P02768	69321.5	5.92				
3	Salivary α-amylase precursor	P04745	57730.9	6.47	1.51 (0.08)	0.72 (0.09)	0.67 (0.15)	2.26 (0.50)
	α-amylase2B precursor	P19961	57673	6.64				
	Pancreatic α-amylase precursor	P04746	57670	6.6				
4	Actin cytoplasmic 2	P63261	41765.8	5.31	0.32 (0.01)	0.20 (0.02)	0.28 (0.02)	0.27 (0.04)
	Actin cytoplasmic 1	P60709	41709.7	5.28				
5	Ig alpha-1 chain C region	P01876	37630.6	6.07	0.32 (0.02)	0.20 (0.02)	0.46 (0.04)	0.27 (0.02)
	Ig alpha-2 chain C region	P01877	36485.1	5.71				
6	Carbonic anhydrase VI precursor	P23280	35342.6	6.65	0.27 (0.02)	0.20 (0.01)	0.56 (0.06)	0.27 (0.01)
	Zinc-alpha-2-glycoprotein precursor	P25311	33850.9	5.57				
7	COP9 signalosome complex subunit 7 b	Q9H9Q2	29603.4	5.83	1.85 (0.11)	0.2 (0.02)	0.52 (0.04)	0.35 (0.05)
8	Cystatin SN precursor	PC01037	16351.3	6.82	1.14 (0.52)	0.3 (0.02)	0.79 (0.02)	1.24 (0.36)
	Cystatin S precursor	P01036	16204	4.95				

** indicates the ratio V_{band}/V_{st} where V_{band} is the volume of each band and V_{st} is the volume of the band at 97 kDa in the marker; standard deviation is reported in parenthesis.*

dimension of 2DE [56]. The large amount of lysozyme (pI~ 10.5) and β-lg (pI~ 4.9) in samples containing SPs associated with the emulsion droplets disturbs the separation and masks SPs with similar pIs, as well. Moreover, only non-ionic and zwitterionic detergents can be used to solubilize proteins and allow their correct separation according to their pI. SDS was used for its property of being an efficient remover of mucous films from polar and

nonpolar surfaces [57]. Dialysis to remove SDS is known to cause sample losses. In consideration of the mentioned drawbacks of 2DE, we decided to use a one-dimensional gel electrophoresis followed by MS to gain insight into the identity of SPs associating with emulsion droplets. Mucins are excluded from the molecular-weight range of separation in most commonly used polyacrylamide gels and therefore do not interfere with the separation of smaller proteins. Furthermore, the concentration of SDS used in the procedure is compatible with this technique, since the samples were diluted in gel electrophoresis sample buffer containing 12.5% SDS.

As shown in Figure 4, interaction between emulsion droplets and saliva is not limited to MUC5B and MUC7, but also involves other salivary proteins in the M_r range 10-100 kDa such as polymeric Ig receptor precursor, amylase and low-molecular-weight protein fractions (M_r <20 kDa). As for the salivary mucins, differences in the electrophoretic patterns of the two PA samples from β-lg and lysozyme stabilized emulsion/saliva mixtures are clearly visible. These findings indicate that the emulsifier adsorbed at the oil-water interface is affecting the interaction of SPs with the emulsion droplets likely via a selective adsorption of SPs to the droplets surface.

Similar results have been obtained for SPs in the M_r range 0.8-20 kDa which has been previously investigated with LC-MS (Chapter 5) [39]. For example, among other proteins, cyclo-statherin Q37, collagen alpha 6 chain precursor and acidic PRP 1 were associated with lysozyme stabilized emulsion droplets, while histatins 1 and cystatin S with emulsion droplets of β-lactoglobulin stabilized emulsions (Chapter 5) [39].

A known paradigm of selective adsorption of salivary proteins onto surfaces in the oral cavity is the formation of the acquired enamel pellicle on the teeth [58]. Several studies have shown that a large number of proteins and peptides is involved in pellicle formation, including MUC5B, MUC7 and salivary micelles [59-63]. Proteomic analysis of *in vivo* enamel pellicle has so far resulted in the identification of about 130 different proteins [59, 60]. The composition of the pellicle changes with time [64]. Salivary proteins such as proline-rich proteins, cystatins and statherin, dominate the early stages of pellicle formation, while mucins seem to be most abundant in the late pellicle [28, 64, 65]. Adsorption experiments of polished tooth enamel incubated with saliva showed that mucins were not among the first proteins to adsorb [65, 66]. In addition, although interaction of mucins with lysozyme has been observed in human bronchial secretions [67], it was recently reported that the layer-by-layer mucus assembly *in vitro* at pH 7 via interaction of MUC5B with

other positively charged proteins including lysozyme could not be achieved [68]. Similarly to the enamel pellicle, it is possible that adsorption of saliva components to emulsion droplets could involve a multi-layer process with low-molecular-weight proteins (M_r <18 kDa) adsorbed in early stage, followed by adsorption of mucins. A multi-layer adsorption mechanism of salivary proteins at oil-water interfaces was hypothesized in chapter 5 based on results from a LC-MS study conducted on the same samples [39].

It is plausible that the SPs adsorbed both as single molecules and as complexes onto the droplet surface. Literature reports that 4-20% of the total salivary protein is present in saliva in the form of micellar aggregates [25, 26, 50, 69-71]. MUC5B isolated from human submandibular/sublingual secretion forms heterotypic complexes with α-amylase, prolin-rich protein, statherin and histatin [26]. Analogously to the high-molecular-weight mucin, MUC7 participates in the formation of the salivary micelles together with sIgA, lactoferrin, α-amylase, glycosylated prolin-rich protein and lysozyme [25] or forms small heterodimeric complexes with lactoferrin in submandibular/sublingual secretion [50]. The observation that MUC5B and α-amylase segregated similarly in the PA and PNA fractions of lysozyme and β-lg emulsion/saliva mixtures, might be an indication for the interaction of SPs complexes with droplet surfaces. Another example of adsorption of SPs to the droplets as complexes might be represented by the co-fractionation of MUC7 and α-amylase in the PNA fraction of the lysozyme stabilized emulsion/saliva mixtures. However, none of the other proteins in salivary micelles was identified in the same PNA fraction.

Knowledge of the interaction between emulsion droplets and SPs may be helpful to understand the perception of these two emulsions and the compositional characteristics of saliva in the oral cavity upon interaction with food, as shown in Figure 4a. The removal of the SPs from the oral fluid, such as MUC5B in lysozyme emulsion/saliva mixtures, could induce a variation is saliva properties, e.g., reduced lubrication, which is believed to determine the perception of astringency [43, 72-76]. Moreover, similarly to our results, the interaction of mucins of the tongue mucus layer with the droplets can explain the observation that lysozyme and isolated whey protein stabilized emulsion droplets were present on the tongue after emulsions intake and even after rinsing with water (Chapter 7) [35].

To conclude, this work showed that interaction between saliva and food emulsion droplets involves a large number of salivary proteins in the whole analyzed molecular mass range.

Moreover, the results suggest that adsorption of salivary proteins onto emulsion droplets plays an important role in saliva-induced flocculation and consequently in perception.

Conclusions

We presented the results of a study of the association of salivary mucins and proteins in the M_r range 10-100 kDa with emulsion droplets stabilized by lysozyme and β-lactoglobulin. To our knowledge, this is the first time that salivary proteins have been reported to adsorb onto the oil-water interface of β-lactoglobulin stabilized emulsions.

Our results indicate that adsorption/association of salivary protein and peptides onto the droplets surface is related to the type of protein at the emulsion oil-water interface. In case of lysozyme stabilized emulsions, MUC5B was found only in the PA fraction while MUC7 was found in both PA and PNA fractions. Oppositely, in β-lg stabilized emulsions, MUC5B was found in both PA and PNA fractions and MUC7 was only present in the PA fraction.

Almost all proteins in the M_r range 10-100 kDa were associated with the emulsion droplets of lysozyme stabilized emulsions, while in case of β-lg stabilized emulsions SP were quite evenly distributed between the PNA and PA fractions.

These findings point to the importance of studying the interaction between saliva and food emulsions at a molecular level, in order to understand the oral behavior of emulsions and subsequently sensory perception.

Acknowledgments

The authors would like to thank Prof. dr. Enno Veerman (Vrije Universiteit, Amsterdam, The Netherlands) for kindly donating the monoclonal antibodies, Marijke Beenes for performing the Western blotting and George van Aken and Willem Norde for critically reading of the manuscript.

References

1. Mandel, I.D., *A contemporary view of salivary research.* Critical Reviews in Oral Biology and Medicine 1993. **4**: p. 599.
2. Humphrey, S.P. and Williamson, R.T., *A review of saliva: Normal composition, flow and function.* Journal of Prosthetic Dentistry, 2001. **85**: p. 162.
3. van Nieuw Amerongen, A. and Veerman, E.C.I., *Saliva - the defender of the oral cavity.* Oral Diseases, 2002. **8**: p. 12.
4. De Smet, K. and Contreras, R., *Human antimicrobial peptides: Defensins, cathelicidins and histatins.* Biotechnology Letters, 2005. **27**: p. 1337.
5. Troxler, R.F., Offner, G.D., Xu, T., Vanderspek, J.C., and Oppenheim, F.G., *Structural relationship between human salivary histatins.* Journal of Dental Research, 1990. **69**: p. 2.
6. Van Nieuw Amerongen, A., Bolscher, J.G.M., and Veerman, E.C.I., *Salivary proteins: Protective and diagnostic value in cariology?* Caries Research, 2004. **38**: p. 247.
7. Raynal, B.D.E., Hardingham, T.E., Thornton, D.J., and Sheehan, J.K., *Concentrated solutions of salivary MUC5B mucin do not replicate the gel-forming properties of saliva.* Biochemical Journal, 2002. **362**: p. 289.
8. Levine, M.J., Reddy, M.S., Tabak, L.A., Loomis, R.E., Bergey, E.J., Jones, P.C., Cohen, R.E., Stinson, M.W., and Al-Hashimi, I., *Structural aspects of salivary glycoproteins.* Journal of Dental Research, 1987. **66**: p. 436.
9. Loomis, R.E., Prakobphol, A., Levine, M.J., and Jones, P.C., *Biochemical and biophysical comparison of two mucins from human submandibular-sublingual saliva.* Archives of Biochemistry and Biophysics, 1987. **258**: p. 452.
10. Zalewska, A., Zwierz, K., Zólkowski, K., and Gindzienski, A., *Structure and biosynthesis of human salivary mucins.* Acta Biochimica Polonica, 2000. **47**: p. 1067.
11. Bobek, L.A., Tsai, H., Biesbrock, A.R., and Levine, M.J., *Molecular cloning, sequence and specificity of expression of the gene encoding the low molecular weight salivary mucin (MUC7).* Journal of Biological Chemistry, 1993. **268**: p. 20563.
12. Reddy, M.S., Bobek, L.A., Haraszthy, G.G., Biesbrock, A.R., and Levine, M.J., *Structural features of the low-molecular-mass human salivary mucin.* Biochemical Journal, 1992. **287**: p. 639.
13. Reddy, M.S., Levine, M.J., and Prakobphol, A., *Oligosaccharide structures of the low-molecular-weight salivary mucin from a normal individual and one with cystic fibrosis.* Journal of Dental Research, 1985. **64**: p. 33.
14. Thornton, D.J., Khan, N., Mehrotra, R., Howard, M., Veerman, E.C.I., Packer, N.H., and Sheehan, J.K., *Salivary mucin MG1 is comprised almost entirely of different glycosylated forms of the MUC5B gene product.* Glycobiology, 1999. **9**: p. 293.
15. Veerman, E.C.I., van den Keijbus, P.A.M., Valentijn-Benz, M., and van Nieuw Amerongen, A., *Isolation of different high-M_r mucin species from human whole saliva.* Biochemical Journal, 1992. **283**: p. 807.
16. Bolscher, J.G.M., Groenink, J., van der Kwaak, J.S., van den Keijbus, P.A.M., van 't Hof, W., Veerman, E.C.I., and van Nieuw Amerongen, A., *Detection and quantification of MUC7 in submandibular, sublingual, palatine and labial saliva by anti-peptide antiserum.* Journal of Dental Research, 1999. **78**: p. 1362.
17. Wickström, C., Davies, J.R., Eriksen, G.V., Veerman, E.C.I., and Carlstedt, I., *MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: Identification of glycoforms and c-terminal cleavage.* Biochemical Journal, 1998. **334**: p. 685.
18. Veerman, E.C.I., van den Keijbus, P.A.M., Nazmi, K., Vos, W., van der Wal, J.E., Bloemena, E., Bolscher, J.G.M., and van Nieuw Amerongen, A., *Distinct localization of MUC5B glycoforms in the human salivary glands.* Glycobiology, 2003. **13**: p. 363.
19. Thomsson, K.A., Prakobphol, A., Leffler, H., Reddy, M.S., Levine, M.J., Fisher, S.J., and Hansson, G.C., *The salivary mucin MG1 (MUC5B) carries a repertoire of unique oligosaccharides that is large and diverse.* Glycobiology, 2002. **12**: p. 1.
20. Schipper, R.G., Silletti, E., and Vingerhoeds, M.H., *Saliva as research material: Biochemical, physicochemical and practical aspects. Archives of Oral Biology, 2007.* ***52****: p.1114*
21. Banderas-Tarabay, J.A., Zacarias-D'Oleire, I.G., Garduno-Estrada, R., Aceves-Luna, E., and Gonzalez-Begne, M., *Electrophoretic analysis of whole saliva and prevalence of dental caries. A study in mexican dental students.* Archives of Medical Research, 2002. **33**: p. 499.

22. Beeley, J.A., Sweeney, D., Lindsay, J.C.B., Buchanan, M.L., Sarna, L., and Khoo, K.S., *Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of human parotid salivary proteins.* Electrophoresis, 1991. **12**: p. 1032.
23. Veerman, E.C.I., van den Keijbus P.A.M., Vissink, A., and van Nieuw Amerongen, A., *Human glandular salivas: Their separate collection and analysis.* European Journal of Oral Science, 1996. **104**: p. 346.
24. Minaguchi, K., Madapallimattam, G., and Bennick, A., *The presence and origin of phosphopeptides in human saliva.* Biochemical Journal, 1988. **250**: p. 171.
25. Soares, R.V., Lin, T., Siqueira, C.C., Bruno, L.S., Li, X., Oppenheim, F.G., Offner, G.D., and Troxler, R.F., *Salivary micelles: Identification of complexes containing MG2, sIgA, lactoferrin, amylase, glycolsylated proline-rich protein and lysozyme.* Archives of Oral Biology, 2004. **49**: p. 337.
26. Iontcheva, I., Oppenheim, F.G., and Troxler, R.F., *Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins.* Journal of Dental Research, 1997. **76**: p. 734.
27. Schwartz, S.S., Zhu, W.X., and Sreebny, L.M., *Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of human whole saliva.* Archives of Oral Biology, 1995. **40**: p. 9499.
28. Jensen, J.L., Lamkin, M.S., and Oppenheim, F.G., *Adsorption of human salivary proteins to hydroxyapatite: A comparison between whole saliva and glandular salivary secretions.* Journal of Dental Research, 1992. **71**: p. 1569.
29. Proctor, G.B., Hamdan, S., Carpenter, G.H., and Wilde, P.J., *A statherin and calcium enriched layer at the air interface of human parotid saliva* Biochemical Journal, 2005. **389**: p. 111.
30. Veerman, E.C., Bolscher, J.G., Appelmelk, B.J., Bloemena, E., van den Berg, T.K., and van Nieuw Amerongen, A., *A monoclonal antibody directed against high M_r salivary mucins recognizes the SO_3-3Galβ1-3GlcNAc moiety of sulfo-Lewis^a: A histochemical survey of human and rat tissue.* Glycobiology, 1997. **7**: p. 37.
31. Rayment, S.A., Liu, B., Offner, G.D., Oppenheim, F.G., and Troxler, R.F., *Immunoquantification of human salivary mucins MG1 and MG2 in stimulated whole saliva: Factors influencing mucin levels.* Journal of Dental Research, 2000. **79**: p. 1765.
32. Skingsley, D.R., *Fourier-transform infrared (FTIR) spectroscopic methods for low volume analysis of oral mucus* European Respiratory Journal, 2003. **22** p. 446.
33. Skingsley, D.R. and Sheffield, D., *Investigation of human oral mucus using fourier-transform infrared (FTIR) spectroscopy.* European Respiratory Journal, 2002. **20**: p. 175.
34. Skingsley, D.R., White, A.J., and Weston, A., *Analysis of pulmonate mucus by infrared spectroscopy.* Journal of Molluscan Studies, 2000. **66**: p. 363.
35. Vingerhoeds, M.H., Silletti, E., de Groot, J., Schipper, R.G., and van Aken, G.A., *Relating the effect of saliva-induced emulsion flocculation on rheological properties and adherence to the tongue surface with sensory perception.* Submitted for publication, 2007.
36. de Jongh, H.H.J. and Janssen, A.M., *Differential clearance kinetics of adhered layer constituents from the oral cavity as modulator for afterfeel of dressings: ATR FTIR measurements of localized oral coatings.* Journal of Texture Studies, 2007. **38**: p. 70.
37. Gambuti, A., Rinaldi, A., Pessina, R., and Moio, L., *Evaluation of aglianico grape skin and seed polyphenol astringency by SDS-PAGE electrophoresis of salivary proteins after the binding reaction.* Food Chemistry, 2006. **97**: p. 614.
38. de Freitas, V. and Mateus, N., *Structural features of procyanidin interactions with salivary proteins.* Journal of Agricultural and Food Chemistry, 2001. **49**: p. 940.
39. Silletti, E., Vitorino, R.M.P., De Groot, J., Vingerhoeds, M.H., and Amado, F.M.L., *Analysis and identification of salivary peptides interacting with β-lactoglobulin and lysozyme stabilized emulsions by SELDI-TOF-MS and RP-HPLC coupled to MS-TOF-TOF.* Submitted, 2007.
40. Silletti, E., Vingerhoeds, M.H., Norde, W., and van Aken, G.A., *The role of electrostatics in saliva-induced emulsion flocculation.* Food Hydrocolloids, 2007. **21**: p. 596.
41. Silletti, E., Vingerhoeds, M.H., van Aken, G.A., and Norde, W., *Rheological behaviour of food emulsions mixed with saliva: Effect of oil content, salivary protein content and saliva type.* Submitted for publication, 2007.
42. Mela, D.J., Langley, K.R., and Martin, A., *Sensory assessment of fat content: Effect of emulsion and subject characteristics* Appetite, 1994. **22**: p. 67.
43. Lu, Y. and Bennick, A., *Interaction of tannin with human salivary proline-rich proteins.* Archives of Oral Biology, 1998. **43**: p. 717.
44. Charlton, A.J., Baxter, N.J., Lilley, T.H., Haslam, E., McDonald, C.J., and Williamson, M.P., *Tannin interactions with a full-length human salivary proline-rich protein display a stronger affinity than with single proline-rich repeats.* FEBS Letters, 1996. **382**: p. 289.

45. Silletti, E., Vingerhoeds, M.H., Norde, W., and van Aken, G.A., *Complex formation in mixtures of lysozyme-stabilized emulsions and human saliva.* Journal of Colloid and Interface Science, 2007. **313**: p. 485.
46. de Jongh, H.H.J., Groneveld, T., and de Groot, J., *Mild isolation procedure discloses new protein structural properties of beta-lactoglobulin.* Journal of Dairy Science, 2001. **84**: p. 562.
47. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C., *Measurement of protein using bicinchoninic acid.* Analytical Biochemistry, 1985. **150** p. 76.
48. Schipper, R., Loof, A., de Groot, J., Harthoorn, L., Dransfield, E., and van Heerde, W., *SELDI-TOF-MS of saliva: Methodology and pre-treatment effects.* Journal of Chromatography B, 2007. **847**: p. 45.
49. Kapitany, R.A. and Zebrowski, E.J., *A high resolution PAS stain for polyacrylamide gel electrophoresis.* Analytical Biochemistry, 1973. **56**: p. 361.
50. Soares, R.V., Siqueira, C.C., Bruno, L.S., Oppenheim, F.G., Offner, G.D., and Troxler, R.F., *MG2 and lactoferrin form a heterotypic complex in salivary secretions.* Journal of Dental Research, 2003. **82**: p. 471.
51. Hu, S., Denny, P., Denny, P., Xie, Y., Loo, J.A., Wolinsky, L.E., Li, Y., McBride, J., Ogorzalek Loo, R.R., Navazesh, M., and Wong, D.T., *Differentially expressed protein markers in human submandibular and sublingual secretions.* International Journal of Oncology, 2004. **25**: p. 1423.
52. Rabilloud, T., *Two-dimensional gel electrophoresis in proteomics: Old, old fashioned, but it still climbs up the mountains.* Proteomics, 2002. **2**: p. 3.
53. Vitorino, R., Lobo, M.J.C., Ferre-Correira, A.J., Dubin, J.R., Tomer, K.B., Domingues, P.M., and Amado, F.M.L., *Identification of human whole saliva protein components using proteomics.* Proteomics, 2004. **4**: p. 1109.
54. Huang, C.-M., *Comparative proteomic analysis of human whole saliva.* Archives of Oral Biology, 2004. **49**: p. 951.
55. Hardt, M., Thomas, L.R., Newport, G., Agabian, N., Prakobphol, A., Hall, S.C., Witkowska, H.E., Fisher, S.J., and Dixon, S.E., *Toward defining the human parotid gland salivary proteome and peptidome: Identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry.* Biochemistry, 2005. **44**: p. 2885.
56. Amado, F.M.L., Vitorino, R.M.P., Domingues, P.M.D.N., Lobo, M.J.C., and Duarte, J.A.R., *Analysis of the human saliva proteome.* Expert Review of Proteomics, 2005: p. 521.
57. Dedinaite, a., Lundin, M., Macakova, L., and Auletta, T., *Mucin-chitosan complexes at the solid-liquid interface: Multilayer formation and stability in surfactant solution.s* Langmuir, 2005. **21**: p. 9502.
58. Lendenmann, U., Grogan, J., and Oppenheim, F.G., *Saliva and dental pellicle. A review.* Advances in Dental Reserach, 2000. **14**: p. 22.
59. Yao, Y., Berg, E.A., Costello, C.E., Troxler, R.F., and Oppenheim, F.G., *Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches.* The Journal of Biological Chemistry, 2003. **278**: p. 5300.
60. Siqueira, W.L., Helmerhorst, E.J., Zhang, W., Salih, E., and Oppenheim, F.G. (2007) *Acquired enamel pellicle and its potential role in oral diagnostics*. Annals of the New York Academy of Sciences.**1098**: p. 504.
61. Lamkin, M.S., Migliari, D., Yao, Y., Troxler, R.F., and Oppenheim, F.G., *New in vitro model for the acquired enamel pellicle: Pellicles formed from whole saliva show inter-subject consistency in protein composition and proteolytic fragmentation patterns.* Journal of Dental Research, 2001. **80**: p. 385.
62. Lamkin, M.S., Jensen, J.L., Setayesh, M.R., Troxler, R.F., and Oppenheim, F.G., *Salivary cystatin SA-III, a potential precursor of the acquired enamel pellicle, is phosphorylated at both its amino- and carboxyl-terminal regions.* Archives of Biochemistry and Biophysics, 1991. **288**: p. 664.
63. Hay, D.I., *Some observations on human saliva proteins and their role in formation of acquired enamel pellicle.* Journal of Dental Research, 1969. **48**: p. 806.
64. Lamkin, M.S., Arancillo, A.A., and Oppenheim, E.G., *Temporal and compositional characteristics of salivary protein adsorption to hydroxyapatite.* Journal of Dental Research, 1996. **75**: p. 803.
65. Embery, G., Heaney, T.G., and Stanbury, J.B., *Studies on the organic polyanionic constituents of human acquired dental pellicle.* Archives of Oral Biology, 1986. **31**: p. 623.
66. Green, D.R.J. and Embery, G., *Structural studies on sulphated glycoprotein preparation isolated from human saliva.* Archives of Oral Biology, 1985. **30**: p. 859.
67. Creeth, J.M., Bridge, J.L., and Horton, J.R., *An interaction between lysozyme and mucus glycoproteins. Implications for density-gradient separations.* Biochemical Journal, 1979. **181**: p. 717.

68. Lindh, L., Svendsen, I.E., Svensson, O., Cárdenas, M., and Arnebrant, T., *The salivary mucin MUC5B and lactoperoxidase can be used for layer-by-layer film formation.* Journal of Colloid and Interface Science, 2007. **310**: p. 74.
69. Helmerhorst, E.J. and Oppenheim, F.G., *Saliva: A dynamic proteome.* Journal of Dental Research, 2007. **86**: p. 680.
70. Yao, Y., Lamkin, M.S., and Oppenheim, E.G., *Pellicle precursor proteins: Acidic proline-rich proteins, statherin, and histatins, and their crosslinking reaction by oral transglutaminase.* Journal of Dental Research, 1999. **78**: p. 1696.
71. Yao, Y., Lamkin, M.S., and Oppenheim, F.G., *Pellicle precursor protein crosslinking: Characterization of an adduct between acidic proline-rich protein (PRP-1) and statherin generated by transglutaminase.* Journal of Dental Research, 2000. **79**: p. 930.
72. Carvalho, E., Mateus, N., Plet, B., Pianet, I., Dufourc, E., and de Freitas, V., *Influence of wine pectic polysaccharides on the interactions between condensed tannins and salivary proteins.* Journal of Agricultural and Food Chemistry, 2006. **54**: p. 8936.
73. de Freitas, V. and Mateus, N., *Nephelometric study of salivary protein-tannin aggregates.* Journal of Science of Food and Agriculture, 2002. **82**: p. 113.
74. Mateus, N., Carvalho, E., Luis, C., and de Freitas, V., *Influence of the tannin structure on the disruption effect of carbohydrates on protein-tannin aggregates.* Analytica Chimica Acta, 2004. **513**: p. 135.
75. Rossetti, G., Yakubov, G.E., Stokes, J.R., Williamson, A.M., and Fuller, G.G., *The interaction of human whole saliva (HWS) and astringent compounds investigated by interfacial shear rheology.* Food Hydrocolloids, 2007. **doi:10.1016/j.foodhyd.2007.1005.1014.**
76. Siebert, K.J. and Chassy, A.W., *An alternate mechanism for the astringent sensation of acids.* Food Quality and Preference, 2003. **15**: p. 13.

Chapter 7

Relating the effect of saliva-induced emulsion flocculation on rheological properties and retention to the tongue surface with sensory perception

E. Silletti, M.H.Vingerhoeds, J. de Groot, R.G. Schipper and G.A.van Aken

Submitted as modified version

Abstract

The perception of food emulsions often cannot be directly related to the structure of the products before consumption. Taking into account the changes in the product structure upon oral processing might increase understanding of the relation between perception and product properties.

This study aimed to gain insight into the effect of saliva-induced flocculation on perception of emulsions at neutral pH. Therefore, we compared emulsions stabilized by whey protein (WPI), which reversibly flocculated by saliva, with lysozyme stabilized emulsions which showed irreversible flocculation upon addition of saliva. Besides the emulsifying protein (WPI vs. lysozyme), we studied the influence of oil content (2.5% w/w vs. 10% w/w) and emulsion thickening with guar gum (at 10% w/w oil) on perception. To relate perception to the processes occurring in the oral cavity, the emulsions were characterized before and after oral processing with respect to rheological properties (viscosity, storage and loss moduli). In addition, insight into retention of emulsion droplets to the tongue surface was obtained by measuring emulsifier and oil content in tongue swabs.

Saliva-induced emulsion flocculation clearly shows a large effect on perception of the studied emulsions. WPI stabilized emulsions showed little retention to the tongue surface and perception was characterized by creaminess, fattiness and thickness. Guar gum thickening further increased perception of these attributes. Perception of lysozyme stabilized emulsions, instead, was largely related to attributes like dryness, roughness and astringency. In addition, a large viscosity increase upon oral processing and clear retention of emulsion droplets on the tongue surface was observed. The behavior of these emulsions resembles the astringent perception of tannins, which precipitate salivary proteins and cause losses in lubrication. The addition of guar gum decreases the effects of lysozyme, likely because of its lubricating properties.

Introduction

Perception of food products, as for example emulsions, is becoming increasingly important in relation to product design and evaluation since food perception often cannot be related to the structural properties of the product before consumption. Oral processing of food emulsions involves mixing with saliva, shearing between palate and tongue, heating or cooling to body temperature, and in some cases changing the pH. Taking into account the changes in the product structure upon oral processing might increase the understanding of the relation between perception and product properties.

Human saliva is a complex biological fluid involved in maintaining oral health, protection of the teeth and mucosal surfaces [1] and facilitating food manipulation and bolus formation [2]. Moreover, saliva plays a role in perception [3-10] and in flavour release [8, 11-17].

Several researchers focused on the identification of measurable physical-chemical properties to predict perceived texture and mouth-feel. For example, it is generally accepted that viscosity enhancement plays an important role in sensory perception of fluid and semi-solid foods [18, 19]. The sensory attribute "creaminess" has been shown to be sensed only if a certain viscosity threshold is met [20]. Furthermore, Kokini and van Aken suggested that other emulsion parameters, such as volume fraction of oil, droplet-size distribution, droplet deformability, rheology of the dispersed and the continuous phase play a role in perception [19].

Previously, we have shown that saliva induces emulsion flocculation, thereby increasing the viscosity of the mixture (Chapter 2) [21, 22]. Chapter 2 showed that the occurrence of flocculation, as well as flocculation behavior upon dilution and shear, was largely dependent on the emulsifier used, with a clear role of emulsifier charge [21]. Strong negatively charged emulsions stabilized by sodium sulfate (SDS) did not flocculate with saliva, while weakly negatively charged emulsions stabilized by whey protein isolate (WPI) or β-lactoglobulin (β-lg) flocculated reversibly. In case of positively charged emulsions stabilized by lysozyme, irreversible flocculation, caused by complex formation between the emulsifier at the oil-water interfaces and salivary proteins, was observed (Chapter 3) [23].

First insight into the effect of irreversible saliva-induced flocculation on sensory perception was gained when WPI stabilized emulsions were compared at neutral and acidic pH [24]. Emulsions stabilized by β-lg result in irreversible flocculation at acid pH and reversible

flocculation at neutral pH (Chapter 2) [21]. Attributes related to dryness, roughness and astringency were rated higher for the irreversible flocculating emulsions (at low pH) than for the reversible flocculating products (at neutral pH). However, the large difference in the pH of the emulsions made it difficult to pinpoint the observed phenomena to (i) difference in flocculation, (ii) the effect of the low pH on saliva properties and/or (iii) to on perception itself. For this reason, we studied saliva-induced flocculation and its relation with perception of emulsions at neutral pH.

In view of the above considerations, it is hypothesized that differences in saliva-induced emulsion behavior (flocculated vs. non flocculated emulsions or (ir)-reversibly flocculated emulsions) could affect emulsion perception. Flocculation of emulsions and entrapment of droplets in the mucous layer at the tongue surface is expected to influence after-feel attributes as coating, dryness or astringency. Unfortunately, food-grade emulsions, which did not flocculate upon mixing with saliva, could not be prepared. Therefore we focused on the comparison between WPI- and lysozyme stabilized emulsions. In addition to the emulsifier type, we investigated the effect of oil-volume fraction, the concentration of the emulsifier in the continuous phase and the use of guar gum as thickening agent. To relate perception to processes occurring in the oral cavity, the emulsions were characterized before and after oral processing with respect to morphology and rheological properties (viscosity, storage and loss moduli). Insight into the emulsion droplets retention at the tongue surface was obtained by evaluating the recovery of emulsifiers, oil and mucins in tongue swabs.

Materials and Methods

Materials

Sunflower oil was refined winterized sunflower oil (RWSFO) donated by Cargill BV (The Netherlands). Lysozyme HCl (batch 1378) was kindly donated by Belovo S.A. (Bastogne, Belgium). Whey protein isolate (WPI, Bipro) was obtained from Davisco Foods International (US). Guar gum powder (Hansacoll) and vanilla flavour (vanilla flavouring powder T03912) were a gift from Brenntag Specialties (The Netherlands) and Danisco Holland BV (The Netherlands), respectively. Sugar (CSM, The Netherlands) and NaCl (NEZO, Akzo Nobel, The Netherlands) were purchased from a local retailer.

Emulsion preparation

An overview of the emulsions is given in Table 1. Possible off-flavours from the oil phase were masked by the addition of sucrose and vanilla flavour. All products had a pH of 6.7. The emulsions were prepared under clean and hygienic conditions 1 day before panel evaluation and stored at 4-7 ˚C.

WPI stabilized emulsions

1 % w/w WPI, not corrected for purity of the protein, was dissolved at room temperature in the continuous phase containing 10 mM NaCl, 2 % w/w sucrose and 0.033 % w/w vanilla flavour. Stock emulsions contained 40 % w/w sunflower oil. The pre-emulsion was prepared using a rotor-stator type mixer at 4,500 min^{-1} (Ultra Turrax, IKA, T25-basic, Germany). This pre-emulsion is homogenized through a Panda homogenizer (Panda 2K, Niro Soavi S.p.A., Parma Italy) at 20 bar for the first step and 200 bar for the second step of homogenization to obtain emulsions with a droplet size of about 1 μm.

The stock emulsion is diluted with continuous phase to 10% w/w oil (emulsion 3) and 2.5% w/w oil (emulsion 7) as listed in Table 1. Emulsion 7 has both a reduced oil and protein content compared to emulsion 3. Based on the assumption that 2 mg m^{-2} protein was adsorbed on the oil droplets surface (1 μm droplets) [25, 26], it was calculated that the protein concentration in the continuous phase of emulsion 3 was 0.14% w/w. In order to obtain an emulsion with the protein content in the continuous phase similar to emulsion 3, emulsion 5 is prepared by diluting emulsion 3 with the continuous phase containing 0.14 % w/w WPI.

Guar gum (1% w/w) was dissolved in the continuous phase by heating the dispersion to 70°C and subsequently cooling down. To prepare emulsion 1, the stock emulsion was diluted with the guar gum solution to a concentration of 10% w/w oil and 0.4% w/w guar (in the continuous phase).

Lysozyme stabilized emulsions

Emulsion preparation was similar as described above for WPI emulsions, except that the stock emulsion contained 20% w/w oil. Also in this case, the stock emulsion was diluted with continuous phase to 10% w/w oil (emulsion 4) and 2.5% w/w oil (emulsion 8) as reported in Table 1. Emulsion 8 has both a reduced oil and protein content compared to emulsion 4. Similarly to WPI emulsions, assuming 2 mg m^{-2} protein adsorbed on the oil droplets surface, it was calculated that the protein concentration in the continuous phase of

emulsion 4 was 0.42 % w/w. Therefore, in emulsion 6, we kept the protein content in the continuous phase similar to emulsion 4 by diluting emulsion 4 with continuous phase containing 0.42 % w/w lysozyme. The guar gum thickened lysozyme stabilized emulsion (emulsion 2) was prepared according to the same procedure as described for the WPI emulsions.

Emulsion characterization

Samples, diluted to 10% w/w oil content, were analyzed by light microscopy (Olympus BX 60 Microscope equipped with an Olympus DP 70 camera; Olympus Nederland BV, The Netherlands) at 10 and 50 times magnification.

The droplet-size distribution, the volume-over surface diameter (d_{32}) and volume weighted mean diameter (d_{43}) were determined after dilution in demineralised water (dilution factor about 3000) by laser diffraction with the Mastersizer Hydro 2000S (Malvern Instruments, Southborough, UK). A refractive index of 1.469 (sunflower oil) was used to calculate the particle-size distribution.

As previously described (Chapters 2 and 4) [21, 27], rheological measurements of emulsion viscosity (η), storage (G') and loss moduli (G'') were carried out using a Paar Physica MCR 300 rheometer (Anton Paar BVBA, Sint Martens Latem, Belgium) with cone-and-plate geometry CP75-1 and a Vilastic-3 capillary viscoelasticity analyzer (Vilastic Scientific Inc., Austin, Texas, USA).

Oral processing of 4 mL emulsion was performed by moving the tongue up and down (approximately 15 times) for 15 seconds. The samples were carefully spat out in pre-weighed, cups and directly analyzed by microscopy and rheology as described above.

Microbial analysis of the emulsions on the day of panel evaluation was conducted at Silliker BV (Ede, The Netherlands) and showed negligible counts of aerobic mesophilic bacteria (i.e. Bacillus cereus, Staphylococcus aureus and Listeria monocytogenes).

Oil and emulsifier retention to the tongue surface

The term oral retention denotes the presence of the compound of interest on oral surfaces upon in-mouth processing [28]. Similarly, we use the term retention in relation to sunflower oil and the emulsifiers to indicate the presence of emulsions droplets on the tongue after processing and rinsing with water. Briefly, after spitting out of the emulsion, the mouth is

rinsed twice with 20 mL water. Then, a sample of the whole tongue surface is taken with a pre-moistened cotton swab (100 µl water). As a control, a sample of the clean tongue is also analyzed. The swabs were immediately frozen in liquid nitrogen and stored at – 40°C.

Oil was extracted from the swab, saponified and methylated according to Metcalfe et al [29]. The fatty acid methylesters were separated by gas chromatography (GC; 6890 N GC-system from Agilent Technologies, Inc, Santa Clara, CA, USA), using a 25 m WCOT fused silica column (inside diameter 0.25 mm) coated with 0.2 µm CP Wax 58 (Chrompack, Middelburg, The Netherlands). The concentrations of fatty acids were calculated in relation to the internal standard (C17). Attenuated Total Reflection Fourier Transform Infrared spectroscopy (ATR-FTIR; Bio-Rad FTS 6000, USA) was used to analyse the swabs with respect to oil content. Silicon oil (Merck, Germany, cat 7742) was employed as internal standard to reduce the inaccuracy introduced by transfer of the sample from the swab onto a germanium crystal (1x8cm). Sunflower oil displays a sharp vibration band at 1750 cm^{-1} due to the ester bond (>C═O) in lipids, whereas in silicon oil a sharp vibration band at 800 cm^{-1} due to the carbon silicon bond in the $Si(CH_3)_2$ group is seen. To normalize the sample, the height of the peak at 1750 cm^{-1} is divided by the height of the peak at 800 cm^{-1} (H_{1750}/H_{800}). Spectra were accumulated in the spectral region of 4000-700 cm^{-1} with a spectral resolution of 2 cm^{-1}, using a speed of 5 kHz, a filter of 1.2 kHz and UDR of 2.

The protein composition was also qualitatively assayed by SELDI-TOF-MS [30]. Swabs were brushed over the spots on a normal phase chip (NP20, Bio-Rad, USA). The spots were washed with 4 µl Milli Q water and air dried. Then, 0.8 µL matrix solution (12.5 mg sinapinic acid in 50% acetonitril/0.1% trifluoracetic acid) was added twice to the spots and air dried prior to reading on a Protein Chip Reader IIC instrument (Bio-Rad, USA).

SDS gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [31]. Briefly, the samples were reduced with β-mercaptoethanol and run on a 4–20% gradient gel (Pierce, USA, cat no 25244) with 0.1M Tris, 0.1M HEPES, 3 mM SDS pH 8 (Pierce, USA, cat no 28398) as the running buffer. The gels were stained for proteins with the PageBlue Fast protein staining (Fermentas, Canada; abbreviated as CBB staining). The proteins in each band were quantified using Quantity One software (Bio-Rad, USA) and the amount is indicated by the measured volume, i.e. optical density (OD)*mm^2.

Western blotting was performed, as previously described (Chapter 5) [32], to determine mucins using antibodies against MUC5B [33] and MUC7 [34]. After SDS-PAGE, each sample was transferred to a polyvinylidene difluoride membrane and washed briefly with

Tris Buffered Saline (TBS; 20 mM Tris, 150 mM NaCl pH 7.6). Membranes were incubated at room temperature for 1 hour with a blocking solution (3% bovine serum albumin and 1% casein in TBS) before being washed with TTBS buffer (20 mM Tris, 150 mM NaCl pH 7.6, 0.1% Tween 20). Incubation with a primary antibody anti MUC5B and anti MUC7 (dilution 1:1000 in TBS) was performed overnight at 4 ^{o}C under gentle agitation. After washing steps with TTBS, the membranes were incubated with secondary antibodies (dilution of 1: 2000 in TBS) against anti-MUC5B (polyclonal alkaline phosphatase (AP)-Rabbit anti-Mouse IgG; Zymed, USA) and anti-MUC7 (AP-Goat Anti-Rabbit IgG; Dako, Denmark) for 2 hours at room temperature. Membranes were washed and color development was obtained by addition of BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitrobluetetrazolium) according to the manufacturer's instructions (Sigma, USA).

Sensory analysis

Subjects

The sensory properties of the emulsions were investigated with the use of a paid panel trained according to the principles of Quantitative Descriptive Analysis (QDA) [35]. All subjects had previously been screened to exclude taste disorders and to select on good olfactory senses. The panel, which was constituted of 9 female panellists with a mean age of 48.1 years and a standard deviation of 9.4, had no scientific background and was informed that the study aimed to evaluate the effect of saliva on perception. An informed consent form was signed at the first training session to confirm the awareness of the presence and possible risks of lysozyme in some products.

Procedure

The panellists, which have been trained on low viscosity emulsions in at least four training sessions of 1.5-2 hours [24], received two extra training sessions to familiarize with the specific emulsions for this study. The panel evaluation began at 13 PM. The participants have been asked not to consume any coffee or tea for 2 hours and not to eat for at least 1 hour before the panel test. The panel judged the set of model emulsions in a semi-monadic assessment procedure, in duplicate (two sessions). The presentation order was randomly designed over panellists (optimally balanced design for order- and carry over effects; balanced Latin square design). Each session started with a warm-up sample, excluded from

the data analysis. Then, per session, eight products, which were poured in 60 cc covered-lid cups at least 60 minutes before evaluation, were tasted in three blocks with short breaks in between (5-10 minutes). All samples were offered at room temperature.

The participants were seated in sensory booths with slightly red light and over-pressure to cancel out carrying over effects of odor residuals. During profiling, the attributes, which were generated in Dutch and subsequently translated in English (Appendix A), appeared per category on a monitor with the attributes on the left and a 100-point response scale anchored at the extremes, on the right (Fizz software, Biosystems, v2.21a, 2006). Sensory attributes were assessed, with the use of a mouse to indicate the perceived strength of each attribute on the visual analogue scales, in the chronological order as they are perceived. Upon opening of the cup, each product was smelled (odor attributes, O), and at least three 4 mL portions were taken using a 5 mL-syringe. After evaluation of taste (T) and mouth-feel (M) attributes, the products were carefully spat out and aftertaste (AT) and after-feel (AF) were judged. Between each product evaluation, the panellists cleaned their mouth with cream crackers, tap water, and optionally warm tap water.

Data analysis

Data analyses were carried out using Fizz calculations (Biosystems, v2.20h 2006), SPSS (version 12.01, SPSS Inc., Chicago, US) and Unscrambler (version 7.5, Camo Inc., Corvallis, USA).

The effect of emulsifier, oil content, and guar gum addition on sensory attributes was analyzed uni-variately using repeated measures ANOVA (SPSS) with the Huynh-Feldt value as epsilon, carried out on the sensory data averaged across replicates. In addition, Analysis of Variance was applied to all sensory attributes separately, to test any differences between the products for statistical significance (Fizz). In case of significant differences, a post-hoc test Least Significant Difference (LSD) was performed at 95% confidence interval to reveal the nature of the differences (Fizz).

Correlation analysis ($p<0.05$; Excel) was done between instrumental measurements and sensory attributes. Principal Component Analysis (PCA) was carried out using panel averaged data (Unscrambler) to identify relations between products, attributes and instrumental measurements to elucidate the configuration of products in the perceptual space as defined by the first two principal components.

Results

Emulsion characteristics before and after oral processing

Table 1 provides an overview of the emulsifiers, oil and protein content used to prepare the emulsions and Table 2 summarizes the emulsion characteristics, i.e. droplet size (d_{32} and d_{43}), viscosity, storage and loss moduli before and after oral processing. The droplet size is only shown before oral processing as the presence of salivary proteins and bacteria after oral processing contaminated the equipment resulting in unreliable measurements. Microscopic images and viscosity values of the prepared emulsions measured at a shear rate of 90 s^{-1} on the day of panel evaluation before and after oral processing are illustrated in Figure 1 and Figure 2 respectively.

The droplet sizes of low viscosity emulsions were reasonably constant, with d_{32} values varying between 0.97 and 1.3 µm. As expected, WPI stabilized emulsions did not flocculate (Figure 1C) and had d_{43} ranging between 2.5 and 3.2 µm.

Table 1 – Overview of the products. All systems contained 2 % w/w sucrose, 0.033 % w/w vanilla flavour, 10 mM NaCl and pH was adjusted to 6.7.

No	Product name	Emulsifier	Oil (% w/w)	Protein continuous phase (% w/w)*	Total Protein (% w/w)	Parameter(s) [#]
1	*W-O10-P0.14-H*	WPI	10	0.14	0.25	↑ viscosity
2	*L-O10-P0.42-H*	Lysozyme	10	0.42	0.5	↑ viscosity
3	*W-O10-P0.14-L*	WPI	10	0.14	0.25	
4	*L-O10-P0.42-L*	Lysozyme	10	0.42	0.5	
5	*W-O2.5-P0.14-L*	WPI	2.5	0.14	0.17	↓ oil content
6	*L-O2.5-P0.42-L*	Lysozyme	2.5	0.42	0.44	↓ oil content
7	*W-O2.5-P0.033-L*	WPI	2.5	0.033	0.0625	↓ oil and protein content
8	*L-O2.5-P0.097-L*	Lysozyme	2.5	0.097	0.125	↓ oil and protein content

** Based on the assumption that 2 mg m^{-2} protein was adsorbed on the oil droplets surface (1 µm droplets); [#] in comparison with emulsions 3 and 4, ↑ and ↓ indicate and an increase or decrease of the reported parameter(s).*

Lysozyme stabilized emulsions, as shown in Figure 1G, were slightly flocculated and exhibited higher d_{43} values (4.5-8.3 μm). Flocculation appeared to be induced during analyses and did not result in phase separation. Guar gum thickened emulsions were flocculated due to depletion induced by the polysaccharide thickener (Figure 1A and 1E). This flocculation combined with the presence of guar gum particles increased the resulting d_{32} and d_{43} values to 1.7-9.2 μm and 20.9-39.6 μm, respectively.

Viscosity of non-thickened emulsions, before oral processing, was low and comparable for both cone-and-plate geometry and capillary set-up, i.e. on average respectively 1.46±0.19 mPas at 90 s^{-1} and 1.30±0.21 mPas at 100 s^{-1}. Addition of guar gum increased the viscosity of both emulsions (Table 2) and induced a shear-thinning behavior (not shown). The observed deviations between the two rheological methods might be due to differences in the measuring shear rate ranges, or, in case of thickened samples, to the high viscosity of the emulsions for which the usage of a wider capillary might be more appropriate. The storage and loss moduli of low viscosity emulsions were largely in line with previous results (Chapter 4) [27], i.e. an average *G'* of 0.51±0.31 mPa and *G"* of 16.3±2.7 mPa. Addition of guar gum largely increased both *G'* and *G"* to approximately the same values with thickened lysozyme stabilized emulsions exhibiting highest storage and loss moduli (Table 2).

In line with expectations based on previous studies (Chapter 2) [21, 22], oral processing induced emulsion flocculation, with lysozyme stabilized emulsions resulting in larger flocs than WPI emulsions (Figure 1F and 1H). Flocculation of lysozyme emulsions thickened by guar gum was enhanced by saliva, whereas the extra effect of saliva-induced flocculation of the guar gum thickened WPI emulsions is less clear (Figure 1B and 1F). Microscopic images from tongue scrapings performed prior rinsing with water, confirmed the presence of flocculated emulsions on the tongue as well (data not shown).

Figure 2 shows the viscosities measured with cone-and-plate geometry for WPI and lysozyme stabilized emulsions before and after oral processing. In-mouth mixing with saliva increased the viscosity of all low viscosity emulsions, with the largest effect on lysozyme stabilized emulsions, as expected due to irreversible flocculation. Capillary measurements of orally processed non-thickened emulsions showed an increase of viscosity, as well as of *G'* and *G"*, for 10% w/w oil emulsions, while at 2.5% w/w oil contents, only *G'* increased (Table 2). The results point to an increase in elastic behavior of the emulsions due to oral processing, in particular for low oil content emulsions.

Table 2 – Emulsion characteristics before and after oral processing

Product name	Droplet size before oral processing		Rheological properties before oral processing				Rheological properties after oral processing			
	d_{32} (μm)	d_{43} (μm)	η*(mPa.s)	$\eta^{\#}$ (mPa.s)	G' (mPa)	G'' (mPa)	η *(mPa.s)	$\eta^{\#}$ (mPa.s)	G' (mPa)	G'' (mPa)
W-O10-P0.14-H	1.70±0.22	20.9±8.2	80.60±8.19	39.11±19.52	438.1±272.0	491.4±245.3	62.79±50.41	39.00±5.41	165.0±37.6	490.1±68.0
L-O10-P0.42-H	9.23±5.74	39.6±5.4	111.2±12.5	69.70±36.94	953.5±107.5	875.8±464.2	66.68±4.57	39.98±8.61	191.8±78.7	502.4±108.2
W-O10-P0.14-L	1.31±0.35	3.17±1.21	1.66±0.09	1.47±0.04	0.41±0.08	18.44±0.56	11.58±1.14	3.48±0.40	32.29±24.50	43.74±5.03
L-O10-P0.42-L	1.03±0.32	4.58±1.85	1.74±0.16	1.66±0.56	0.90±0.85	20.88±7.10	27.59±20.24	4.34±3.21	16.84±24.92	54.55±40.38
W-O2.5-P0.14-L	1.17±0.20	2.85±0.42	1.38±0.09	1.15±0.02	0.43±0.07	14.40±0.30	3.67±0.00	0.93±0.76	8.98±4.53	11.68±9.30
L-O2.5-P0.42-L	0.97±0.25	6.60±6.77	1.38±0.15	1.24±0.11	0.47±0.08	15.62±1.42	44.44±25.65	1.50±0.44	3.86±6.90	18.80±5.49
W-O2.5-P0.033-L	1.08±0.20	2.51±0.58	1.24±0.03	1.12±0.02	0.42±0.07	14.08±0.02	3.50±0.00	1.74±0.10	4.84±1.30	21.92±1.29
L-O2.5-P0.097-L	0.99±0.29	8.33±6.80	1.37±0.19	1.14±0.07	0.40±0.10	14.30±0.91	40.95±47.29	1.07±0.00	0.46±0.00	13.46±.0.00

*Average ± stdev of at least two samples is given. * Viscosity measured with cone-and-plate geometry at 90 s^{-1}; # Viscosity measured with the capillary set-up at 100 s^{-1}.*

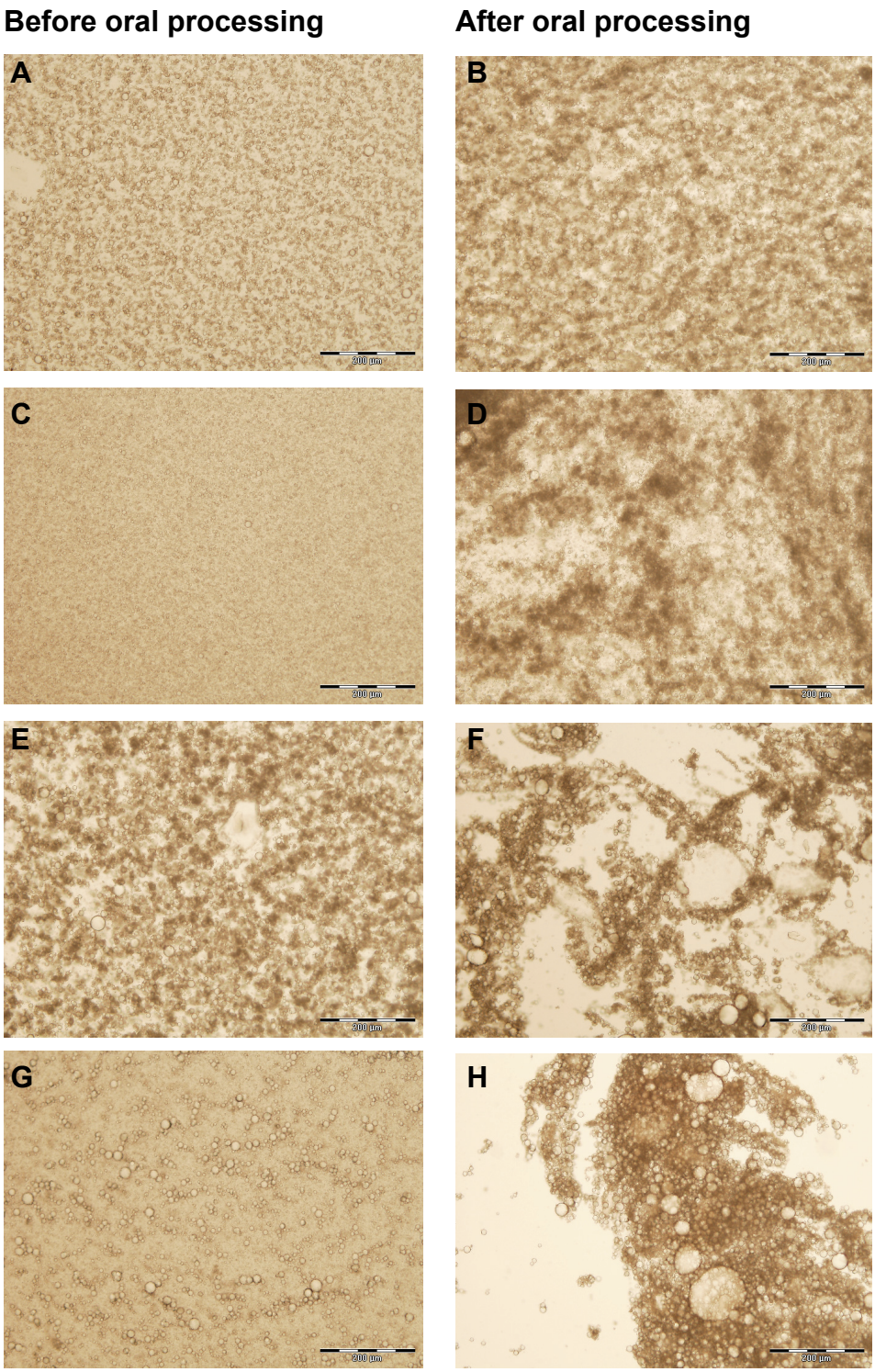

Figure 1 – Microscopic images of 10% w/w oil containing WPI (A-D) and lysozyme (E-F) emulsions before (left) and after (right) oral processing on day of panel evaluation at 10 times magnification. A and B: guar gum thickened WPI emulsions; C and D: low viscosity WPI emulsions. E and F: guar gum thickened lysozyme emulsions; G and H: low viscosity lysozyme emulsions. The scale bar represents 200 µm.

Viscosity, storage and loss moduli of guar gum thickened emulsions, upon *in vivo* oral processing, seemed to be independent of the type of emulsifier (as illustrated in Figure 2 and Table 2). A general reduction of viscosity, storage and loss moduli was observed, presumably due to the dilution effect of the saliva and/or due to in-mouth shearing.

In summary, before oral processing the low viscosity WPI and lysozyme stabilized emulsions had similar viscosity and were, despite the slight flocculation of lysozyme emulsions, reasonably comparable in droplet size. Although the thickened lysozyme emulsions had a slightly higher viscosity than the WPI emulsions, in our opinion the emulsions were sufficiently comparable for evaluation of the effect of saliva-induced flocculation on sensory perception.

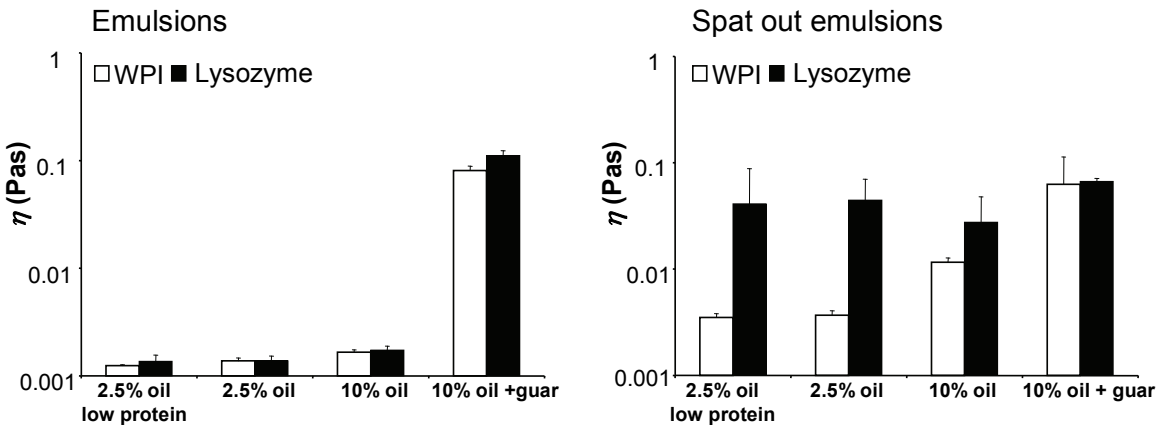

Figure 2 – Emulsion viscosity (η) before and after oral processing at a shear rate of 90 s^{-1}. Error bars represent the standard deviation.

Oil and emulsifiers retention and mucin recovery at the tongue surface

Gas chromatography (GC) and ATR-FTIR were performed to detect sunflower oil, while SELDI-TOF-MS and SDS-PAGE were used to obtain information on the presence of the emulsifiers (WPI and lysozyme) on the tongue surface. An overview of the results is provided in Table 3. GC analysis shows that larger amounts of oil are retrieved from the tongue after intake of lysozyme stabilized emulsions compared to WPI stabilized emulsions. As expected, increasing the oil-volume fraction in the original emulsions enhances the amount of oil found on the tongue. Addition of guar gum to the 10% w/w emulsions

Table 3 – Emulsion retention and mucin characterization on the tongue surface after oral processing.

Product name	Oil		Emulsifiers		MUC5B	MUC7
	GC *	FTIR #	SELDI-TOF-MS	SDS-PAGE	WB	WB
W-O10-P0.14-H	0.34	0.08	±	± (0.67)	+	±
L-O10-P0.42-H	1.62	0.67	+	++++ (9.56)	+	++
W-O10-P0.14-L	0.39	0.23	±	± (0.24)	+	±
L-O10-P0.42-L	2.66	0.91	+	+++ (6.64)	+	++
W-O2.5-P0.14-L	0.17	0.05	±	± (0.30)	+	±
L-O2.5-P0.42-L	1.22	0.45	+	++ (4.57)	+	++
W-O2.5-P0.033-L	0.05	0.05	Nd	-	+	+
L-O2.5-P0.097-L	0.70	0.67	Nd	+ (1.93)	+	++
Clean tongue	0.02	0	-	-	+	++

** mg fatty acids/swab; # peak height at 1750 cm^{-1}/peak height at 800 cm^{-1}, average of two individuals; ( ) indicates the OD*mm^2 measured for the band of β-lg (18 kDa) and lysozyme (14 kDa); + indicates clearly detectable; - is not detectable; Nd: not determined. WB: Western blotting.*

slightly decreased, instead, the amount of oil retained at the tongue surface, especially in case of lysozyme stabilized emulsions. Results of ATR-FTIR were in line with those of GC. Figure 3a illustrates, as an example, SELDI-TOF-MS profiles in the range between *m/z* 10000 and 20000 performed on proteins extracted from the tongue swabs for 10% w/w low viscosity emulsions. The intensity scale is optimized to offer a good visualization of the peaks. Several peaks, including those assigned to the emulsifiers i.e. β-lg (*m/z* 18402) and α-lactalbumin (*m/z* 14187) in WPI and lysozyme (*m/z* 14293), were detected. In addition, two peaks were detected at about *m/z* 10845 and at *m/z* 14682 (probably human lysozyme). The observed profile from lysozyme emulsions showed higher intensity peaks than the profile from WPI emulsions.

SDS-PAGE showed that both β-lactoglobulin and lysozyme were recovered from the tongue surface, as indicated by the presence of both 18 kDa and 14 kDa bands in lane 2 and 3, respectively (Figure 3b). Analysis of the OD of these bands revealed that lysozyme was recovered in larger quantity compared to β-lactoglobulin (Table 3). These results were in line with the findings obtained by SELDI-TOF-MS. The large amount of lysozyme compared to WPI is not caused by the higher protein concentration of the original lysozyme emulsion (emulsion 4), since the same results were obtained when comparing lysozyme and WPI emulsions both containing 10% w/w oil and 0.5% w/w emulsifier (not shown).

Mucin recovery from the tongue surface after oral processing of emulsions was determined using antibodies against MUC5B and MUC7. In case of lysozyme stabilized emulsions, both MUC5B and MUC7 were detected (Figure 3b). The results of the Western blotting indicated, although only qualitatively, that the amount of salivary mucin MUC5B recovered from the tongue surface was not affected by oral processing (Table 3). MUC7B was unaffected by oral processing in lysozyme stabilized emulsions as well, while unexpectedly, it was reduced in WPI emulsions.

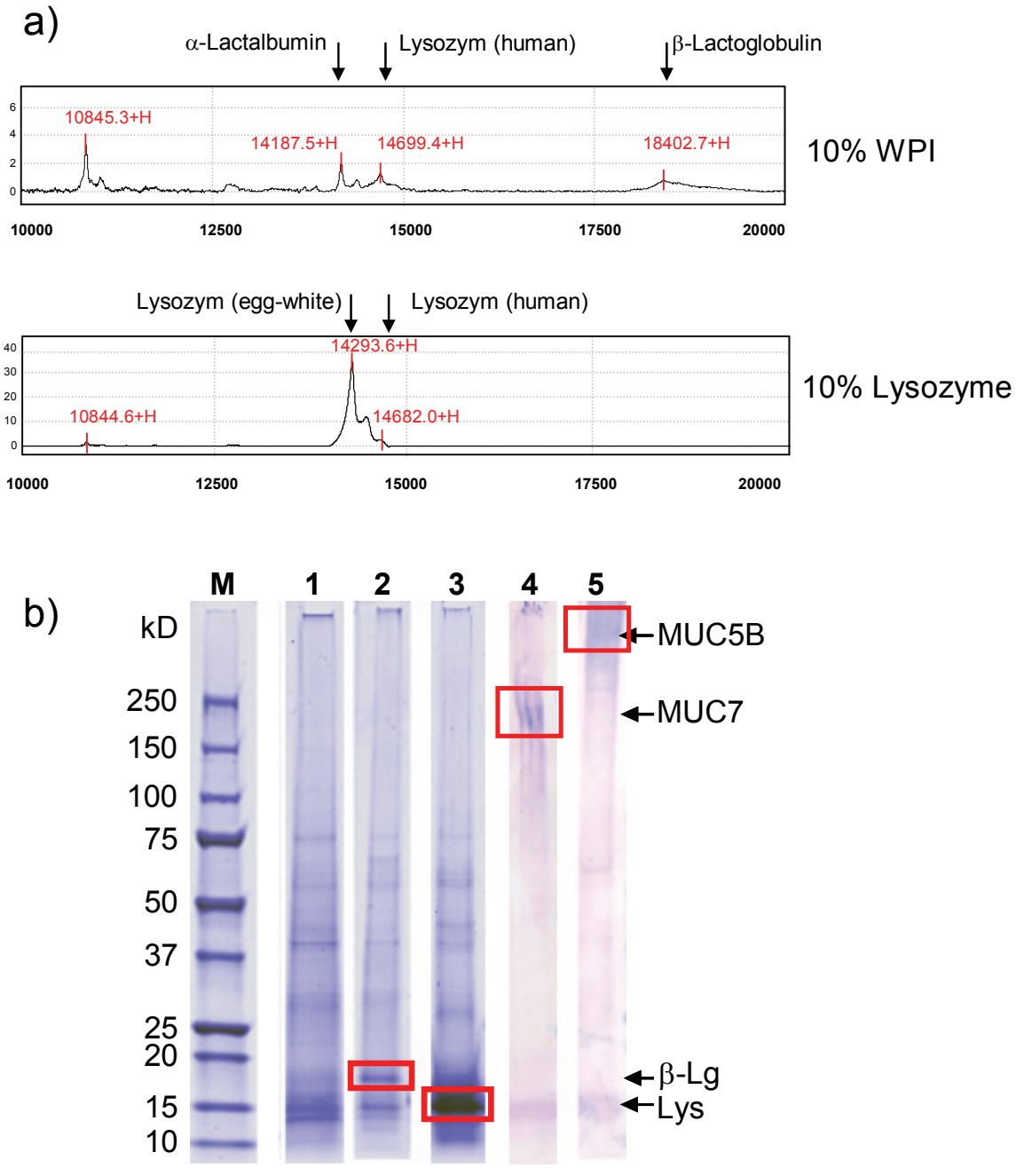

Figure 3 – (a) SELDI-TOF-MS, (b) SDS gel electrophoresis (lanes 1-3) and Western blotting (lanes 4 and 5) for 10% w/w oil containing low viscosity emulsions. Marker (lane M), tongue swab of clean tongue (lane 1), tongue swab of WPI emulsion (lane 2), tongue swab of lysozyme emulsion (lane 3). Western blotting of tongue swab of lysozyme emulsion (lanes 4 and 5) performed with anti-MUC7 (lane 4) and anti-MUC5B (lane5).

Sensory perception

Analysis of variance shows that 34 of the 38 sensory attributes discriminate between the products (at 5% level; Table 4).

Table 4 – Average sensory ratings and significant differences as determined by ANOVA analysis.

Attribute	**W-O10-P0.25-H**	**L-O10-P0.5-H**	**W-O10-P0.25-L**	**L-O10-P0.5-L**	**W-O2.5-P0.25-L**	**L-O2.5-P0.5-L**	**W-O2.5-P0.0625-L**	**L-O2.5-P0.125-L**	**Probability[#]**
Ointensity	45.51	35.62	38.83	37.45	43.21	39.76	38.82	40.47	0.5266
	!	!	!	!	!	!	!	!	
Ovanilla	23.81	20.19	28.59	31.3	33.51	26.42	31.27	36.75	0.0776
	!	!	!	!	!	!	!	!	
Ochalk	44.03	43.16	43.03	32.17	35.96	34.79	28.76	37.92	0.2359
	!	!	!	!	!	!	!	!	
Tintensity	42.9	73.08	56.52	81.85	39.6	79.63	44.4	66.11	<0.0001
	D	*AB*	*C*	*A*	*D*	*A*	*D*	*B*	***
Tvanilla	24.1	39.18	46.6	34.02	40.15	30.65	36.16	36.9	0.0159
	C	*AB*	*A*	*BC*	*AB*	*BC*	*AB*	*AB*	*
Tsweet	26.24	71.94	48.27	70.3	40.87	67.29	40.11	63.91	<0.0001
	C	*A*	*B*	*A*	*B*	*A*	*B*	*A*	***
Tsour	13.8	36.84	23.15	40.28	20.01	42.09	17.32	36.42	<0.0001
	C	*A*	*B*	*A*	*BC*	*A*	*BC*	*A*	***
Tsalty	27.66	78.66	38.62	78.71	16.57	72.33	23.43	66.78	<0.0001
	D	*A*	*C*	*A*	*E*	*AB*	*DE*	*B*	***
Tbitter	18.61	38.6	23.67	50.44	20.26	48.98	21.14	47.54	<0.0001
	C	*B*	*C*	*A*	*C*	*A*	*C*	*A*	***
Toil	59.16	46.53	42.19	44.22	38.75	32.75	30.51	34	<0.0001
	A	*B*	*BC*	*B*	*BCD*	*CD*	*D*	*CD*	***
Tcreamy	44.37	40.94	53.3	19.52	33.28	13.31	24.58	19.22	<0.0001
	AB	*BC*	*A*	*EF*	*CD*	*F*	*DE*	*EF*	***
Twallpaperglue	52.08	38.51	24.67	21.2	32.4	21.59	25.31	24.95	<0.0001
	A	*B*	*C*	*C*	*BC*	*C*	*C*	*C*	***
Mthick	82.99	87.3	48.48	26.78	32.24	19.66	33.44	22.51	<0.0001
	A	*A*	*B*	*CD*	*C*	*E*	*C*	*DE*	***
Mcreamy	78.73	79.45	61.33	25.37	37.86	13.26	37.03	16.16	<0.0001
	A	*A*	*B*	*D*	*C*	*E*	*C*	*E*	***
Mfatty	76.84	72.03	50.41	28.2	41	31.62	37.15	25.04	<0.0001
	A	*A*	*B*	*E*	*C*	*DE*	*CD*	*E*	***
Mslippery	75.03	73.69	45.46	23.27	31.68	16.87	28.86	15.65	<0.0001
	A	*A*	*B*	*CD*	*C*	*D*	*C*	*D*	***
Msticky	55.19	53.11	36.56	17.84	18	18.66	20.68	18.18	<0.0001

	A	*A*	*B*	*C*	*C*	*C*	*C*	*C*	***
Mdry	12.9	32.08	23.89	74.32	25.15	76.35	26.25	68.31	<0.0001
	E	*C*	*D*	*AB*	*D*	*A*	*CD*	*B*	***
Mrough	9.25	22.05	20.33	67.75	21.52	72.03	27.4	69.61	<0.0001
	D	*BC*	*C*	*A*	*BC*	*A*	*B*	*A*	***
Mmouthfilling	86.4	87.87	55.8	35.14	36.96	34.21	36.3	30.81	<0.0001
	A	*A*	*B*	*C*	*C*	*C*	*C*	*C*	***
Mmelting	29.71	26.84	23.54	10.96	16.18	9.81	18.42	11.32	<0.0001
	A	*A*	*AB*	*DE*	*CD*	*E*	*BC*	*DE*	***
ATintensity	33.3	75.85	43.24	79.32	34.02	80.45	34.42	66.49	<0.0001
	D	*A*	*C*	*A*	*D*	*A*	*D*	*B*	***
ATsweet	38.4	62.9	45.85	61.53	31.81	65.22	29.51	52.29	<0.0001
	CD	*A*	*BC*	*A*	*DE*	*A*	*E*	*B*	***
ATbitter	13.57	46.33	25.41	50.63	22.61	49.92	24.51	41.28	<0.0001
	C	*A*	*B*	*A*	*BC*	*A*	*B*	*A*	***
ATsalty	18.24	74.11	27.38	70.07	18.38	67.07	19.36	57.79	<0.0001
	D	*A*	*C*	*A*	*D*	*A*	*CD*	*B*	***
AToxidised	18.46	47.99	29.04	43.89	26.47	47.12	31.12	49.96	<0.0001
	C	*A*	*B*	*A*	*BC*	*A*	*B*	*A*	***
ATduration	35.51	80.63	40.23	79.92	32.04	79.48	44	70.53	<0.0001
	CD	*A*	*CD*	*AB*	*D*	*AB*	*C*	*B*	***
AFastringent	15.09	29.22	26.81	67.96	24	60.6	37.36	53.51	<0.0001
	E	*CD*	*D*	*A*	*DE*	*AB*	*C*	*B*	***
AFdry	13.24	34.6	31.74	72.71	29.66	69.13	35.8	65.94	<0.0001
	C	*B*	*B*	*A*	*B*	*A*	*B*	*A*	***
AFrough	13.77	35.45	28.25	68.93	25.95	67.76	35.01	67.85	<0.0001
	D	*B*	*BC*	*A*	*C*	*A*	*B*	*A*	***
AFraw tongue	10.87	28.88	26.07	54.55	24.02	55.35	27.58	52.96	<0.0001
	C	*B*	*B*	*A*	*B*	*A*	*B*	*A*	***
AFgrainy	20.45	21.69	17.34	18.12	15.38	26.09	14.59	18.73	0.2734
	!	!	!	!	!	!	!	!	
AFcreamy	59.57	45.15	46.38	14.28	23.25	13.69	19.42	13.61	<0.0001
	A	*B*	*B*	*C*	*C*	*C*	*C*	*C*	***
AFslimy	53.46	50.5	34.93	29.03	24.97	25.01	25.07	22.9	<0.0001
	A	*A*	*B*	*BC*	*C*	*C*	*C*	*C*	***
AFcoating	54.24	58.9	40.97	40.02	24.86	35.69	31.84	36.81	<0.0001
	A	*A*	*B*	*BC*	*D*	*BC*	*CD*	*BC*	***
AFsticky	42.63	44.47	30.28	18.98	25.09	25.25	25.99	19.2	<0.0001
	A	*A*	*B*	*C*	*BC*	*BC*	*BC*	*C*	***
AFsatiation	69.28	71.42	47.79	34.91	26.57	36.1	27.6	34.47	<0.0001
	A	*A*	*B*	*CD*	*D*	*C*	*CD*	*CD*	***
AFprickling	19	38.72	26.24	30.01	19.44	33	32.9	36.32	0.0065
	C	*A*	*BC*	*ABC*	*C*	*AB*	*AB*	*AB*	**

[#] *Significant differences are indicated with asterisks: * p<0.05, **, p<0.01, *** p<0.001. Different letters indicate a significant difference at p< 0. 05 calculated with the post hoc LSD analysis; !: not computed as no significant differences were revealed from the ANOVA analysis.*

An overview of the sensory space of the attributes and products based on the PCA analysis is given in Figure 4, where the upper plot depicts odor, taste and after taste attributes and the lower plot the texture attributes.

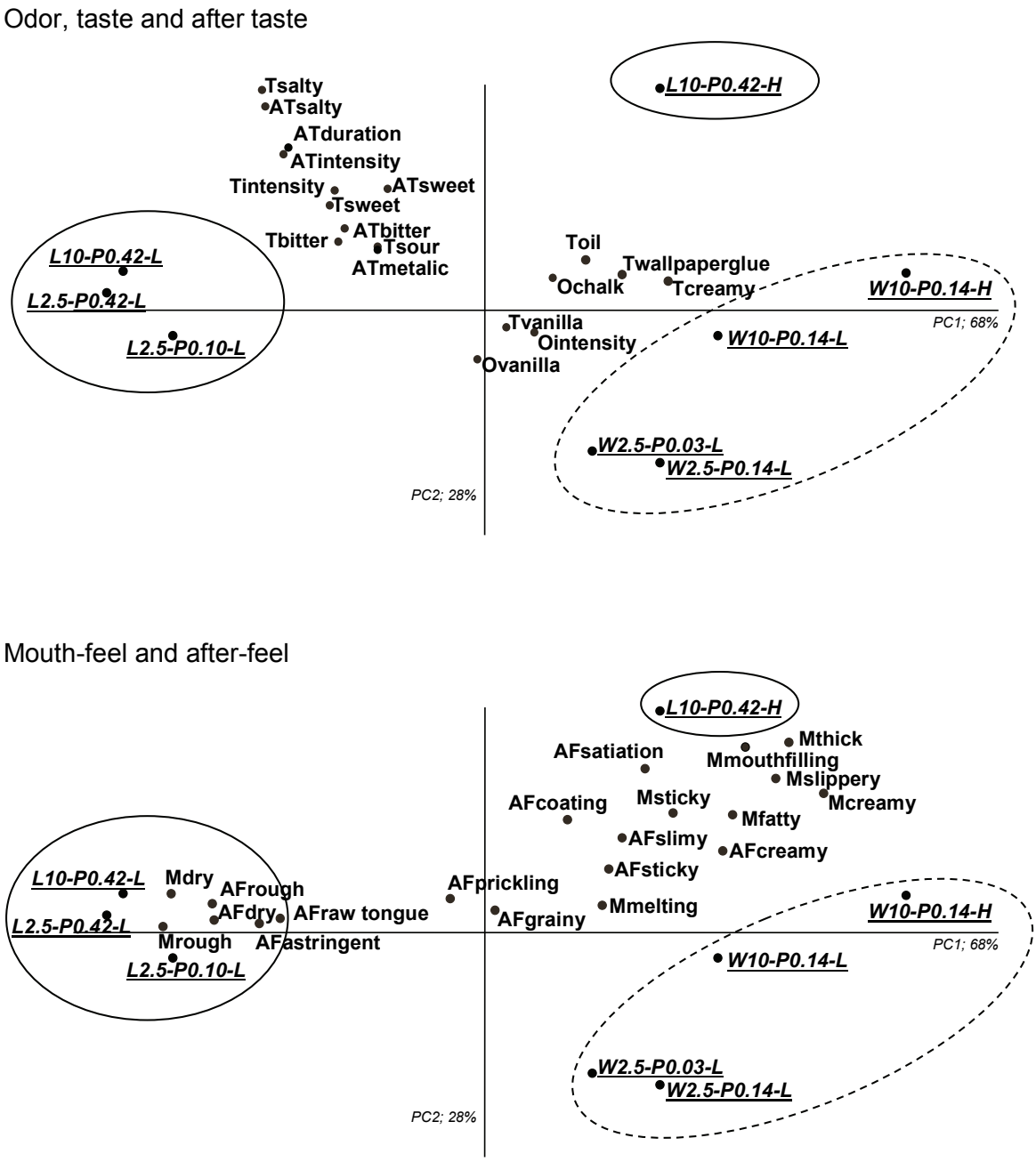

Figure 4 – PCA plot of all products (for the underlined codes see Table 1) and attributes. For clarity, the plot is divided in odor (O), taste (T) and after taste (AT) attributes (top) and mouth-feel (MF) and after-feel (AF) attributes (bottom). Solid and dashed circles are used for emulsions stabilized by lysozyme and WPI, respectively.

The biplot, explaining 96% of the variance, reveals three groups of emulsions. WPI stabilized emulsions (both low viscosity and guar gum thickened) are located on the right hand side of the plot, low viscosity lysozyme stabilized emulsions on the left hand side and the thickened lysozyme emulsion on the upper right part. Clearly, there is a large effect of the type of emulsifier on perception of low viscosity emulsions, indicated by the PC1 axis. The low viscosity WPI stabilized emulsions are situated close to attributes related to creamy, fatty, and slippery mouth-feel whereas lysozyme stabilized emulsions are situated close to attributes related to astringency, dryness, roughness and a series of taste attributes. The set-up of this study made it possible to evaluate the effect of the emulsifier with ANOVA analysis in three different scenarios: at 10% w/w oil content (low viscosity and thickened emulsions), at 2.5% w/w oil content (varying protein content) and at equal protein content, but differing oil content. The results (Table 5) were very similar for all the three comparisons indicating a major effect of the emulsifiers on attributes such as Mcreamy, M/AFdry and M/AFrough, to name a few. WPI stabilized emulsions had significantly higher ratings on attributes like Mthick (only significant in case of low viscosity emulsions), M/AFcreamy, Mfatty and Mslippery, while lysozyme stabilized emulsions are characterized by significantly higher ratings on attribute M/AFdry, M/AFrough, AFastringent, AFraw tongue (Table 4).

Table 5 – Effect of varying emulsion parameters on sensory attributes.

Attribute	**Emulsifier**			**Guar gum**	**Oil content**	**Protein content**
Emulsion	3,5 vs. 4,6	5,7 vs. 6,8	1,3 vs. 2,4	1,2 vs. 3,4	3,4 vs. 5,6	5,6 vs. 7,8
Ointensity						
Ovanilla				*		
Ochalk						
Tintensity	***	***	***	**	*	
Tvanilla				*		
Tsweet	**	**	***	*		
Tsour	*	*	*			
Tsalty	***	***	***		**	
Tbitter	*	*	*	**		
Toil				*		
Tcreamy	**	*	**		*	
Twallpaperglue				*		
Mthick	**	**		***	**	
Mcreamy	***	***	***	***	**	
Mfatty	**	***	**	***		*
Mslippery	**	***	*	***	*	
Msticky				***	*	
Mdry	***	***	***	***		
Mrough	***	***	***	***		
Mmouthfilling				***	**	
Mmelting						
ATintensity	***	***	***	*	*	*
ATsweet	**	**	*			*
ATbitter	*	**	***			
ATsalty	***	***	***			
AToxidised	*	*	*			
ATduration	***	***	***			
AFastringent	***	*	***	**	*	
AFdry	***	***	***	***	**	
AFrough	***	***	***	***		
AFraw tongue	***	***	***	**		
AFgrainy	***				*	
AFcreamy	***	*	***	*	*	
AFslimy				***	**	
AFcoating				*	**	
AFsticky				**		
AFsatiation				***		
AFprickling						

*Significant differences are indicated with asterisks: * p<0.05, ** p<0.01, *** p<0.001, based on ANOVA analysis using repeated measures.*

Thickening of the emulsions with guar gum leads for both emulsions to a shift in the PCA plot in the direction of creaminess, thickness and related attributes, and minimises the differences between the two emulsifiers. The effect of guar gum addition on perception was the largest for the lysozyme emulsions, as illustrated, as an example, in Figure 5 for perceived dry mouth-feel.

In line with previous results [24], perceived creaminess and fattiness increased conjointly with increased viscosity. This becomes clear from increased ratings for Mthick, M/AFcreamy, Mfatty, Mslippery, Msticky, Mmouth filling, AFslimy, AFcoating, AFsticky and AFsatiation. On the other hand astringency, roughness and dryness (Figure 5) largely decreased by guar gum thickening.

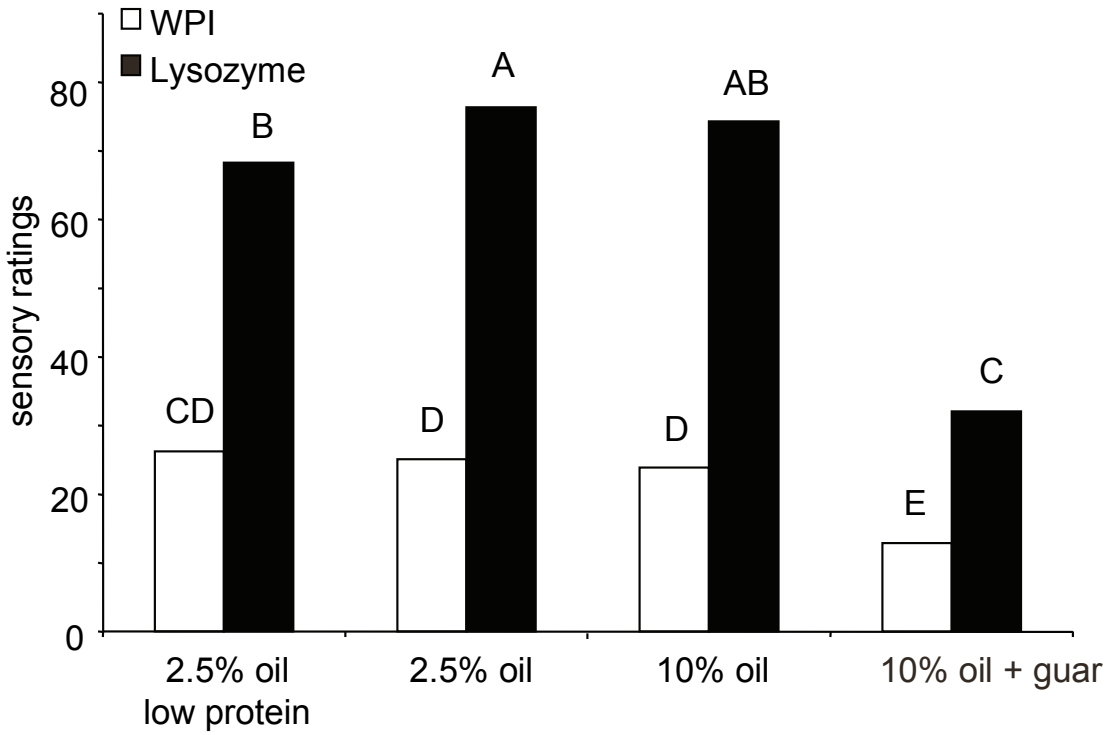

Figure 5 – Effect of guar gum thickening on perception of dry mouth-feel (average). Different letters indicate a significant difference at $p< 0.05$.

Another investigated variable was the oil content, which was studied for low viscosity emulsions. As shown in Tables 4 and 5, the effect of oil content is largely in line with expectations. Increasing the oil content from 2.5% w/w to 10% w/w increased the

perception of T/ATintensity and fat-related attributes like Mthick, M/AFcreamy, Mslippery, AFcoating, and AFsatiation, but also dryness and astringency slightly increased with increasing oil content. Reducing the oil content with an aqueous phase without protein reduced also the protein content in the continuous phase of the emulsions (emulsions 7 and 8). The extent of this effect was minor as as illustrated in Figure 4 and Table 5 by comparing low oil content emulsions diluted with a protein solution (emulsions 5 and 6).

Relation between emulsion characteristics, tongue retention and sensory perception

The relation between emulsion rheological characteristics (η, G' and G'') before and after oral processing, oil and protein retention at the tongue surface and sensory perception was investigated. Emulsion viscosity is one of the parameters that has often been correlated, for example, with perceived thickness and creaminess. The correlation between emulsion viscosity and texture attributes was separately calculated for each emulsifier. As reported in Table 6, high positive correlations were found for WPI emulsion viscosity with Mthick, Mfatty, Mslippery, M/AFsticky, Mmouthfilling, AFgrainy and AFslimy while negative correlations were obtained with M/AFdry, M/AFrough and AFraw tongue. Interestingly, correlations with WPI emulsions were improved by using viscosity data after oral processing as shown by the 3 now significant correlations with Mcreamy, Mmelting and AFcoating. For lysozyme emulsions all texture attributes, except AFgrainy and AFprickling showed high correlations, either positive or negative, with emulsion viscosity. Opposite to the observations with WPI emulsions, correlations of sensory attributes with lysozyme viscosity decreased when values after oral processing were used. For example, Mthick, Mcreamy and Mmelting, did not show significant correlations with the viscosity of the spat out emulsions. The results indicate that oral processing has a great impact on the correlation between emulsion viscosity and sensory attributes and appeared to be emulsifier dependent (WPI vs. lysozyme).

Table 6 – Correlation between viscosity of emulsions, before and after oral processing, and texture attributes.

Attribute	WPI emulsions		Lysozyme emulsions	
	Before oral processing	After oral processing	Before oral processing	After oral processing
Mthick	**0.951**	**0.983**	**0.996**	0.850
Mcreamy	0.831	**0.895**	**0.987**	0.807
Mfatty	**0.952**	**0.982**	**0.992**	**0.899**
Mslippery	**0.941**	**0.976**	**0.993**	0.837
Msticky	**0.880**	**0.933**	**1.000**	**0.902**
Mdry	**-0.988**	**-0.998**	**-0.986**	**-0.888**
Mrough	**-0.914**	**-0.940**	**-0.998**	-0.862
Mmouthfilling	**0.925**	**0.966**	**0.998**	0.877
Mmelting	0.861	**0.916**	**0.997**	0.875
AFastringent	-0.781	-0.800	**-0.935**	**-0.957**
AF dry	**-0.967**	**-0.965**	**-0.987**	**-0.938**
AFrough	**-0.902**	**-0.910**	**-0.999**	**-0.908**
AFrawtongue	**-0.982**	**-0.971**	**-0.997**	**-0.891**
AFgrainy	**0.898**	**0.945**	0.096	0.403
AF creamy	0.785	0.857	**1.000**	**0.886**
AFslimy	**0.939**	**0.975**	**0.980**	0.799
AFcoating	0.856	**0.908**	**0.986**	0.806
AFsticky	**0.961**	**0.988**	**0.970**	**0.941**
AFsatiation	0.877	**0.931**	**0.999**	**0.901**
AFprickling	-0.549	-0.544	0.735	**0.880**

Significant correlations ($p<0.05$) are indicated in bold.

Correlation analysis between oil and protein retention on the tongue surface and texture attributes, calculated for all emulsions as well as for WPI and lysozyme emulsions separately (not shown), revealed that these oil and protein retention hardly correlate with texture perception. Excluding guar gum thickened emulsions improved the correlations, i.e.

protein retention showed a significant correlation (for combined results of WPI and lysozyme) with Mdry, Mmelting (negative), AFastringent, AFdry, AFrough and AFraw tongue (not shown). In conjunction, oil retention significantly correlated with the texture attributes M/AFdry and AFastringent. This result indicates that for the evaluated low viscosity emulsions there is a strong relation between protein retention and sensations of roughness and dryness.

As thickened emulsions largely dominate the outcome of viscosity measurements as well as sensory perception, we limited further analysis aiming to relate emulsion characteristics, retention and sensory perception to the low viscosity emulsions. In addition, since correlation analysis revealed that a large number of physical data was correlated (not shown), the investigation was limited to rheological properties of emulsions and spat out samples measured at 90 s^{-1}, oil- and protein retention and the perception of texture attributes. In line with Figure 4, Figure 6 shows that WPI stabilized emulsions were situated in proximity of the fat/creamy cluster on the right hand side and lysozyme stabilized emulsions in proximity of the rough/dry cluster on the left-hand side of the plot.

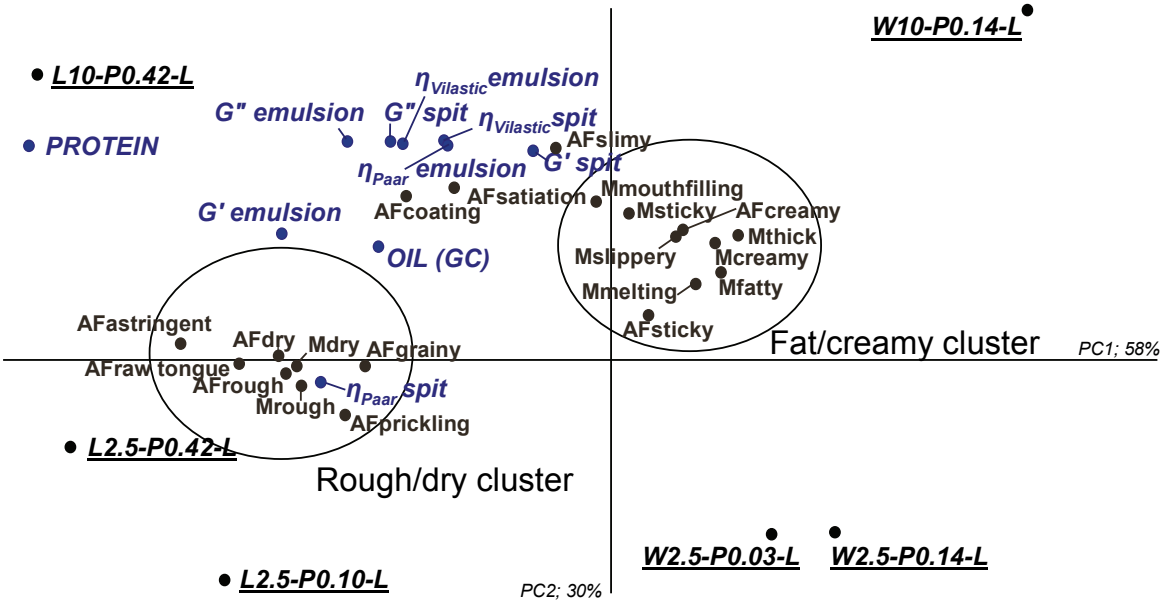

Figure 6 – Biplot of PCA analysis of rheological parameters at 90 s^{-1}, oral retention of oil and protein based on GC and SDS electrophoresis (italics), and texture attributes for low viscosity lysozyme and WPI stabilized emulsions (underlined). The codes for the emulsions are clarified in Table 1. The circled areas indicate the rough/dry and the fat/creamy clusters.

The PC1 axis can be viewed as the emulsifier axis. Protein and oil retention are located on the left, near the rough/dry cluster, indicating that they are related to perceived dryness and roughness. The PC2 axis can be viewed as oil content and viscosity axis, with 10% w/w emulsion on top and 2.5% w/w at the bottom of the biplot. Emulsion viscosity was in close proximity of the attributes AFcoating, AFsatiation and AFslimy. Considering the orally processed emulsions, the shear rate dependent viscosity measured with the cone-and-plate geometry (η_{Paar}spit) is situated in the rough/dry cluster and near the lysozyme stabilized emulsions. This observation suggests that the viscosity of flocculated spat out emulsions relate to the perception of roughness and dryness more than to creaminess. The other rheological characteristics ($\eta_{Vilastic}$spit, *G'* and *G")* of spat out emulsions, instead, were in the biplot not clearly located in close proximity of one of the emulsifier types. Furthermore it is worth to mention that while *G'* of emulsions was near the rough/dry cluster, the *G'* of the spat out was close to the fat/creamy cluster and near to the attribute AFslimy. Oppositely to the viscosity finding, *G'* of flocculated spat out emulsions seems to relate to the perception of creaminess and fattiness rather than to roughness and dryness.

Discussion

The aim of this study was to understand how saliva-induced flocculation behavior of emulsions influences perception and to correlate physical properties of orally processed emulsions with sensory attributes. Previously, we have reported that the usage of WPI or lysozyme as emulsifiers influenced saliva-induced flocculation behavior and physical-chemical characteristics of the emulsion/saliva mixtures, such as viscosity, storage and loss moduli (Chapters 2 and 4) [21, 27].

WPI emulsions, reversibly flocculating with saliva, were perceived as creamy and fatty, opposite to lysozyme emulsions, irreversibly flocculating with saliva, that were perceived as rough, dry and astringent. The large effect of the emulsifier on saliva-induced flocculation behavior and sensory perception underlines the importance of understanding structure changes of emulsions during oral processing in relation to perception.

Emulsion viscosity is a recognized dominant factor in perceived fattiness and creaminess [6, 18, 36-42]. The sensory effect obtained for WPI emulsions in our study is largely in line with previous results [24, 39]. The viscosity of WPI emulsions positively correlated with fat-related attributes like Mthick, Mfatty, Mmouthfilling, AFslimy, and negatively with

perceived roughness and dryness (Table 6). Correlations were improved when viscosity measurements were used of orally processed and subsequently spat out emulsions, resembling the in-mouth emulsion viscosity. This is for example illustrated by the now significant correlation with the complex attribute creaminess (0.895; Table 6). Based on these results, it is advisable to also take into account rheological properties after oral processing when seeking relations between emulsion properties and sensory perception.

Lysozyme is known as one of the sweet tasting proteins [43], which was also clear from this study with high scores on sweet taste and after taste. Unexpectedly, both lysozyme stabilised emulsions and viscosities of orally processed emulsions (η_{Paar}spit) are in the PCA biplot in or in close proximity of the rough/dry cluster. The results resemble the perception of other astringent ingredients like tannins. Tannins interact with salivary proteins, leading to precipitation and withdrawal of lubricating praline-rich proteins and histatins from saliva. The loss of lubrication and elasticity of saliva results in perceived astringency [44-48]. Lysozyme is also known to interact with salivary proteins (Chapter 3) [23, 49] leading to complex formation and irreversible flocculation of emulsions (Chapters 2 and 3) [23]. Similarly to tannins, flocculation of lysozyme stabilized emulsions induced by complexes with proteins in saliva as well as in the mucous layer, might reduce lubrication and perhaps even remove patches of the mucous layer from the tongue surfaces. This could result in a drop in the lubrication properties of saliva and oral surfaces leading to the perception of attributes as astringent, dry and rough mouth- and after-feel. Identification of salivary proteins and peptides involved in emulsion flocculation in emulsion/saliva mixtures (Chapters 5 and 6) [32, 50] demonstrated that a number of peptides and proteins, including MUC5B and MUC7, are associated with emulsion droplets stabilized by lysozyme and β-lactoglobulin. In the current work, we focused on the recovery of MUC5B and MUC7 from the tongue surface after emulsion intake and processing. When compared to the clean tongue, i.e. before oral processing, no differences were observed in recovered MUC5B and MUC7 upon consumption of lysozyme stabilized emulsions. These findings suggest that removal of mucins from the tongue mucous layer is not the main mechanism explaining the astringency induced by these emulsions. Surprisingly, in case of oral processing of WPI emulsions, the amount of MUC7 recovered from the tongue surface decreased compared to a clean tongue, which might indicate that this mucin is removed from the tongue surface after emulsion intake and subsequent rinsing. Possibly the association of MUC7 with WPI emulsion droplets (Chapter 6) [32] leads to clearance of this mucin from the oral cavity.

Another explanation could be that after the formation of the emulsion coating, the underlying tongue mucus layer is less accessible for sampling of the mucins. However, this latter explanation is not supported by MUC5B recovery, which was unaffected. Ongoing research using repeated sampling and/or sampling at different time points after oral processing might clarify this issue.

Another sensory attribute which was hypothesized to be correlated with viscosity is thickness. The correlation between emulsion viscosity and mouth-feel thick improved when considering the viscosity of orally processed flocculated WPI emulsions compared to emulsions before oral processing. However, perceived thickness of lysozyme stabilized emulsions, with a higher viscosity after oral processing, was lower than the corresponding WPI emulsions. This suggests that perception of thickness occurs directly after taking the emulsion in the mouth, with a minor contribution of saliva. This is underlined by the observation that the lysozyme emulsion viscosity correlates much better with perceived thickness when measured in the absence of saliva (Table 6). Another possible explanation for the lower perceived thickness of lysozyme stabilized emulsions is that this attribute is negatively affected by the strong dryness and roughness of these emulsions.

Polysaccharides are often applied to emulsions, mostly because of their thickening effect [51] and/or their role in fat replacement [52]. Previously, it was hypothesized that fatty mouth-feel is related to the formation of a thin lubricating layer of liquid fat on the surfaces of the tongue and palate [53]. Guar gum might form a similar thin lubricating layer which might be sensed as more slimy and sticky than fatty [24]. In case of lysozyme emulsions, guar gum addition reduced the rough, dry and astringent sensations, as well as oil retention to the tongue surface. Although saliva-induced flocculation still occurs in thickened emulsions (Figure 1), it is possible that the complex formation between the emulsion droplets and salivary proteins is influenced by the guar gum. The presence of polysaccharides have been reported to prevent complex formation between salivary proteins and tannins, with consequent reduction of the astringent after-feel [46, 54]. However, we believe that the main reason for the reduced perceived dryness and roughness is the viscous guar gum layer substituting the lubricating properties of saliva. This is in line with the use of hydrocolloids, e.g. carboxymethyl cellulose, in artificial saliva to treat dry mouth syndrome [55-58]. The results stress the importance of the lubrication activity of saliva, also for sensory perception of food products and the possibilities for hydrocolloids to add to salivary lubrication and for ingredients as lysozyme to reduce it.

To gain understanding in after-feel sensations, we used several biochemical techniques to analyze the retention of oil and emulsifiers on the tongue surface. As coalescence of WPI stabilized emulsion was not observed upon oral processing [59] and flocculated emulsions were observed in tongue scrapings, it is likely that the presence of emulsion droplets at the tongue surface plays an important role in after-feel sensations of food emulsions. There was a large difference between the two emulsions, with lysozyme stabilized emulsions showing more retention than WPI stabilized emulsions. In the PCA biplot this resulted in a location of recovered protein emulsifier and oil in the biplot in close proximity of dryness and roughness, indicating a relation between perceived dryness and roughness and oral retention. Opposite to expectations, no clear relations were found between protein and oil retention with after-feel coating. This implies that the large differences in the degree of oral retention, as seen for lysozyme and WPI, do not add to the understanding of this attribute.

Conclusions

Perception of emulsions is affected by saliva-induced flocculation of the emulsion droplets. WPI emulsions show reversible flocculation with saliva, with little retention on the tongue surface and perception characterized by creaminess, fattiness and thickness. Guar gum thickening further increases perception of these attributes. On the other hand, irreversible flocculation with saliva of lysozyme stabilized emulsions leads to a large viscosity increase upon oral processing, retention of emulsions droplets on tongue surface and is related to dryness and roughness sensations. Guar gum thickening decreases the effects of lysozyme, likely because of the improved lubrication related to the increased viscosity. The behavior of lysozyme emulsions reminds one of astringency perception of e.g. tannins that precipitate salivary proteins.

Acknowledgements

We thank Cargill BV for donating high quality sunflower oil and Belovo for their kind gift of lysozyme. In addition, we acknowledge all companies who generously donated the ingredients, as indicated in the material section. We thank Hetty Kroese-Hoedeman, Nancy Holthuysen, and Jos Mojet (Agrotechnology and Food Sciences Group, The Netherlands) for performing the panel tests and assistance in writing the informed consent. René de Wijk

is thanked for the statistical analyses and discussion on interpretation of the sensory results. Franklin Zoet, Jerry van Maanen, Eefjan Timmerman are acknowledged for their help in setting up measuring methods, developing the emulsion systems, emulsion preparation and characterization. Marijke Beenes is thanked for mucin determination in tongue swabs. The valuable discussions with Els de Hoog and Jan Benjamins were highly appreciated.

References

1. van Nieuw Amerongen, A. and Veerman, E.C.I., *Saliva - the defender of the oral cavity.* Oral Diseases, 2002. **8**: p. 12.
2. Prinz, J.F. and Lucas, P.W., *An optimization model for mastication and swallowing in mammals.* Proceedings of the Royal Society B: Biological Sciences, 1997. **264**: p. 1715.
3. Bardow, A., Moe, D., Nyvad, B., and Nauntofte, B., *The buffer capacity and buffer systems of human whole saliva measured without loss of CO2.* Archives of Oral Biology, 2000. **45**: p. 1.
4. Christensen, C.M., Brand, J.G., and Malamud, D., *Salivary changes in solution pH: A source of individual differences in sour taste perception.* Physiology & Behavior, 1987. **40**: p. 221.
5. De Wijk, R.A., Prinz, J.F., Engelen, L., and Weenen, H., *The role of alpha-amylase in the perception of oral texture and flavour in custards.* Physiology & Behavior, 2004. **83**: p. 81.
6. De Wijk, R.A., Prinz, J.F., and Janssen, A.M., *Explaining perceived oral texture of starch-based custard desserts from standard and novel instrumental measurements.* . Food Hydrocolloids, 2006. **20**: p. 24.
7. Engelen, L., de Wijk, R.A., Prinz, J.F., and Bosman, F., *The relation between saliva flow after different stimulations and the perception of flavor and texture attributes in custard desserts.* Physiology & Behavior, 2003. **78**: p. 165.
8. Engelen, L., De Wijk, R.A., Prinz, J.F., Janssen, A.M., Van der Bilt, A., Weenen, H., and Bosman, F., *A comparison of the effects of added saliva, alpha-amylase and water on texture perception in semisolids.* Physiology & Behavior, 2003. **78**: p. 805.
9. Lugaz, O., Pillias, A.M., Boireau-Ducept, N., and Faurion, A., *Time-intensity evaluation of acid taste in subjects with saliva high flow and low flow rates for acids of various chemical properties.* Chemical Senses, 2005. **30**: p. 89.
10. Sanz, T. and Luyten, H., *Effect of thickening agent in the in vitro mouth, stomach and intestine release of tyrosol from enriched custards.* Food Hydrocolloids, 2006. **20**: p. 703.
11. Barylko-Pikielna, N., Martin, A., and Mela, D.J., *Perception of taste and viscosity of oil-in-water and water-in-oil emulsions.* Journal of Food Science, 1994. **59**: p. 1318.
12. Doyen, K., Carey, M., Linforth, R.S.T., Marin, M., and Taylor, A.J., *Volatile release from an emulsion: Headspace and in-mouth studies.* Journal of Agriculture and Food Chemistry, 2001. **49**: p. 804.
13. Odake, S., Roozen, J.P., and Burger, J.J., *Effect of saliva dilution on the release of diacetyl and 2-heptanone from cream style dressings.* Nahrung, 1998. **42**: p. 385.
14. Odake, S., Roozen, J.P., and Burger, J.J., *Flavor release of diacetyl and 2-heptanone from cream style dressings in three mouth model systems.* Bioscience, Biotechnology and Biochemistry, 2000. **64**: p. 2523.
15. Prinz, J.F. and De Wijk, R.A., *The role of oral processing in flavour perception*, in *Flavor perception*, A.J. Taylor and D.D. Roberts, Editors. 2004, Blackwell Publishing Ltd: Oxford. p. 39.
16. Ruth, S.M.v. and Roozen, J.P., *Influence of mastication and saliva on aroma release in a model mouth system.* Food Chemistry, 2000. **71**: p. 339.
17. van Ruth, S.M., Grossmann, I., Geary, M., and Delahunty, C.M., *Interactions between artificial saliva and 20 aroma compounds in water and oil model systems.* Journal of Agricultural and Food Chemistry, 2001. **49**: p. 2409.
18. Akhtar, M., Stenzel, J., Murray, B.S., and Dickinson, E., *Factors affecting the perception of creaminess of oil-in-water emulsions.* Food Hydrocolloids, 2005. **19**: p. 521.
19. Kokini, J. and Aken, G.v., *Discussion session on food emulsions and foams.* Food Hydrocolloids, 2006. **20**: p. 438.
20. Daget, N., Joerg, M., and Bourne, M., *Creamy perception in model dessert creams.* Journal of Texture Studies, 1987. **18**: p. 367.
21. Silletti, E., Vingerhoeds, M.H., Norde, W., and van Aken, G.A., *The role of electrostatics in saliva-induced emulsion flocculation.* Food Hydrocolloids, 2007. **21**: p. 596.
22. Vingerhoeds, M.H., Blijdenstein, T.B.J., Zoet, F., and Aken, G.A.v., *Emulsion flocculation induced by saliva and mucin.* Food Hydrocolloids, 2005. **19**: p. 915.
23. Silletti, E., Vingerhoeds, M.H., Norde, W., and van Aken, G.A., *Complex formation in mixtures of lysozyme-stabilized emulsions and human saliva.* Journal of Colloid and Interface Science, 2007. **313**: p. 485.

24. Vingerhoeds, M.H., De Wijk, R.A., Zoet, F.D., Nixdorf, R.R., and Van Aken, G.A., *How emulsion composition and structure affect sensory perception of low viscosity model emulsions* Food Hydrocolloids, in press. **doi:10.1016/j.foodhyd.2007.02.011**.
25. Blijdenstein, T.B.J., van Vliet, T., van der Linden, E., and van Aken, G.A., *Suppression of depletion flocculation in oil-in-water emulsions: A kinetic effect of beta-lactoglobulin.* Food Hydrocolloids, 2003. **17**: p. 661.
26. Hunt, J.A. and Dalgleish, D.G., *Adsorption behavioour of whey protein isolate and caseinate in soya oil-in-water emulsions.* Food Hydrocolloids, 1994. **8**: p. 175.
27. Silletti, E., Vingerhoeds, M.H., van Aken, G.A., and Norde, W., *Rheological behaviour of food emulsions mixed with saliva: Effect of oil content, salivary protein content and saliva type.* Submitted for publication, 2007.
28. de Jongh, H.H.J. and Janssen, A.M., *Differential clearance kinetics of adhered layer constituents from the oral cavity as modulator for afterfeel of dressings: Atr ft-ir measurements of localized oral coatings.* Journal of Texture Studies, 2007. **38**: p. 70.
29. Metcalfe, L.D., Schmitz, A.A., and Pelka, J.R., *Rapid preparation of fatty acids esters from lipids for gaschromatographic analysis.* . Analytical Chemistry, 1966. . **318**: p. 514.
30. Schipper, R., Loof, A., de Groot, J., Harthoorn, L., Dransfield, E., and van Heerde, W., *SELDI-TOF-MS of saliva: Methodology and pre-treatment effects.* Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 2007. **847**: p. 45.
31. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T* . Nature, 1970. **227**: p. 680.
32. Silletti, E., Vitorino, R.M.P., Schipper, R.G., Amado, F.M.L., and Vingerhoeds, M.H., *Characterization of salivary proteins interacting with lysozyme and β-lactoglobulin stabilized emulsions.* Manuscript in preparation., 2007
33. Veerman, E.C., Bolscher, J.G., Appelmelk, B.J., Bloemena, E., van den Berg, T.K., and van Nieuw Amerongen, A., *A monoclonal antibody directed against high M_r salivary mucins recognizes the SO_3-3Galβ1-3GlcNAc moiety of sulfo-Lewis^a: A histochemical survey of human and rat tissue.* . Glycobiology, 1997. **7**: p. 37.
34. Bolscher, J.G.M., Groenink, J., van der Kwaak, J.S., van den Keijbus, P.A.M., van 't Hof, W., Veerman, E.C.I., and van Nieuw Amerongen, A., *Detection and quantification of MUC7 in submandibular, sublingual, palatine and labial saliva by anti-peptide antiserum.* Journal of Dental Research, 1999. **78**: p. 1362.
35. Stone, H. and Sidel, J.L., *Sensory evaluation practices*. 3rd ed. Food science and technology, ed. S.L. Taylor. 2004, New York: Elsevier Academic Press. 377.
36. Akhtar, M., Murray, B.S., and Dickinson, E., *Perception of creaminess of model oil-in-water dairy emulsions: Influence of the shear-thinning nature of a viscosity-controlling hydrocolloid.* Food Hydrocolloids, 2006. **20**: p. 839.
37. de Wijk, R.A. and Prinz, J.F., *Fatty versus creamy sensations for custard desserts, white sauces, and mayonnaises.* Food Quality and Preference, 2007. **18**: p. 641.
38. De Wijk, R.A., Terpstra, M.E.J., Janssen, A.M., and Prinz, J.F., *Perceived creaminess of semi-solid foods.* Trends in Food Science & Technology, 2006. **17**: p. 412.
39. Mela, D.J., Langley, K.R., and Martin, A., *Sensory assessment of fat content: Effect of emulsion and subject characteristics* Appetite, 1994. **22**: p. 67.
40. Janhøj, T., Frøst, M.B., and Ipsen, R., *Sensory and rheological characterization of acidified milk drinks.* Food Hydrocolloids, 2007. **doi:10.1016/j.foodhyd.2007.03.006**
41. Kilcast, D. and Clegg, S., *Sensory perception of creaminess and its relationship with food structure.* Food Quality and Preference, 2002. **13**: p. 609.
42. Tarrega, A. and Costell, E., *Effect of composition on the rheological behaviour and sensory properties of semisolid dairy dessert.* Food Hydrocolloids, 2006. **20**: p. 914.
43. Masuda, T., Ide, N., and Kitabatake, N., *Structure-sweetness relationship in egg white lysozyme: Role of lysine and arginine residues on the elicitation of lysozyme sweetness* Chem. Senses, 2005. **30**: p. 667.
44. de Freitas, V. and Mateus, N., *Nephelometric study of salivary protein-tannin aggregates.* J. Science of Food and Agriculture, 2002. **82**: p. 113.
45. Lu, Y. and Bennick, A., *Interaction of tannin with human salivary proline-rich proteins.* Archives of Oral Biology, 1998. **43**: p. 717.
46. Mateus, N., Carvalho, E., Luis, C., and de Freitas, V., *Influence of the tannin structure on the disruption effect of carbohydrates on protein-tannin aggregates.* Analytica Chimica Acta, 2004. **513**: p. 135.

47. Prinz, J.F. and Lucas, P.W., *Saliva tannin interactions.* Journal of Oral Rehabilitation, 2000. **27**: p. 991.
48. Siebert, K.J. and Chassy, A.W., *An alternate mechanism for the astringent sensation of acids.* Food Quality and Preference, 2003. **15**: p. 13.
49. Soares, R.V., Lin, T., Siqueira, C.C., Bruno, L.S., Li, X., Oppenheim, F.G., Offner, G.D., and Troxler, R.F., *Salivary micelles: Identification of complexes containing mg2, siga, lactoferrin, amylase, glycolsylated proline-rich protein and lysozyme.* Archives of Oral Biology, 2004. **49**: p. 337.
50. Silletti, E., Vitorino, R.M.P., De Groot, J., Vingerhoeds, M.H., and Amado, F.M.L., *Analysis and identification of salivary peptides interacting with β-lactoglobulin and lysozyme stabilized emulsions by SELDI-TOF-MS and RP-HPLC coupled to MS-TOF-TOF.* Submitted, 2007.
51. Tarrega, A. and Costell, E., *Effect of composition on the rheological behaviour and sensory properties of semisolid dairy dessert.* Food Hydrocolloids, 2006. **20**: p. 914.
52. Frøst, M.B., Dijksterhuis, G., and Martens, M., *Sensory perception of fat in milk.* Food Quality and Preference, 2001. **12**: p. 327.
53. Van Aken, G.A., De Hoog, E.H.A., Nixdorf, R.R., Zoet, F.D., and Vingerhoeds, M.H., *Aspects of sensory perception of food emulsions thickened by polysaccharides*, in *Gums and stabilisers for the food industry*, P.A. Williams and G.O. Phillips, Editors. 2006, The Royal Society of Chemistry: Cambridge. p. 449
54. Carvalho, E., Mateus, N., Plet, B., Pianet, I., Dufourc, E., and de Freitas, V., *Influence of wine pectic polysaccharides on the interactions between condensed tannins and salivary proteins.* Journal of Agricultural and Food Chemistry, 2006. **54**: p. 8936.
55. Kelly, H.M., Deasy, P.B., Busquet, M., and Torrance, A.A., *Bioadhesive, rheological, lubricant and other aspects of an oral gel formulation intended for the treatment of xerostomia.* International Journal of Pharmaceutics, 2004. **278**: p. 391.
56. Levine, M.J., Aguirre, A., Hatton, M.N., and Tabak, L.A., *Artificial salivas: Present and future.* Journal of Dental Research, 1987. **66**: p. 639.
57. Reeh, E.S., Douglas, W.H., and Levine, M.J., *Lubrication of human and bovine enamel compared in an artificial mouth.* Archives of Oral Biology, 1995. **40**: p. 1063.
58. van der Reijden, W.A., Veerman, E.C.I., and Van Nieuw Amerongen, A., *Rheological properties of commercially available polysaccharides with potential use in saliva substitutes.* Biorheology, 1994. **31**: p. 631.
59. Dresselhuis, D.M., De Hoog, E.H.A., Cohen Stuart, M.A., Vingerhoeds, M.H., and Van Aken, G.A., *The occurrence of in-mouth coalescence of emulsion droplets in relation to perception of fat.* Food Hydrocolloids, 2007. **doi:10.1016/j.foodhyd.2007.06.013**.

Appendix A - Description of sensory attributes

Attribute	Description
Odor attributes	
Ointensity	Intensity of the odor
Ovanilla	Vanilla odor; baby food
Ochalk	Odor of chalk; pancake batter; meal, milk
Taste attributes	
Tintensity	Intensity of the taste; taste-explosion
Tvanilla	Taste of vanilla
Tsweet	Sweet taste
Tsour	Sour taste
Tsalty	Slightly salt taste, like liquorice, sea water
Tbitter	Bitter taste
Toil	Taste of salad oil
Tcreamy	Taste of cream; dairy taste
Twallpaperglue	Cardboard taste, starch, wall paper glue
Mouth-feel attributes	
Mthick	From water to yoghurt thickness
Mcreamy	Velvety; warm; soft
Mfatty	Fatty oily layer in mouth and on lips
Mslippery	Slippery feeling, smooth, like syrup
Msticky	Tacky, sticky feeling
Mdry	Dry feeling in the mouth; saliva is absorbed; swallowing difficult
Mrough	Rough feeling on the teeth and palate
Mmouthfilling	Feeling that whole the mouth is filled up
Mmelting	Thinning of the product in the mouth, structure disappears
After taste attributes	
ATintensity	Intensity of the after taste
ATsweet	Sweet aftertaste
ATbitter	Bitter; taste like aspirin; sparkling feeling in the rear of the mouth
ATsalty	Slight salty after taste; like liquorice or seawater
AToxidised	Metal; iron like
ATduration	Duration of taste perception in the mouth
After-feel attributes	
AFastringent	Astringent; contracting after feel
AFdry	Saliva absorbing; dry tongue
AFrough	Rough feeling on the teeth
AFraw tongue	Raw feeling; sandpaper or cat's tongue
AFgrainy	Very fine granules; mealy, powdery, flowery
AFcreamy	Velvety; warm; soft
AFslimy	Feeling of threads at palate; keep swallowing
AFcoating	Fatty coating on tongue, lips or cheek
AFsticky	Syrupy; stickily
AFsatiation	Hunger alleviation; rich, satisfactory
AFprickling	Tingling prickling feeling on lips and/or tongue

Chapter 8

Summary and general discussion

The aim of this work was to examine the role of saliva in the oral behavior of food emulsions. With oral behavior of emulsions we denote the changes in the emulsion properties in the oral cavity (environment) due to the presence of saliva. Other aspects of oral behavior such as the effect of the tongue movement or the shearing of emulsions between oral surfaces are beyond the scope of this thesis.

Extensive literature is available on various aspects of emulsions and saliva separately, a glimpse of which is provided in the introduction of this thesis (Chapter 1). Furthermore, the reported studies [1-7] about the influence of saliva on sensory perception of food (emulsions) indicate an increasing interest in this topic. Nevertheless, at the beginning of this research, very little information was available on the impact of saliva on emulsion characteristics and on how and/or which parameters are relevant in determining the physical properties and texture of food emulsions when mixed with saliva.

Saliva-induced emulsion flocculation showed similarity with physical chemical studies conducted on mixtures of emulsions and polysaccharides. The first mechanistic investigation on the effect of salivary proteins, namely mucins, reported by Vingerhoeds and co-authors [8], offered the starting point for this research. The authors hypothesized mucin-induced depletion as the main flocculation mechanism, although they suggested that other salivary components could play a role in emulsion flocculation as well. Based on the above-mentioned hypotheses, and aiming to understand the *in vivo* situation, we decided to use human saliva, collected from different donors. Although stimulated saliva can be easily collected in relatively large quantity, it is constituted mostly of parotid saliva, which does not contain mucins. In the scope of the suggested hypothesis, the use of unstimulated saliva was more appropriate. As the amount of saliva from one person was not sufficient to conduct repeated measurements, unstimulated saliva was pooled from a group constituted of about ten volunteers. We do realize that, in this way, the presented results reflect an averaged response. However, even if it would have been possible to use individual saliva, drawing general conclusions on emulsion oral behavior would be equally difficult because of the well-known large variability of saliva properties between different persons.

The complexity of saliva imposed limitations on the type of physical chemical techniques that could be used (Chapters 2-4). In the search for alternative approaches, biochemical and proteomic methods seemed to be a suitable choice. As a result, we were able to provide valuable information at molecular level of the salivary components involved in emulsion flocculation (Chapters 5 and 6). To conclude this work, we performed a sensory study (Chapter 7) to investigate the influence of the different flocculation behavior on perception

and to explain the perception of food emulsions based on the information obtained from physical-chemical and biochemical studies.

Summary of the main results

The preceding observation that saliva induces emulsion flocculation [8] was further investigated considering the effect of emulsion parameters on the flocculation reversibility (Chapter 2), rheological properties (Chapters 2 and 4) and on sensory perception (Chapter 7). Chapter 2 describes the influence of using different emulsifiers, both surfactants and proteins, in emulsion/saliva mixtures. The variation in the observed behavior of emulsion/saliva mixtures clearly depends on the adsorbed layer of emulsifier at the oil-water interface of the emulsion droplets and was attributed to the differences in ζ-potential induced by the emulsifiers. When emulsions were prepared with SDS and diacetyl tartaric acid ester of monoglyceride (Panodan), resulting in emulsions with a highly negative ζ-potential (i.e. -90 mV and -75 mV, respectively), no flocculation was observed. When emulsions showed weakly negative or positive ζ-potential, saliva-induced flocculation occurred. "Reversible" or "irreversible" flocculation, upon water dilution and shearing experiments, was linked to the charge sign on the droplet surface. Reversible flocculation was observed for emulsions stabilized by β-lg and Tween 20, which showed a slightly negatively ζ-potential. Upon mixing with saliva, an increase in the viscosity (η) of these emulsions by a factor of two to three was observed (Chapter 2). Irreversible flocculation of positively charged emulsions stabilized by lysozyme and CTAB, resulted in even larger increases in η, storage modulus (G') and loss modulus (G'') (Chapters 2 and 4) compared to reversible flocculation. The behavior of emulsion/saliva mixtures was further studied considering, the effect of oil content or salivary protein concentration (Chapter 4). Increased η, G' and G'' were observed upon increasing both oil content and salivary protein concentration. Lastly, the effect of the types of saliva (stimulated vs. unstimulated) was analyzed as well. The results indicate that stimulated saliva causes a smaller increase in the η of β-lg stabilized emulsion compared to unstimulated saliva. A decrease in G' and G'' was observed upon mixing stimulated saliva with β-lg stabilized emulsions. In case of lysozyme stabilized emulsion/saliva mixture, stimulated saliva increased G' and G'', but did not significantly affect the viscosity of this mixture.

By means of a sensory panel study, the effect of some of the investigated parameters, e.g. emulsifier type, on perception was evaluated (Chapter 7). Clearly, a major difference in the perception was related to the type of emulsifiers used. Emulsions stabilized by whey isolated protein (WPI), which flocculate reversibly with saliva, were perceived as creamy and fatty while lysozyme stabilized emulsions, which flocculate irreversibly upon mixing with saliva, were perceived as rough, dry and astringent.

In Chapters 3, 5 and 6 we further investigated the mechanism responsible for emulsion flocculation. It was hypothesized that a depletion mechanism was responsible for flocculation of weakly negatively charged emulsions while complex formation between salivary proteins and emulsifiers at the oil-water interface could be responsible for floc formation in positively charged emulsions (Chapter 2). Indeed, complexes between lysozyme and salivary proteins in solution were observed by CLSM (Chapter 3). Furthermore, CLSM images of lysozyme emulsion/saliva mixtures showed that flocs were constituted of droplets embedded in a proteinaceous structure. In anticipation of the forthcoming discussion, it was noted that CLSM images of β-lg stabilized emulsion/saliva mixtures, did not show similar results. To investigate the nature of flocculation and establish the protein composition both in the emulsion's continuous and droplet phase, we used different biochemical and proteomic techniques. Chapters 5 and 6 showed that a large number of salivary proteins and peptides (SPs) in the whole studied M_r range are associated with lysozyme and β-lg stabilized emulsion droplets. Moreover, the results indicate that the type of emulsifying protein at the oil-water interface determines which SPs associate with the droplets in the flocs.

Interaction of salivary components with emulsion droplets

The large amount of SPs associated with the droplets raises several questions regarding the nature of the interactions involved. Information about the percentage of secondary structure (e.g. α-helix) or surface hydrophobicity upon adsorption of lysozyme and β-lg to air-water interfaces is available in literature [9-12]. However, detailed information, for example, of which amino acids of the proteins at the oil-water interfaces are exposed to the aqueous phase is not available yet, although in case of lysozyme adsorbed onto an air-water interface there are indications that the C-terminal end of the molecule is positioned facing the aqueous solution [11]. The study of the conformation of used proteins at the oil-water

interface lies beyond the scope of this thesis. Some speculations are therefore needed to discuss the interaction mechanism.

Analysis of the salivary components associated with the emulsion droplets showed very complex profiles due to the large number of detected species and M_r range (from 1000 Da to > 250 kDa). Based on results in Chapters 5 and 6, the emulsifier type determines which SPs are associated with the droplets. For example, the findings that hydrophilic and hydrophobic peptides with M_r < 2000 Da were respectively associated to lysozyme and β-lg stabilized emulsion droplets, suggest that interactions of these SPs might be driven by specific motives on the emulsifier molecule exposed to the aqueous phase. In addition, biochemical evidences from experiments conducted on β-lg emulsion/saliva mixtures at pH 4 point to a major role of the emulsifier in affecting the association of SPs with the droplets (Figure 1). At pH 4, both RP-HPLC and SDS-PAGE show patterns similar to those obtained at neutral pH, indicating only a minor pH effect on the association of SPs (with M_r < 100 kDa) with the droplets (Figure 1a and 1b, respectively). Differences were, instead, observed for the salivary mucins MUC5B and MUC7. At pH 4, both MUC5B and MUC7 were associating with droplets (Figure 1c), whereas, at neutral pH, MUC5B was found in the continuous phase of β-lg emulsion/saliva mixtures as well. These results confirm that the emulsifier protein layer largely influences the type of adsorbed SPs. Moreover the electrostatic effect as driving force for adsorption due to pH changes seems to be limited, in β-lg emulsion/saliva mixtures, to large molecular weight proteins as MUC5B.

Another point of discussion concerns the structure of the associated protein layer, which might be of particular interest for future investigations. For example, SPs might interact with the emulsifiers at the oil-water interfaces, as single molecules but also as part of larger complexes. The presence of complexes between several SPs [13-16] could allow proteins which do not have affinity to the emulsion droplets to be associated with the oil droplets. Moreover, it is possible that several SPs layers are present on the droplets and that the adsorption of each protein is influenced by the nature of the previous layer [17, 18]. A summary of the possible interaction mechanisms of salivary proteins and peptides with emulsion droplets is schematically illustrated in Figure 2.

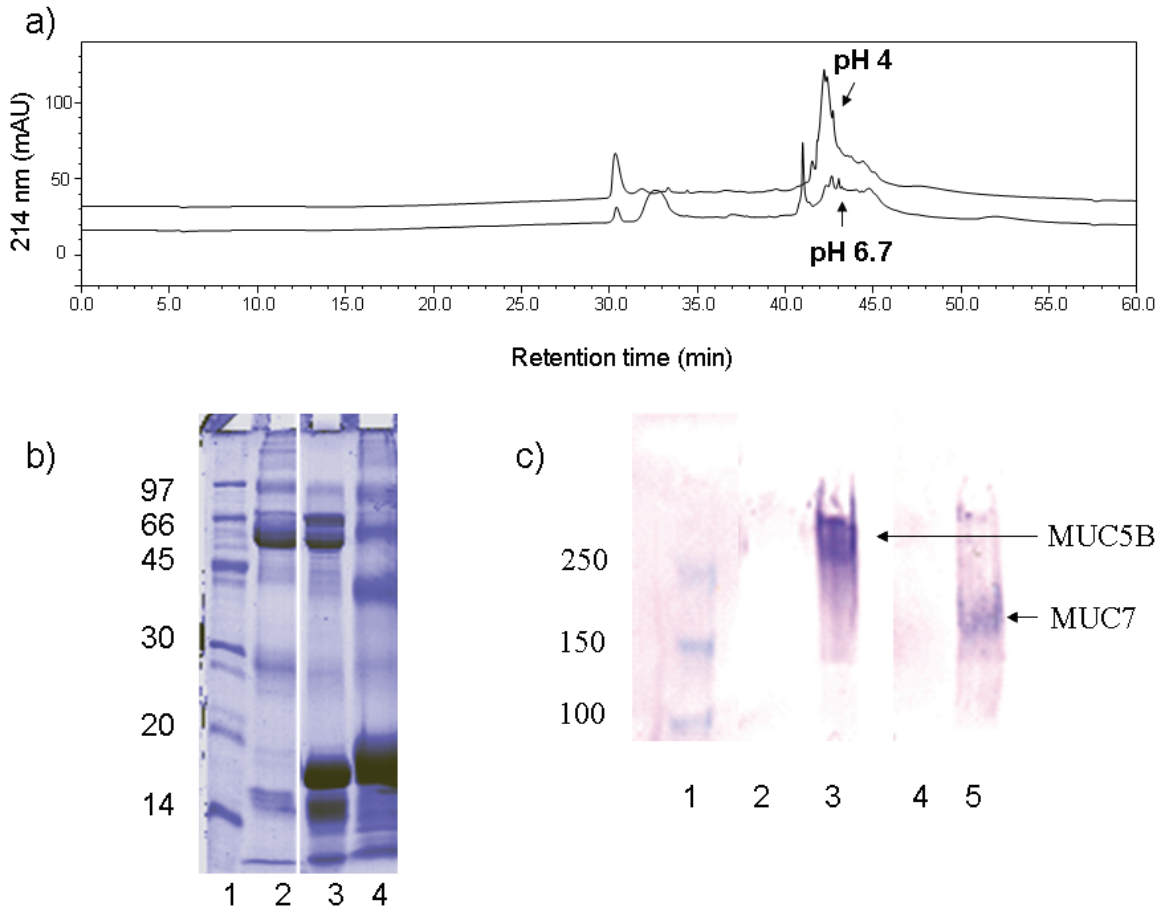

Figure 1 – (a) RP-HPLC profiles PA of β-lg stabilized emulsions mixed with saliva at pH 4 and pH 6.7 (for comparison); (b) SDS-PAGE on a 15 % gel after Coomassie staining of marker (lane 1), saliva, (lane 2), PNA (lane 3) and PA (lane 4) obtained from β-lg emulsions/saliva mixture at pH 4; (c) Western blot performed with monoclonal antibodies anti-MUC5B (lanes 2 and 3) and anti-MUC7 (lanes 4 and 5) of samples PNA (lanes 2 and 4) and PA (lanes 3 and 5). Lane 1 shows the marker. PNA and PA indicate proteins non-associated and protein-associated with emulsion droplets, respectively, upon mixing emulsions with saliva.

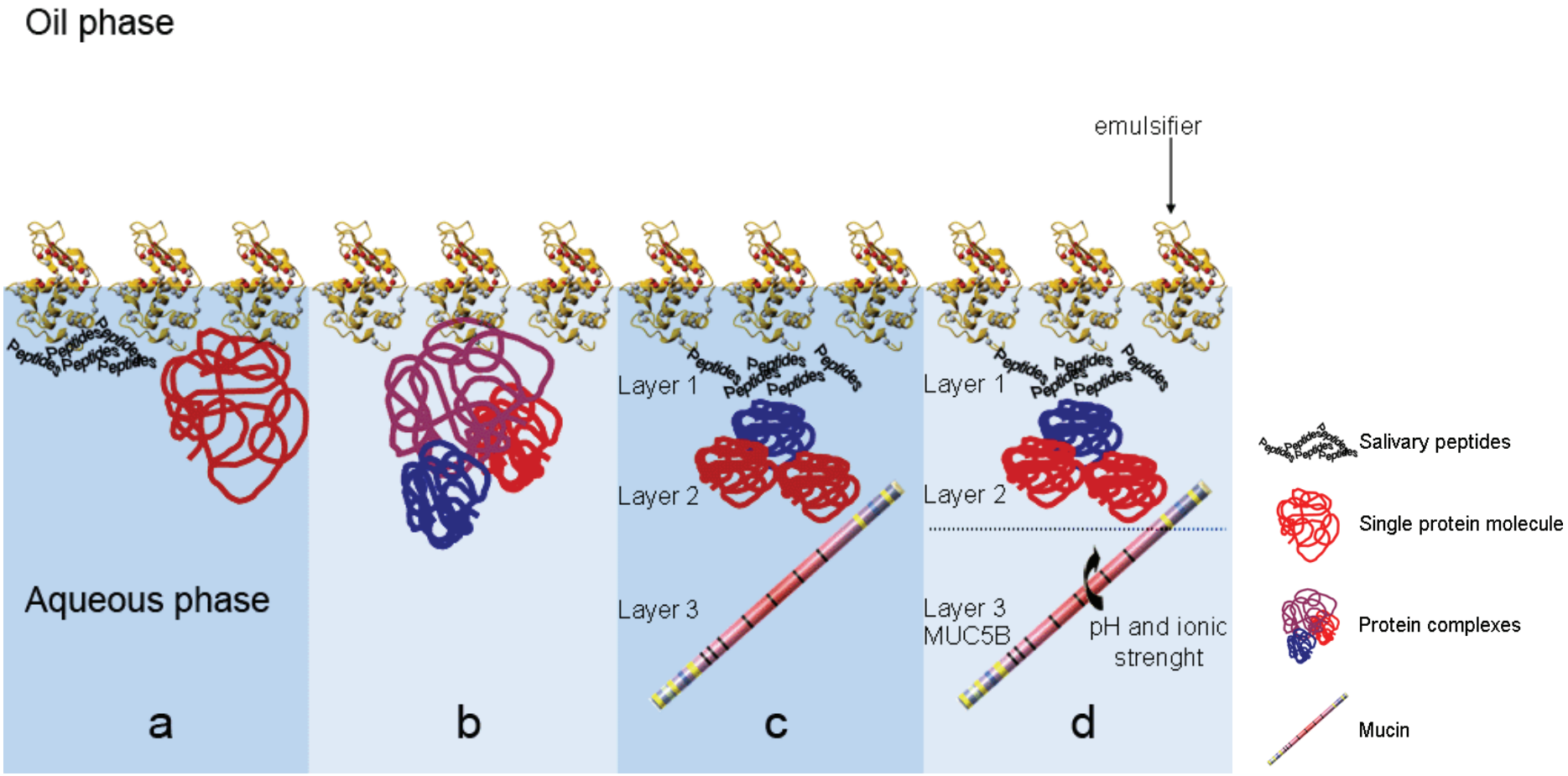

Figure 2 – Schematic representation of the possible interaction of SPs with the emulsifiers at the oil-water interfaces (based on Chapters 5 and 6). Interaction of SPs as peptide molecule or single protein (a), as part of complexes (b) or as components of multi-layers (c). The effect of the pH (Chapters 2 and 8) and ionic strength (Chapter 3) in affecting SPs adsorption maybe be confined to the outer SPs layer and involved proteins such as MUC5B (d). At neutral pH (for β-lg) and high ionic strength (for lysozyme) a decrease of SPs in this layer may explain the presence of smaller flocs and observed viscosity of the mixtures.

Depletion or bridging flocculation?

In view of the findings presented in Chapters 2 and 3, both depletion and bridging flocculation mechanisms were initially hypothesized. According to Chapters 5 and 6, the crucial step in determining the emulsion flocculation behavior is the interaction/adsorption of SPs with the oil-water interface stabilized by the emulsifiers. CLSM images of lysozyme emulsions after mixing with saliva, showed emulsion droplets embedded in a matrix of proteinaceous salivary structure. Unfortunately, microscopic investigation using SEM and CLSM conducted on β-lg emulsion/saliva mixtures did not provide valuable indication to support the two hypothesized flocculation mechanisms. As illustrated in Figure 3, it is not clear whether SPs are in contact with the droplets or are just located in their proximity. Moreover, these techniques do not offer sufficient resolution for a close analysis of the droplet surface. The depletion mechanism for β-lg emulsions was initially proposed based on a previous study [8] and on laser diffraction and rheology measurements (Chapter 2). The sizes of the flocs in flocculated emulsions obtained with laser diffraction were smaller than those observed with CLSM and light microscopy. It is likely that dilution and shearing during the measurements lead to break up of the large flocs into smaller ones. This suggestion, underlined by the hysteresis observed during rheological experiments, with lower viscosity values for the backward curves compared to the forward curves, points to irreversible break up of the flocs upon shear.

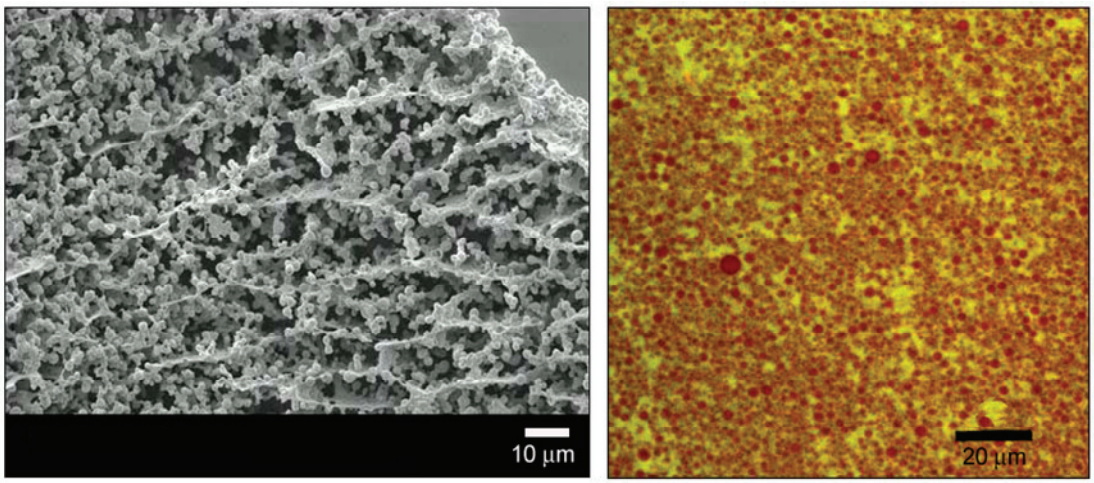

Figure 3 – Cryo-SEM (left) and CLSM (right) of β-lg emulsion/saliva mixtures (10% w/w oil; 0.6 mg mL^{-1} SPs) stained with Nile-Red (oil phase, in dark) and Oregon Green (SPs in white).

According to the definition of depletion, flocculation is induced by non-adsorbing polymers, because the center of mass of the molecule cannot approach the droplet surface closer than a distance of about its radius of gyration. In view of the large M_r and three-dimensional structure, mucins resemble other polysaccharides, such as dextran, which are causing emulsion flocculation by depletion [19, 20]. If salivary mucins are responsible for depletion flocculation of β-lg emulsions, they should not be found present in the droplets phase. As shown, instead, both MUC5B and MUC7 were associated with β-lg emulsion droplets at physiological pH (Chapter 6). We propose that bridging flocculation plays a dominant role in the observed flocculation of β-lg and lysozyme stabilized emulsions. Therefore, differences in emulsion behavior upon mixing with saliva as described in Chapters 2, 4 and 7 are attributed to variations in complex formation/adsorption of salivary proteins to the emulsion droplets. The (ir-)reversibility can be explained assuming differences in the interaction forces due to the different SPs adsorbed to the droplets. Upon dilution and higher shear rates, SPs-droplets complexes are broken down. We, therefore, suggest that in case of β-lg emulsion/saliva mixtures, the particle-size distribution obtained with laser diffraction measurements reflects the presence of single droplets with small salivary proteins and peptides attached. Figure 4 illustrates the effect of shear and water dilution on the flocculated lysozyme and β-lg emulsions, according to the proposed flocculation mechanism.

In this thesis the mechanism of saliva-induced flocculation was further investigated by means of biochemical techniques only for emulsions stabilized by proteins. However, it is likely that SPs adsorption, as driving force for flocculation, occurred with emulsions stabilized by surfactant as CTAB and Tween 20 as well. Similarly to complex formation in Chapter 3, complexes between CTAB in solution and saliva have been observed with light microscopy (data not shown). Moreover, irreversible flocculation took place in a mixture of saliva and CTAB stabilized emulsions (Chapter 2). Previously, irreversible flocculation in Tween 20 stabilized emulsion/saliva mixtures was reported when saliva from different individuals was used [8]. These observations, which suggest adsorption of SPs onto CTAB and Tween 20 oil-water interfaces, point to a predominant role of bridging in the saliva-induced flocculation of the studied emulsions.

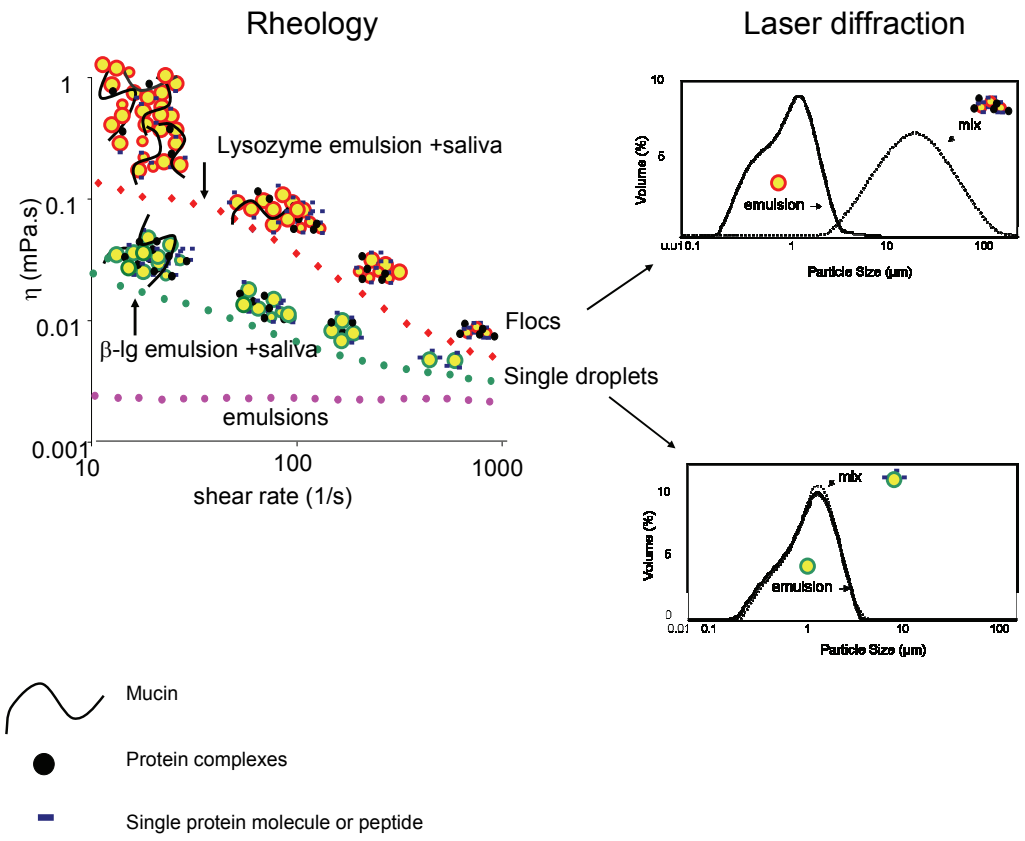

Figure 4 – Variation of the shear rate dependent viscosity and measured particle size for lysozyme and β-lg stabilized emulsions mixed with saliva as consequence of the different SPs associated with emulsion droplets.

A different perspective for sensory perception

It is generally accepted that the sensory perception of food in the mouth is related to physical-chemical properties of the products [21-25]. For instance, viscosity is thought to be one of the important parameters to explain the perception of creaminess and thickness [24-27]. During this study we have shown that rheological properties of liquid emulsions drastically change upon mixing with saliva as a consequence of association with SPs. An increase in η, G' and G'' was observed as a consequence of flocculation.

Sensory perception is also known to be affected by the processes occurring in the mouth during mastication and by mixing with saliva [1, 4, 5, 28]. We showed that the differences in saliva-induced flocculation behavior can be clearly perceived with astringent and rough attributes from one side, and creamy, fatty attributes from the other.

Our results show that different SPs are involved in adsorption with emulsion droplets which results in emulsion flocculation. Our suggestion is that the different SPs present at the

droplet surface are responsible for the characteristics of the flocs as, for example, the floc size. These features ultimately determine the bulk properties of the studied emulsion/saliva mixtures such as the viscosity and the shear thinning behavior (Figure 4). Moreover, upon adsorption onto the droplets, saliva is deprived of proteins and peptides as well (Chapters 5 and 6) which may lead to changes in saliva properties. For example, the complete removal of the lubricating mucins from saliva, as seen in lysozyme emulsion/saliva mixtures might affect lubrication at oral surfaces and ultimately be responsible for the astringent sensation [29-31]. Furthermore, it is likely that flocculation at the tongue and palate surfaces influences the friction forces which are known to be important for perception [32, 33]. In view of these considerations, SPs-droplets complexes most likely influence the perception of low viscosity emulsions in several ways rather than affecting only the bulk rheological properties of the emulsion/saliva mixtures. The schematic representation of this new proposed mechanism ultimately responsible for the sensory perception of non-thickened emulsions is illustrated in Figure 5. To understand the cause of the sensorial experience of food emulsions in the mouth, it might be important to further investigate at a molecular level the interaction with salivary components.

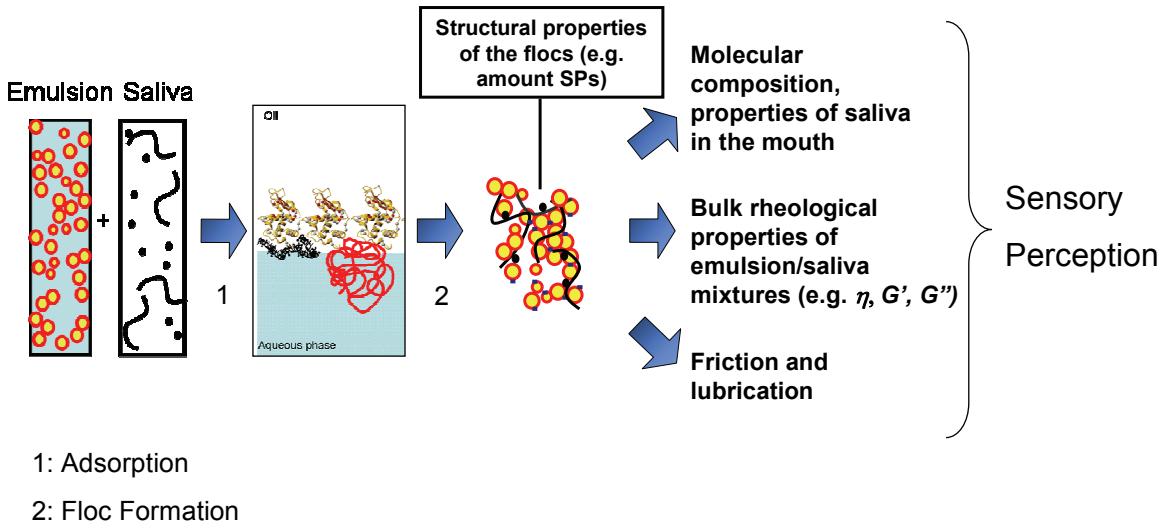

Figure 5 – Schematic representation of the different steps occurring upon mixing the studied emulsions with saliva. Adsorption of salivary protein and peptide to the emulsion droplets, followed by floc formation with characteristic structural features, is the key factor influencing sensory perception. As a consequence of the floc formation, changes in saliva properties, rheology of emulsion/saliva mixtures, friction and lubrication in the mouth might determine differences in perception of low viscosity emulsions.

Suggestions for future research

As research is performed new results lead to new insights while many questions are still waiting to be answered. Future research could focus on the molecular interaction between the surface of emulsion droplets and saliva in order to better understand the flocculation mechanism and the possibility of controlling it. We show that by varying conditions, as type of emulsifiers and pH, flocculation can be changed. As the experiments have been conducted using predominantly unstimulated pooled saliva from different donors, it is of interest to evaluate the effect of unstimulated saliva collected from different individuals. Moreover, it would be useful to further study association of SPs from stimulated saliva with emulsion droplets. As an alternative, instead of using an *in vitro* mixture of emulsions with saliva, spat out samples could be used. As salivary components were found on the emulsion droplets, it could be of interest to relate perception to the amount of SPs at the droplets surface. It is surely fascinating to study a relationship between the different amount of SPs at the droplets and the sensory rating by different persons. Moreover, the same type of study could be performed to evaluate the link of SPs with measured rheological parameters, e.g. viscosity, from spat out samples.

To further confirm the role of flocculation in perception, the investigation of the perception of emulsions which do not flocculate upon mixing with saliva could be informative. Unfortunately, our attempt to prepare food grade low viscosity emulsions with such characteristics did not succeed.Moreover, since this research was mostly conducted on liquid model emulsions, it is worthwhile to investigate the effect of saliva on more complex emulsions with composition similar to commercial products.

References

1. Engelen, L., de Wijk, R.A., Prinz, J.F., and Bosman, F., *The relation between saliva flow after different stimulations and the perception of flavor and texture attributes in custard desserts.* Physiology & Behavior, 2003. **78**: p. 165.
2. Engelen, L., de Wijk, R.A., Prinz, J.F., Janssen, A.M., van der Bilt, A., Weenen, H., and Bosman, F., *A comparison of the effects of added saliva, alpha-amylase and water on texture perception in semisolids.* Physiology & Behavior, 2003. **78**: p. 805.
3. Engelen, L., van der Keybus, P.A.M., de Wijk, R.A., Veerman, E.C.I., van Nieuw Amerongen, A., van der Bilt, A., Prinz, J.F., and Bosman, F., *The effect of saliva composition on texture perception in semi solids.* Archives of Oral Biology, 2007. **52**: p. 518.
4. Kallithraka, S., Bakker, J., Clifford, M.N., and Vallis, L., *Correlations between saliva protein composition and some T-I parameters of astringency.* Food Quality and Preference, 2001. **12**: p. 145.
5. Guinard, J.-X., Zoumas-Morse, C., and Walchak, C., *Relation between parotid saliva flow and composition and the perception of gustatory and trigeminal stimuli in foods.* Physiology & Behavior, 1998. **63**: p. 109.
6. De Wijk, R.A., Prinz, J.F., Engelen, L., and Weenen, H., *The role of alpha-amylase in the perception of oral texture and flavour in custards.* Physiology & Behavior, 2004. **83**: p. 81.
7. Christensen, C.M., Brand, J.G., and Malamud, D., *Salivary changes in solution pH: A source of individual differences in sour taste perception.* Physiology & Behavior, 1987. **40**: p. 221.
8. Vingerhoeds, M.H., Blijdenstein, T.B.J., Zoet, F., and van Aken, G.A., *Emulsion flocculation induced by saliva and mucin.* Food Hydrocolloids, 2005. **19**: p. 915.
9. Lechevalier, V., Croguennec, T., Pezennec, S., Guerin-Dubiard, C., Pasco, M., and Nau, F., *Ovalbumin, ovotrasferrin, lysozyme: Three model proteins for structural modifications at the air-water interface.* Journal of Agricoltural and Food Chemistry, 2003. **51**: p. 6354.
10. de Jongh, H.H.J., Kosters, H.A., Kudryashova, E., Meinders, M.B., Trofimova, D., and Wierenga, P.A., *Protein adsorption at air-water interfaces: A combinations of detailes.* Biopolymers, 2004. **74**: p. 131.
11. Lu, J.R., Su, T.J., Thomas, R.K., Penfold, J., and Webster, J., *Structural conformation of lysozyme layers at the air-water interfaces studied by neutron reflection.* Journal of the Chemical Society Faraday Transactions, 1998. **94**: p. 3279.
12. Martin, A.H., Meinders, M.B.J., Bos, M.A., Cohen Stuart, M.A., and van Vliet, T., *Conformational changes of proteins at the air/water interface studied by IRRAS.* Langmuir, 2002. **19**: p. 2922.
13. Helmerhorst, E.J. and Oppenheim, F.G., *Saliva: A dynamic proteome.* Journal of Dental Research, 2007. **86**: p. 680.
14. Yao, Y., Lamkin, M.S., and Oppenheim, F.G., *Pellicle precursor protein crosslinking: Characterization of an adduct between acidic proline-rich protein (PRP-1) and statherin generated by transglutaminase.* Journal of Dental Research, 2000. **79**: p. 930.
15. Iontcheva, I., Oppenheim, F.G., and Troxler, R.F., *Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins.* Journal of Dental Research, 1997. **76**: p. 734.
16. Oppenheim, F.G., Hay, D.I., and Franzbla.C, *Proline-rich proteins from human parotid saliva .1. Isolation and partial characterization.* Biochemistry, 1971. **10**: p. 4233.
17. Lindh, L., Svendsen, I.E., Svensson, O., Cárdenas, M., and Arnebrant, T., *The salivary mucin MUC5B and lactoperoxidase can be used for layer-by-layer film formation.* Journal of Colloid and Interface Science, 2007. **310**: p. 74.
18. Svensson, O., Lindh, L., Cárdenas, M., and Arnebrant, T., *Layer-by-layer assembly of mucin and chitosan. Influence of surface properties, concentration and type of mucin.* Journal of Colloid and Interface Science, 2006. **299** p. 608.
19. Blijdenstein, T.B.J., van Winden, A.J.M., van Vliet, T., van der Linden, E., and van Aken, G.A., *Serum separation and structure of depletion- and bridging-flocculated emulsions: A comparison.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2004. **245**: p. 41.
20. Blijdenstein, T.B.J., Zoet, F.D., van Vliet, T., van der Linden, E., and van Aken, G.A., *Dextran-induced depletion flocculation in oil-in-water emulsions in the presence of sucrose.* Food Hydrocolloids, 2004. **18**: p. 857.
21. Akhtar, M., Stenzel, J., Murray, B.S., and Dickinson, E., *Factors affecting the perception of creaminess of oil-in-water emulsions.* Food Hydrocolloids, 2005. **19**: p. 521.

22. Daget, N., Joerg, M., and Bourne, M., *Creamy perception in model dessert creams.* Journal of Texture Studies, 1987. **18**: p. 367.
23. Barylko-Pikielna, N., Martin, A., and Mela, D.J., *Perception of taste and viscosity of oil-in-water and water-in-oil emulsions.* Journal of Food Science, 1994. **59**: p. 1318.
24. Kilcast, D. and Clegg, S., *Sensory perception of creaminess and its relationship with food structure.* Food Quality and Preference, 2002. **13**: p. 609.
25. Akhtar, M., Murray, B.S., and Dickinson, E., *Perception of creaminess of model oil-in-water dairy emulsions: Influence of the shear-thinning nature of a viscosity-controlling hydrocolloid.* Food Hydrocolloids, 2006. **20**: p. 839.
26. Mela, D.J., Langley, K.R., and Martin, A., *Sensory assessment of fat content: Effect of emulsion and subject characteristics* Appetite, 1994. **22**: p. 67.
27. Tarrega, A. and Costell, E., *Effect of composition on the rheological behaviour and sensory properties of semisolid dairy dessert.* Food Hydrocolloids, 2006. **20:** p. 914.
28. Engelen, L., de Wijk, R.A., Prinz, J.F., Janssen, A.M., van der Bilt, A., Weenen, H., and Bosman, F., *A comparison of the effects of added saliva, alpha-amylase and water on texture perception in semisolids.* Physiology & Behavior, 2003. **78**: p. 805.
29. Prinz, J.F. and Lucas, P.W., *Saliva tannin interactions.* Journal of. Oral Rehabilitation, 2000. **27**: p. 991.
30. Joslyn, M.A. and Goldstein, J.L., *Astringency of fruits and fruit products in relation to phenolic content.*, in *Advance in food reseach.* C.O. Chichester, M.E. Mrak, and G.F. Stewart, Editors. 1987, Academic Press. p. 179.
31. Carvalho, E., Mateus, N., Plet, B., Pianet, I., Dufourc, E., and de Freitas, V., *Influence of wine pectic polysaccharides on the interactions between condensed tannins and salivary proteins.* Journal of Agricultural and Food Chemistry, 2006. **54**: p. 8936.
32. Kokini, J.L., *The physical basis of liquid food texture and texture-taste interactions.* Journal of Food Engineering, 1987. **6**: p. 51.
33. Prinz, J.F., de Wijk, R.A., and Huntjens, L., *Load dependency of the coefficient of friction of oral mucosa.* Food Hydrocolloids, 2007. **21**: p. 402.

Samenvatting

Tijdens consumptie ondergaan levensmiddelen verscheidene processen in de mond, die de sensorische waarneming beïnvloeden. Eén van die processen is het mengen van het voedsel met speeksel. Veel levensmiddelen, zoals bijvoorbeeld melk, zijn emulsies. Emulsies zijn dispersies van twee onderling niet mengbare vloeistoffen, waarin één vloeistof in de vorm van kleine druppeltjes is gedispergeerd in een andere vloeistof. Deze studie richt zich op het gedrag van levensmiddelemulsies wanneer deze in de mond met speeksel gemengd worden. Vanuit eerder onderzoek is bekend dat olie-in-water emulsies uitvlokken wanneer deze gemengd worden met speeksel, waarbij depletie is voorgesteld als drijvende kracht voor vlokking. Depletievlokking is een gevolg van de verlaging van de osmotische druk in een klein volume tussen twee nabije druppels, waartoe polymere moleculen (zoals grote speekseleiwitten) geen toegang hebben (depletie) vanwege hun te grote afmeting. De osmotische druk van de omringende polymeeroplossing drijft de druppels dan bijeen. Om meer inzicht te verkrijgen in het mechanisme waarmee speeksel emulsies uitvlokt, is in **hoofdstuk 2** de rol van elektrostatische interacties op het gedrag van emulsie/speeksel mengsels onderzocht. Emulsies, bereid met surfactants en eiwitten variërend in lading, werden gemengd met speeksel. Emulsies met sterk negatief geladen druppels (SDS en Panodan) vlokken niet met speeksel. De meest waarschijnlijke reden hiervoor is dat de elektrostatische afstoting tussen de druppels sterker is dan de som van de aantrekkende krachten als gevolg van depletie en van der Waals interacties. Netto ongeladen en zwak negatief geladen emulsies (Tween 20 en β-lactoglobuline (β-lg) pH 6.7) vlokken met speeksel, waarbij de vlokken weer uit elkaar vallen (omkeerbare vlokking) bij verdunnen met water of bij aanbrengen van afschuivingkrachten. Dit type vlokking kan waarschijnlijk toegeschreven worden aan depletie interacties veroorzaakt door grote speekseleiwitten zoals mucins, in combinatie met van der Waals interacties en lage elektrostatische afstoting tussen de druppels. Positief geladen emulsies (CTAB, lysozym en β-lg pH 3.0) vlokken onomkeerbaar (irreversibel) met speeksel, wat resulteert in een snelle faseschеiding. De elektrostatische aantrekking tussen de negatief geladen speekseleiwitten en de positief geladen emulsiedruppels speelt hierbij een belangrijke rol. De resultaten van **hoofdstuk 2** laten zien dat het teken van de lading en de ladingsdichtheid op het oppervlak van emulsiedruppels significant bijdragen aan het gedrag van emulsies bij mengen met speeksel. Afhankelijk van de lading zijn depletie en elektrostatische krachten voorgesteld als de twee belangrijkste drijvende krachten voor speekselgeïnduceerde emulsievlokking.

In **hoofdstuk 3** is de interactie tussen speeksel en lysozym-gestabiliseerde emulsies (10 % w/w olie, 10 mM NaCl, pH 6.7) bestudeerd om ons inzicht in het mechanisme van vlokking van deze emulsies met speeksel te vergroten. Met behulp van Confocale Laser Scanning Microscopie zijn complexen tussen speekseleiwitten en lysozym aangetoond. Deze complexvorming vindt zowel plaats met lysozym in oplossing als met lysozym geadsorbeerd aan het olie-water grensvlak van de emulsiedruppels.

Om de rol van elektrostatische interacties in emulsie/speeksel mengsels te onderzoeken, zijn laser diffractie en rheologische metingen uitgevoerd bij variërende ionsterkte. Een toename in de ionsterkte resulteerde in een afname van emulsievlokking door speeksel, zoals waargenomen door afnemende vlokgrootte en het effect op de viscositeit. Turbiditeitsexperimenten met variërende pH (3 tot 7) en ionsterkte lieten ook verminderde complexvorming zien in mengsels van speeksel en lysozym in oplossing. Concluderend, de elektrostatische aantrekking tussen lysozym en speekselcomponenten speelt een belangrijke rol voor complexvorming in lysozym-emulsie/speeksel mengsels.

Hoofdstuk 4 bestudeert de invloed van variatie in emulsie- en speekseleigenschappen op de rheologische eigenschappen van β-lg en lysozym gestabiliseerde emulsies gemengd met speeksel bij pH 6.7. Hierbij is het effect onderzocht van de volumefractie olie, de speekseleiwitconcentratie en het gebruik van niet gestimuleerd en gestimuleerd speeksel. Viscositeit, opslag- en verliesmoduli zijn bepaald aan de emulsies (η_{emul}, G'_{emul} en G''_{emul}) en aan emulsie/speeksel mengsels (η_{mix}, G'_{mix} en G''_{mix}). Voor een inschatting van het effect als gevolg van de speekselgeïnduceerde vlokking op het rheologische gedrag van de emulsies zijn de ratio's η_{mix}/η_{emul}, G'_{mix}/G'_{emul} en G''_{mix}/G''_{emul} berekend. Een verhoging van de volumefractie olie en van de speekseleiwitconcentratie resulteerde in beide gevallen in een verhoging van η_{mix}/η_{emul}, G'_{mix}/G'_{emul} en G''_{mix}/G''_{emul}.

Het mengen van gestimuleerd speeksel met β-lg gestabiliseerde emulsies resulteerde in lagere η_{mix}/η_{emul}, G'_{mix}/G'_{emul} en G''_{mix}/G''_{emul} vergeleken met het niet gestimuleerde speeksel. Aan de andere kant werd in het geval van lysozym gestabiliseerde emulsies een toename in zowel G'_{mix}/G'_{emul} als G''_{mix}/G''_{emul} gevonden voor gestimuleerd speeksel vergeleken met niet gestimuleerd speeksel. Er was geen verschil in het effect van gebruik van gestimuleerd of niet gestimuleerd speeksel op de η_{mix}/η_{emul} van lysozym emulsie/speeksel mengsels. Deze resultaten laten zien dat het effect van gestimuleerd speeksel op de rheologische eigenschappen van emulsie/speeksel mengsels sterk afhangt

van het emulsie type. Dit hoofdstuk geeft aan dat zowel speeksel- als emulsie-eigenschappen het rheologische gedrag van emulsie/speeksel mengsels kunnen beïnvloeden. De verschillen in emulsiegedrag als gevolg van mengen met al dan niet gestimuleerd speeksel, geven aan dat er een belangrijke rol is van speekseleiwitten op het rheologische gedrag van emulsies in de mond. Het doel van **hoofdstuk 5 en 6** was om meer inzicht te verkrijgen in welke speekseleiwitten betrokken zijn bij emulsievlokking. In **hoofdstuk 5** zijn twee proteomics technieken gebruikt, te weten SELDI-TOF-MS en RP-HPLC gekoppeld met MS, om de interactie tussen emulsies en speekseleiwitten en peptiden (SP's) met een molekuulgewicht tussen 800 en 20000 Da uit niet gestimuleerd speeksel te bestuderen. Met deze opzet wordt het voordeel van een snel resultaat met weinig monstervoorbewerking en een algemeen overzicht van de betrokken speekseleiwitten door SELDI-TOF-MS gecombineerd met de identificatie en karakterisering van de sequenties met behulp van RP-HPLC-MS. Lysozym en β-lg gestabiliseerde emulsies zijn gemengd met niet gestimuleerd speeksel tot een concentratie van 10% w/w olie en 0.6 mg mL^{-1} SP's. Speekselcomponenten geassocieerd met de emulsiedruppels, zijn geanalyseerd na het verwijderen van de gebonden eiwitten en peptiden van de druppels met SDS. Voor lysozym gestabiliseerde emulsies zijn in totaal 60 verschillende SP's teruggevonden in de fracties geassocieerd met emulsiedruppels, en voor β-lg emulsies zelfs 119. De resultaten duiden er op dat hydrofiliciteit en hydrofobiciteit een rol spelen bij de associatie van SP's < *m/z* 2000 aan respectievelijk lysozym en β-lg gestabiliseerde emulsiedruppels. Gefosforyleerde SP's associëren voornamelijk met β-lg gestabiliseerde emulsiedruppels. De resultaten van dit hoofdstuk suggereren dat de adsorptie/associatie van SP's aan emulsiedruppels gedeeltelijk gedreven wordt door een specifieke interactie van de speekselcomponenten met de naar de waterfase toe geëxposeerde sequenties van de emulgerende eiwitten op de olie-water grensvlakken.

Hoofdstuk 6 bestudeert de interactie tussen lysozym en β-lg gestabiliseerde emulsies met speekseleiwitten en peptiden met een molecuulgewicht (M_r) groter dan 10 kDa met behulp van infrarood spectroscopie, Western blotting, PAS kleuring en SDS-PAGE gekoppeld aan MS. Het hoogmoleculaire mucine MUC5B bindt sterk aan lysozym gestabiliseerde emulsies. Aan β-lg gestabiliseerde emulsies bindt MUC5B matig en MUC7 sterk. Ook speekseleiwitten in de M_r range van 10-100 kDa binden verschillend aan de lysozym en β-lg emulsiedruppels. Merendeel van de SP's binden aan de lysozym emulsiedruppels, terwijl in het geval van β-lg gestabiliseerde emulsies de SP's zich meer evenredig verdelen tussen

de druppels en de continue fase van de emulsie. Een duidelijk voorbeeld is α-amylase (M_r ~ 55 kDa), dat sterk associeert met lysozym gestabiliseerde emulsiedruppels, maar niet met β-lg emulsie druppels. Onze bevindingen duiden er op dat adsorptie/associatie van speekseleiwitten aan het druppeloppervlak afhankelijk is van het type emulgerend eiwit op het olie-water grensvlak.

Emulsieperceptie kan meestal niet direct worden gerelateerd aan de structuur van deze producten voor consumptie. Rekening houden met de veranderde structuur van het product tijdens verwerking in de mond kan het inzicht in de relatie tussen sensoriek en producteigenschappen vergroten. De resultaten van **hoofdstuk 7** laten een duidelijk effect van speekselgeïnduceerde vlokking op perceptie van de onderzochte emulsies zien. WPI gestabiliseerde emulsies (omkeerbare vlokking) blijven nauwelijks achter op de tong en perceptie wordt gekenmerkt door romigheid, vettigheid en dik mondgevoel. Het verdikken van deze emulsies met guar gom versterkt perceptie van deze attributen. Aan de andere kant wordt perceptie van lysozym gestabiliseerde emulsies (onomkeerbare vlokking) gekenmerkt door een ruw, droog en astringent mond- en nagevoel, een sterke toename in de viscositeit na mengen met speeksel en een duidelijke retentie op het tongoppervlak. Het gedrag van deze emulsies lijkt op de astringente perceptie van tannines, die veroorzaakt wordt door complexeren met speekseleiwitten waardoor de lubricerende werking van speeksel achteruit gaat. Verdikken met guar gom verlaagt de typische sensorische effecten van de lysozym emulsie, waarschijnlijk vanwege de lubricerende eigenschappen van guar gom.

Het laatste hoofdstuk, **hoofdstuk 8**, legt de relatie tussen de fysisch-chemische, biochemische en sensorische studies naar de interactie tussen speeksel en emulsies. Er wordt een interactiemechanisme voorgesteld om speekselgeïnduceerde emulsievlokking te verklaren, in samenhang met de waargenomen perceptie.

Vlokking door brugvorming, waarbij speekseleiwitten en peptiden adsorberen aan het olie-water grensvlak en een brug vormen tussen één of meerdere emulsiedruppels, speelt een dominante rol in de waargenomen vlokking. De verschillen in gebonden speekseleiwitten (hoofdstukken 5 en 6) bepalen de eigenschappen van de vlokken, en daarmee de rheologische eigenschappen van de emulsie/speeksel mengsels, zoals beschreven in hoofdstuk 2, 4 en 7.

Door adsorptie van speekseleiwitten aan de druppels, vermindert de concentratie van deze eiwitten en peptiden in speeksel, wat vervolgens leidt tot veranderde

speekseleigenschappen, zoals een verminderde lubricerende werking. Bovendien is het zeer waarschijnlijk dat emulsievlokking aan tong en verhemelte de frictiekrachten beïnvloeden, waarvan bekend is dat deze een rol spelen bij perceptie. Dit proefschrift stelt dat perceptie van laag-visceuze emulsies wordt beïnvloed door complexen tussen speekseleiwitten en emulsiedruppels, wat leidt tot veranderingen in zowel de speekselsamenstelling als ook in de rheologische eigenschappen van de emulsie/speeksel mengsels.

Riassunto

Alcuni prodotti alimentari, come il latte e il burro, sono emulsioni, ossia dispersioni, più o meno stabili, di un liquido sotto forma di minutissime goccioline (fase dispersa) in un altro liquido non miscibile (fase disperdente). A seguito della loro ingestione, questi prodotti, vengono sottosposti nella nostra bocca a diversi processi e modificazioni structturali. Uno dei questi processi e' il mescolamento con la saliva. Una precedente ricerca aveva mostrato che, a seguito del mescolamento con la saliva, le goccioline della fase dispersa aggregavano, formando dei fiocchi di goccioline, probabilmente per "deplezione".

La finalita' di questa tesi di dottorato e' di studiare il ruolo della saliva nella generazione del processo di aggregazione, meglio conosciuto come flocculazione, di emulsioni modello, cioe' emulsioni costituite da goccioline di olio, stabilizzate da proteine o altri emulsionanti.

Nel **capitolo 1** vengono riportate alcune nozioni di base delle emulsioni e della saliva. Il **capitolo 2** affronta il problema del ruolo dell' interazione elettrostatica nella flocculazione delle miscele emulsione/saliva. L'uso di diverse proteine e surfattanti per stabilizzare le emulsioni, induce una differenza di carica sulla superficie delle emulsioni. Miscele di emulsioni stabilizzate da SDS e Panodan, che posseggono un potenziale ζ sulla superficie delle goccioline altamente negativo, non flocculano. In questo caso, la repulsione elettrostatica supera l'effetto delle attrazione generate dallle forze di deplezione e di van der Waals. Miscele di emulsioni stabilizzate da Tween 20 e β-lattoglobulina a pH 6.7, con un potenziale ζ neutro o poco negativo, flocculano reversibilmente a seguito di diluizioni e di forze di scorrimento (shear). In questo caso, la flocculazione e' probabilemte dovuta a effetto di deplezione delle mucine in combinazione con forze di van der Waals e la debole repulsione elettrostatica. Emulsioni con carica positiva, come quelle stabilizzate da CTAB, lisozima e β-lattoglobulina a pH 3.0 flocculano irreversibilimente. I risultati di questo capitolo mostrano che la carica e l'intensita' della carica sulla superficie delle goccioline contribuisce significativamente al comportamento delle miscele emulsioni/saliva portandoci ad ipotizzare ambedue i fenomeni di deplezione e interazione elettrostatica alla base della flocculazione delle miscele analizzate.

Nel **capitolo 3** abbiamo investigato le miscele di saliva e emulsioni stabilizzate da lisozima (10 % w/w olio, 10 mM NaCl, pH 6.7). Con l'ausilio della microscopia (CLSM) abbiamo mostrato la presenza della formazione di complessi tra le emulsioni e le proteine della saliva. La formazione di questi complessi ha luogo sulla superficie delle goccioline di emulsione, all'interfaccia acqua-olio. L'interazione elettorstatica viene studiata usando metodiche di reologia e di diffrazione della luce, dopo aver variato la forza ionica (tra 0 e

168 mM NaCl) e il pH delle miscele (da 3 a 7). L'aumento della forza ionica induce una riduzione dell'incremento della viscosita' delle miscele dovuta alla flocculazione indotta dalla presenza della saliva. Esperimenti di misurazione della turbidita', invece sono stati condotti variando sia la forza ionica che il pH di alcune soluzioni di lisozima e di saliva. I risultati ottenuti sono in linea con aspettative, indicando rispettivamente una diminuzione e un aumento dei complessi tra le molecule di lisozima e le proteine della saliva a seguito della variazione di NaCl (fornza ionica) e di pH. In conclusione, la formazione di fiocchi di goccioline in queste miscele e' indotta largamente dalla interazione elettrostatica e conseguente formazione di complessi tra il lisozima che stabilizza le emulsione e le proteine della saliva.

Il **capitolo 4** riporta gli effetti delle variazione di alcune caratteristiche delle emulsione e della saliva focalizzando l'attenzione sulle miscele di emulsioni stabilizzate da lisozima (potenziale di superficie positivo) e sulle miscele di emulsioni stabilizzate β-lattoglobulina a pH 6.7 (potenziale di superficie negativo). La viscosita', il modulo dellle deformazione elastica e plastica, in inglese viscosity (η), storage modulus (G') e loss modulus (G''), sono stati studiati variando il volume d'olio delle miscele (dal 2.5% w/w al 10% w/w), la concentrazione di saliva (da 0.1 a 0.8 mg mL^{-1}) e usando saliva prodotta con o senza stimolazione meccanica. L'effetto della saliva e' stato valutato utilizzando il rapporto tra η_{mix}, G'_{mix} en G''_{mix} delle miscele e η_{emul}, G'_{emul} en G''_{emul} delle emulsioni di partenza (in assenza di saliva). I risultati mostrano un aumento di η_{mix}/η_{emul}, G'_{mix}/G'_{emul} e G''_{mix}/G''_{emul} a seguito dell'incremento del volume di olio e della concentrazione della saliva per tutte e due le emulsioni analizzate. La miscele di saliva, ottenuta mediante stimulazione, e emulsione stabilizzata da β-lattoglobulina a pH 6.7, mostra una diminuzione di η_{mix}/η_{emul}, G'_{mix}/G'_{emul} en G''_{mix}/G''_{emul} rispetto all'utilizzazione di saliva ottenuta senza stimulazione. Dall'altro lato, la miscele di saliva, ottenuta mediante stimulazione, e emulsione stabilizzata da lisozima, mostra una aumento di G'_{mix}/G'_{emul} en G''_{mix}/G''_{emul} rispetto all'utilizza di saliva ottenuta senza stimilazione. Questi risulati dimostrano chiaramente che sia variazioni delle proprieta' della saliva che delle emulsioni influenzano il comportamento reologico delle miscele.

Nella seconda parte di questa tesi, **capitoli 5 e 6**, sono state utilizzate diverse metodologie di biochimica e di proteomica per meglio caratterizzare quali sono le proteine della saliva attivamente coinvolte nell'induzione della flocculazione delle emulsioni. Nel **capitolo 5** l'utilizzo di SELDI-TOF-MS e RP-HPLC in combinazione con MS, hanno permesso l'

analisi delle proteine e dei peptidi della saliva (SPs), aventi massa molecolare compresa nell'intervallo tra 800 e 20000 daltons (Da). Il vantaggio di utilizzare queste due techniche consiste da un lato nell' ottenere velocemente informazioni relative al numero di masse molecolari (quindi numero di composti) e dall'altro nell'identificazione delle proteine stesse. Dopo aver mescolato saliva con emulsioni stabilizzate da lisozima e β-lattoglobulina a pH 6.7, le SPs che sono rimaste attaccate alle goccioline, sono rimosse con un trattamento con SDS. Nel caso di miscele di saliva con emulsioni di lisozima, 60 differenti SPs sono stati trovati associati alle emulsioni, mentre, nel caso di miscele di saliva con emulsioni di β-lattoglobulina a pH 6.7, 119 differenti SPs sono stati rinvenuti. I risultati indicano che l'idrofobicita' e/o idrofilicita' di peptidi aventi massa molecolare inferiore a 2000 Da, gioca un ruolo importante nel determinare se un peptide assorbira' o meno sulla superficie delle goccie di emulsione. Inoltre e' stato dimostrato che proteine con modificazioni post traduzionali, come ad esempio fosforilazione, associano in prevalenza a goccie di emulsione stabilizzate da β-lattoglobulina a pH 6.7. In generale questo capitolo mostra che l' assorbimento/associazione di SPs con le emulsioni e' guidato da un interazione specifica tra i componenti della saliva e sequenze delle emulsificante esposte verso la fase disperdente. Il **capitolo 6,** continuazione del precedente capitolo, esplora l'interazione delle emulsioni analizzate con proteine aventi massa molecolare (M_r) superiore a 10 kDa. Spettroscopia a infrarosso, Western blotting, colorazione PAS e SDS-PAGE associato a MS sono le metodiche utilizzate in questo capitolo. Tra gli altri risultati, e' stato osservato che la mucina MUC5B si lega fortemente a emulsioni di lisozima, mentre in misura minore a emulsioni stabilizzate da β-lattoglobulina a pH 6.7. Anche altre proteine, aventi M_r tra 10 e 100 kDa sono state identificate, e sono associate, seppure con notevoli diferenze, ad ambedue le emulsioni. Ad esempio, la α-amylase ($M_r \sim 55$ kDa) si lega alle emulsioni di lisozima, ma non a quelle di β-lattoglobulina. In conclusione, anche per le proteine com massa molecolare supeiore a 10 kDa, l'agente emulsificante determina l' assorbimento/associazione con le emulsioni.

Nel **capitolo 7**, invece viene affrontato il problema della percezione delle emulsioni. Spesso la percezione delle emulsioni non puo' essere direttamente correlata alla struttura e proprieta' dei prodotti prima del consumo. Considerare i diversi cambiamenti che avvengono nelle miscele di saliva ed emulsioni durante la persistenza dei prodotti nel cavo orale puo' essere di aiuto nel spiegare la percezione sensoriale. Emulsioni stabilizzate da WPI (costutito per la maggior parte da β-lattoglobulina) e da lisozima, flocculano, come in

precedenza dimostrato, rispettivamente in modo reversibile e irreversibile. Queste emulsioni vengono analizzate in relazione alla percezione. Oltre al diverso tipo di emulsificante usato (WPI vs. lysozyme), abbiamo studiato l'effetto della concentrazone dell'olio (2.5% w/w vs. 10% w/w) e dell'aggiunta di agenti addensanti quali guar gum. I risultati mostrano un chiaro effetto dell'emulsificante usato. Emulsioni stabilizzate da WPI sono percepite come cremose e presentano una stretta correlazione anche con altri attributi spesso associati alla cremosita'. L'aggiunta di agente addensante incrementa la percezione tutti questi attributi. Le emulsioni stabilizzate da lisozima, invece, sono percepite come astringenti. La percezione di queste ultime emulsioni richiama alla percezione di prodotti che contengono i tannini, come il te' o il vino. I tannini formano dei complessi insolubili con alcune delle proteine contenute nella saliva, quali le proteine ricche di prolina e le istatine. Questi complessi sono ritenuti responsabili della sensazione di astringenza che segue l'ingestione di prodotti. L'aggiunta di agente addensante, probabilemente a seguito della sua azione lubrificante, diminuisce la percezione dell'astringenza, mentre aumenta la percezione degli attributi legati alla cremosita'.

L'ultimo capitolo, **capitolo 8**, tenta di correlare i diversi risultati ottenuti dalle metodiche di chimica fisica, biochimica e di percezione sensoriale. Abbiamo visto, nei capitoli 2, 4 e 7 che la flocculazione indotta nelle diverse emulsioni dalle presenza della saliva cambia in modo marcato le proprieta' fisico-chimiche, come ad esempio la viscosita', delle miscele stesse. Nei capitoli 5 e 6 abbiamo dimostrato che diverse proteine e peptidi contenuti nella saliva assorbono/interagiscono con le goccioline di emulsione. Visti i risultati abbiamo ipotizzato che la flocculazione delle emulsioni analizzate e' dovuta a un unico meccanismo di flocculazione, che assomiglia al cosidetto " flocculazione a bridging" dove le proteine delle saliva fanno da ponte tra due o piu' goccie portando le stesse in stretta vicinanza. In questo modo i fiocchi che abbiamo osservato nei capitoli 2 e 7 sono il risultato di goccie tenute insieme da un intreccio di proteine. Inoltre, abbiamo visto che non solo vi sono differenze nel tipo di proteine associate alle goccie di emulsione, ma anche nella loro quantita' relativa. E' facilemente immaginabile che queste differenze influenzino la morfologia e la struttura stessa dei fiocchi, oltre che le properieta' fisico-chimiche. In aggiunta, in seguito alla formazione di complessi con le goccie di emulsione e la rimozione di alcune proteine e peptidi dal fluido salivare, la composizione della saliva stessa risulta alterata. Per esempio le mucine, che sono note per la loro azione lubrificante, assorbono sulla superficie delle goccioline perdendo cosi' le loro proprieta'. Per di piu', dato che la flocculazione delle emulsioni e' stata osservata anche sulla superficie della lingua, e'

possibile che i fiocchi formati dalla saliva, alterino anche le forze di frizione che esistono nella cavita' orale tra palato e la lingua, influenzando in questo modo la percezione dei prodotti alimentari.

In conclusione, questa tesi mostra non solo i mecchanismi di formazione del processo di flocculazione, ma anche ipotizza una diretta correlazione tra flocculazione e percezione.

Acknowledgements

And now it is finally time for the acknowledgements. Allow me to break a general rule of "het Nederlandse dankwoord" and to start with thanking the closest persons in my life which have contributed in different ways to this success.

Arno, without you these last four years would have been surely much more difficult to face. Thank you for your infinite patient and for listening every evening at all the stories of what happened during the day. Still wondering how it is possible that you never had headaches! But most of all, thank you for always being proud of me and having faith in my capabilities. Not to mention the fact that you have contributed so much to this book with the design of the cover, formatting of tables, figures etc. It is so nice that every page of this book talks a little bit about you too.

Mamma, papa', Alberto grazie per il vostro supporto ed interesse. Io sono sicura possiate immaginare cosa significhi per me aver portato a termine questo dottorato! E per una volta tanto, in tempo.

Fanny and Rui Paulo, how can I forget that this adventure started after your convincing brainwash during a dinner we had in Arnhem. Thank you both for being such good friends.

I am also extremely grateful to all the people of WCFS, Wageningen University and the University of Aveiro (Portugal), which have contributed to this research. Willem Norde, my promotor, thank you for your support and enthusiasm. Your comments and encouraging words were very important to me. Even during your trip to Australia, you made time to read and correct this manuscript. George, I'll not forget the long fruitful discussion in front of the blackboard in your office. Monique, my supervisor, you were always helping me out with anything really. It is not possible to mention all what I have learned from you. You are the type of supervisor I wish everyone could have. Francisco and Rui, working with you was a great experience. I'm so glad that I could spend two exciting months in your department. Rui and Sofia, you have been both great teachers in the lab. I remember how nervous I was at my first 2D electrophoresis. But I pulled it through thanks to your experience and suggestions. I also want to thank the whole B-014 group for the nice time I had in these last four years. Franklin, who introduced me to the world of making emulsions, Eefjan and Jerry, for performing several experiments for me and with me. Diane, my roommate, we did it. I'm glad we started this PhD/WCFS adventure together. You know more than anybody how tough these 4 years have been. Guido, mi manchera'venire a trovarti nel tuo ufficio e parlare di tutto e di piu'. E'stato un po' come essere di nuovo in Italia. Spero che troveremo il modo di rimanere in contatto anche al di fuori del lavoro. Raymond, Jolan, Jeroen and Marijke, you made my work at Food Chemistry extremely pleasant. It felt as I was part of the B-017 too. Jolan, thank you for our talks in the lab. Your reassuring attitude made me confident and less worrying of making mistakes. Marijke,

thank you for teaching me the Western blotting and for performing it for me, as well. Raymond it was great to have our meetings and thanks for organizing the work group "Saliva-mucus group". I was always looking forward for the meetings and presentations. I'm grateful to all the participants to the "Saliva-mucus group" for the extremely interesting discussions, exchange of ideas, problem solving attitude. Ton, thanks for the time you spent with me looking at the rheological results and Harmen, it was great to discuss with you about new approaches for this research. Ab, (Ab van der Linden) thanks for teaching me the first notion of the ζ-potential. It was a pity thought that after all the effort, the measurements did not worked out for my samples and I had to go Akzo.

Marcel van Eijk from Akzo Nobel is acknowledged for his assistance in the ζ-potential measurements and Wouter Jansen for the help with some rheological experiments. Edwin (E. Bakx, Food Chemistry Department), I'm glad you introduced me to the mass spectrometry and Arnoud (A. Loof, University Medical Centre St. Radboud), it was lovely to work with you at the SELDI-TOF-MS. I also want to thank Jacqueline (J. Donkers, AFSG) and Jan (J. Klok, Nizo Food Research) for the great SEM and CLSM pictures. I'd like to express my gratitude to Hetty Kroese-Hoedeman, Nancy Holthuysen, Jos Mojet and René de Wijk (AFSG) for their contribution to the sensory chapter. My students Catia da Silva and Dimitris Nitsas for their help with the measurements. It was a very interesting experience being your supervisor. All the people of the "saliva donor group": Bart, Bas H., Bas K., Cristina, Diane, Els, George, Guido, Eefjan, Jerry, Hans, Ilja, Jan B., Jolan, Josie, Anita, Lee Y., Monique, Marijke, Jeroen, Paulo, Renate, Wilfred, Richard, Peter W. Marcel, Wiebe, Saskia, Pascal, Erwin, Jasper, Mieke, Herman, Renko, Remco, Catia, and Dimitri. Hopefully I did not forget anyone. I am grateful to all my colleagues at Fysko for the nice time at lunch, lab uitjes, New Year breakfasts and other numerous occasions. Ilja, together with Diane, we had a nice time in our room. Pity that in the last year we travelled a lot, which made it difficult to be together. Bert, you always had time for scanning my numerous gels and Mara, your support during the crazy ordering-period before going to Portugal was essential. Josie, you are the best secretary ever. Rui, Sofia, Sao, Ana, Joao, Rosario, Pedro, Francisco, Armando, Miguel, Catarina, Gina, Andrea and Hannah you have brought back my Mediterranean spirit. I very much enjoyed my stay in Aveiro. I'll never forget the city of the "ovos moles" and I hope to see you some day. I hope sincerely I haven't left anyone out, and if I have, I'm really sorry. To all of you, once more THANKS !

Erika

List of publications

1. **Silletti, E.**, Vingerhoeds, M.H., Norde, W., and van Aken, G.A., *The role of electrostatics in saliva-induced emulsion flocculation.* Food Hydrocolloids, 2007. **21**: p. 596.
2. **Silletti, E**., Vingerhoeds, M.H., Norde, W., and van Aken, G.A., *Complex formation in mixtures of lysozyme-stabilized emulsions and human saliva.* Journal of Colloid and Interface Science, 2007. **313**: p. 485.
3. **Silletti, E.**, Vingerhoeds, M.H., van Aken, G.A., and Norde, W., *Rheological behaviour of food emulsions mixed with saliva: Effect of oil content, salivary protein content and saliva type.* Submitted, 2007.
4. **Silletti, E.**, Vitorino, R.M.P., De Groot, J., Vingerhoeds, M.H., and Amado, F.M.L., *Analysis and identification of salivary peptides interacting with β-lactoglobulin and lysozyme stabilized emulsions by SELDI-TOF-MS and RP-HPLC coupled to MS-TOF-TOF.* Submitted, 2007.
5. **Silletti, E.**, Vitorino, R.M.P., Schipper, R.G., Amado, F.M.L., and Vingerhoeds, M.H., *Characterization of salivary proteins interacting with lysozyme and β-lactoglobulin stabilized emulsions.* To be submitted, 2007
6. Vingerhoeds, M.H., **Silletti, E.**, de Groot, J., Schipper, R.G, and van Aken, G.A., *Relating the effect of saliva-induced emulsion flocculation on rheological properties and adherence to the tongue surface with sensory perception.* Submitted, 2007.
7. **Silletti, E.**, Vingerhoeds, M.H., Norde, W., and van Aken, G.A., *Saliva-induced emulsion flocculation: The role of droplet charge*, in *Food colloids*, *Self-assembly and Material Science.* E. Dickinson, Royal Society of Chemistry: Cambridge, UK 2007, p.463.
8. Schipper, R.G., **Silletti, E.**, and Vingerhoeds, M.H., *Saliva as research material: Biochemical, physicochemical and practical aspects.* Archives of Oral Biology, 2007. **52**: p.1114
9. Ugolini, R., Ragona, L., **Silletti, E.**, Fogolari, F., Visschers, R.W., Alting, A.C., and Molinari, H. *Dimerization, stability and electrostatic properties of porcine β-lactoglobulin.* European Journal of Biochemistry, 2001. **268**: p.4477.

Curriculum Vitae

Erika Maria Grazia Silletti was born on the 11th of March 1974 in Verona, Italy. She graduated in Agro-Industrial Biotechnology in 2003 at the University of Verona (Italy) with a thesis entitled "Large scale purification and characterization of porcine β-lactoglobulin". This work was conducted mostly at NIZO Food Research back in 1999 and 2000. Since 2001, she moved to live in the Netherlands working for the first two years at NEC International situated in Wijchen, while continuing the university in Italy.

Since November 2003, she was working as a Ph.D. fellow at the Physical Chemistry and Colloids Science department at the Wageningen University, on a Ph.D. project financed by the Wageningen Centre for Food Science, nowadays known as TI Food and Nutrition. This thesis is the result of research aiming to understand the behavior of emulsions in the oral environment, with emphasis on the role of saliva and salivary components.

Overview of the educational activities

Courses and conferences

- Summer Glycoscience course,VLAG, The Netherlands, 2004
- Ph.D. introduction course, VLAG, The Netherlands, 2004
- Food Structure and Food Quality conference, Cork, Ireland, 2004
- Macroion Complexation: Fundamentals and applications, Wageningen The Netherlands, 2005
- Saliva Symposium, Egmond aan Zee, The Netherlands, 2005
- Spring Meeting of the Belgium-Dutch Biopharmaceutical Society, Gent, Belgium, 2005
- Bionanotechnology course,VLAG, The Netherlands, 2006
- Industrial protein course, VLAG, The Netherlands, 2006
- Food Colloids Conference, Montreux, Switzerland, 2006
- Debating course, WCFS, The Netherlands, 2006
- Trainingship at the Mass Spectrometry Group, University of Aveiro, Portugal 2007

Meetings

- PhD students work group, Laboratory of Physical Chemistry and Colloids Science, Wageningen University, The Netherlands, 2004-2007.
- Project meetings WCFS project “Engineered texture of emulsions and foams”, WCFS, 2004-2007.
- Work meeting “Saliva-mucus group”, WCFS, 2004-2007.

Photo: “Cryo-SEM of unstimulated saliva” by Jacqueline Donkers
Cover design: Arno Kusters
Printing: Ponsen & Looijen, Wageningen