Controlling the aggregation and gelation of β-lactoglobulin by the addition of its peptides

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Abstract

In this thesis the effects of peptides, or protein hydrolysates on the heat-induced aggregation and gelation of (concentrated) protein systems were studied. First, it was investigated if specific peptides could influence the heat-induced denaturation and aggregation of intact proteins solutions, and which peptide properties dominated the different interactions. Next, the effects of the peptides on the heat-induced gelation of intact proteins as a model for a potential high protein food system were studied.

It was found that certain peptides in the hydrolysate show binding to native proteins, and some additional peptides bind to unfolded proteins. Since the same peptides were shown to bind to not only β-lactoglobulin, but also other to proteins, it is concluded that the binding does not depend on specific molecular details of the protein. The hydrophobicity and charge were found to be important in determining the binding and the effect on aggregation. With the hydrolysates, as well as with two synthesized peptides (modelled on those found in the hydrolysate) it was confirmed that the addition of these binding peptides has significant effects on the heat-induced aggregation. In the gelation experiments performed in this study a dominant effect was found for peptides containing free SH groups. While it is expected that the changes in aggregation behaviour, induced by the binding of non-cysteine-containing peptides also affects the gel properties, this was not found with the techniques used. Finally, disulfide-containing peptides were found to reduce the presence of sulfurous volatiles formed after heating of β-lactoglobulin, WPI and lysozyme.

Since only certain peptides exhibit binding to intact proteins, it is expected that control over the hydrolysis process and, thereby the concentrations of such specific peptides, can be used to produce hydrolysates with specific functionalities in this respect.

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Chapter 1

General Introduction

Proteins in food products can aggregate during heating steps in the processing. These aggregates can further cluster to form networks or gels. The properties of such protein networks determine the texture and thereby the consumers liking of a food product. An example where this is important, are the high protein foods that currently receive nutritional attention. These products contain approximately twice the amount of proteins compared to related products and are thought to help prevention of obesity and sarcopenia[1, 2] by decreasing the food intake. The sensory characteristics of these products, such as hardness, were found to deteriorate during storage. To improve the quality of such systems it is important to understand the aggregation and gelation, and to find methods to control these processes.

Typically, studies on the aggregation and gelation of proteins focus on the effects of changes in system conditions, such as pH, ionic strength and temperature. However, in food products it is often not practical to vary these conditions. To change these processes under constant system conditions, additives can be added. In a high protein food, it would of course be preferable if this additive could be derived from proteins. It has been shown that protein hydrolysates have different techno-functional properties compared to the parental protein[3-5] and may be used for this purpose. The effect of using hydrolysates as additives to protein systems has been studied using peptides obtained from a hydrolysis of a whey protein isolate (WPI) with a glutamate specific enzyme.[6] Mixing of a 40 mg/mL WPI solution in the presence of 10 mg/mL peptide solution, was found to result in maximum aggregation.[7] The aggregation and gel forming properties of proteins might, therefore, be controlled under constant system conditions (pH, I, etc.) by adding protein hydrolysates. The effects of the hydrolysate can be changed by varying the concentration and/or the composition. The reasons for these effects of peptides on the heat-induced aggregation and gelation are not yet clear. Certain peptides may bind non-covalently to the proteins, thereby participating in and affecting the aggregate formation. Other peptides may bind covalently, e.g. via disulfide bridge formation. The aim of this thesis is to identify

the different contributions of peptides to the effect of the total hydrolysate when they were added to intact protein. In addition, it is investigated whether specific properties of the amino acids, e.g. charge, hydrophobicity, size or a combination of these properties are important. For this, the binding of peptides to β-lactoglobulin and the resulting effects on aggregation and gelation were investigated. During this work, it became apparent that the addition of peptides to intact proteins did not only affect the aggregation and gelation, but also influenced the presence of heat-induced sulfurous volatiles. This observation became part of the study, since here also the question was whether specific peptide properties were needed for the effect observed.

β-Lactoglobulin

β-Lactoglobulin is a globular protein obtained from the whey fraction from bovine milk. Eleven genetic variants of bovine β-lactoglobulin are known, called A, B, C, D, E, F, G, H, I, J, and W[8] of which variants A (molecular mass of 18,363 Dalton) and B (molecular mass of 18,277 Dalton) are the most abundant present in milk of Western breeds of cow's (*Bos taurus*).[9] The primary sequences of these variants consist of 162 amino acids. The A and B variants differ only at residue positions 64(Asp→Gly) and 118(Val→Ala). β-Lactoglobulin contains two disulfide bridges between the cysteine residues at position 106-119 and 66-160 and one free sulfhydryl group at residue position 121. The secondary structure consists of approximately 15% α-helix, 50% β-strands and 35% random coil structure. The tertiary structure (figure 1) is fully elucidated by X-ray crystallography and consist of anti-parallel β-sheet, formed by nine strands wrapped round to form a flattened calyx.[10]

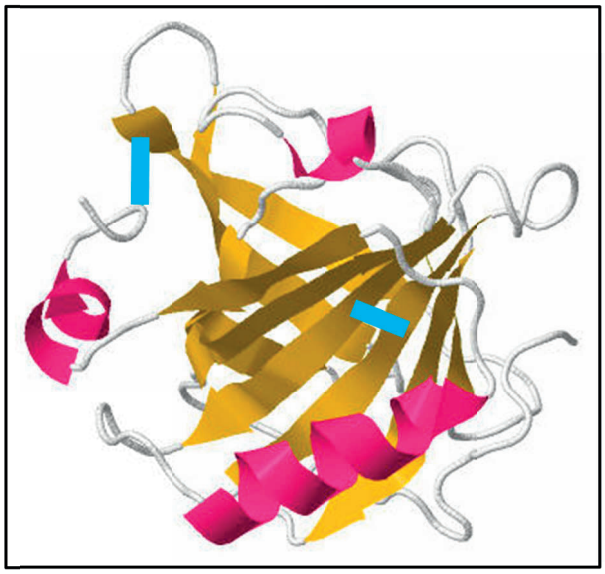

Figure 1. The tertiary structure of β-lactoglobulin (Jmol v 12.0.41 view of 3BLG entry in RCSB Protein Data Bank). The disulfide bridge locations are coloured blue. The yellow arrows indicate the nine β-strands and the helical regions are coloured red.

At neutral pH β-lactoglobulin occurs as a dimer. This dimerization is mainly driven by electrostatic interactions. Above neutral pH and upon heating the dimer dissociates into monomers due to pH-dependent reversible conformational changes reflected by a change in physical, chemical and spectroscopic properties. These conformational changes together are known as the Tanford transition.[11] At elevated temperature (>40 °C) small conformational changes occur and at higher temperatures intramolecular bonds are broken and the protein (partially) unfolds. Unfolding leads to the exposure of hydrophobic regions present in the internal structure of the protein and these regions tend to stick together to form aggregates. The increased reactivity of the free sulfhydryl group on the intramolecular disulfide bridges upon unfolding leads to irreversible aggregation of the protein molecules by the formation of intermolecular disulfide bonds.[12-15] The importance of the

free sulfhydryl group was demonstrated by the absence of polymerization of bovine β-lactoglobulin containing sulfhydryl blocked groups by using *N*-ethylmaleimide (NEM).[16]. Later studies[17] showed that no gelation occurred for porcine β-lactoglobulin, which lacks the free sulfhydryl group present in bovine β-lactoglobulin.

If the concentration of proteins is high enough (e.g. for β-lactoglobulin > 8-10% (w/w) at neutral pH), the aggregates formed can cluster together and form a gel. Gelation of proteins can be induced in various ways. The simplest way, the so-called physically-induced gelation, is by applying heat or pressure. Other ways, the so-called chemically-induced gelation, are enzyme-induced, salt-induced, urea-induced and acid-induced.[18] In this study, the focus was on heat-induced gelation. The strength of the gel depends on system conditions, like temperature, pH and ionic strength. The functionality of a gel is determined by the non-covalent and covalent bonds of the network that result from the intrinsic protein properties (electrostatic interactions, hydrophobicity, molar mass).[19] The resulting gels can consist of random aggregates (turbid gels), string of beads types of aggregates (fine stranded bead) or intermediate structures.[20]

While the heating of β-lactoglobulin has been mostly studied in relation to unfolding, aggregation and gelation, it also results in the formation of sulfurous volatiles, mainly dihydrogen sulfide (H_2S) and methanethiol (MeSH). These volatiles are considered to be responsible for the cooked flavour of milk and milk products.[21-23] The same type of volatiles are also found upon heating of other protein-rich products, such as egg, coffee and meat products.[24] In milk, these volatiles are considered to be formed via a reaction between cysteine or methionine and a diketone.[25]

Since in this thesis the binding of peptides to proteins is studied, it is important to note that β-lactoglobulin binds retinol and shows remarkable similarities to the retinol binding protein (RBP) in human blood. It is considered to be a carrier of retinol (animal vitamin A) from the maternal milk to the neonate.[10] It has been reported that the binding of retinol had a stabilizing effect on the unfolding of β-

lactoglobulin B during heat treatment.[26] It is not known whether such effects also occur during the binding of peptide to β-lactoglobulin.

Use of peptides in foods

Proteins were long believed to be broken down in the digestive system into individual amino acids. Consequently, from a nutritional point of view the value of proteins was primarily attributed to the amino acid composition. Currently, it is known that peptides are not completely digested to single amino acids, resulting in more interest in the biofunctional properties of peptides. Summarizing, three main functions of peptides can be identified:

1) *Nutritional properties*. Peptides are a source of essential amino acids and nitrogen, but they have a better digestibility than the parental protein. This increased digestibility is used in e.g. infant formulae.[27]

2) *Bioactive properties.* The main identified bioactive properties are: mineral-binding, anti-microbial, anti-hypertensive, opioid-like, immuno-modulating, and antithrombotic activities.[28-31]

3) *Techno-functional properties*. Enzymatic hydrolysis is commonly applied to increase the solubility of poorly soluble proteins, e.g. gluten.[32, 33] The increased solubility is typically due to the smaller size of the peptides, together with the increase in the number of charged groups (due to liberation of free carboxylic and amino groups). Furthermore, some exotic properties of peptides were recently reported, such as the inhibition of ice-crystal formation by water binding properties of specific gelatin hydrolysates[34, 35], and the formation of so called nanotubes from partial hydrolysis of α-lactalbumin.[36]

For textural properties, such as aggregation or gelation, most studies focussed on intact proteins. One reason is that the intact proteins are well defined with respect to parameters such as overall charge and hydrophobicity. Moreover, due to their size they can span longer distances. In contrast, protein hydrolysates are often ill-defined. In a lot of cases only an overall description of the hydrolysate is given, e.g. the degree of hydrolysis. In some cases the presence of certain peptides is

established, but very often no complete overview on the abundance of the individual peptides present is provided.

Effects of hydrolysis on functional properties

As stated above, protein gels can be formed by aggregation of proteins when heated close to, or above the denaturation temperature. The gels formed can have different optical (turbid, transparent) and rheological properties. These properties can be affected by system conditions, such as pH and ionic strength. At high ionic strength or near the isolelectric point (pI) of the proteins, the appearance of the protein gel becomes turbid. At low ionic strength and far from the pI protein gels become transparent.[37] In food products, these parameters cannot be varied too much, since the products may become inedible. Therefore, it is of importance to study the possibility to use additives to affect the properties of protein networks. Such effects have been studied in protein films (heat-induced network of proteins in dry state), where additives or plasticizers, such as glycerol, are added to control the macroscopic properties like tensile strength, elongation, etc. of the protein films.[38, 39] Since consumers prefer products without exogenous additives (‘chemicals’), this is not the preferred option. Therefore, the challenge in high protein foods is to find methods that affect the system properties, under constant conditions with only the use of proteinaceous material.

Similar to the use of plasticizers in protein films, peptides could be used to alter the textural properties of the parental protein systems during hydrolysis.[40, 41] and to influence the gel properties of enzyme-induced and heat-induced gels. The effects of enzymatic hydrolysis on gelation at 40°C has been illustrated[42] by the partial hydrolysis of whey protein isolate, which resulted in gel formation at 12% (w/w) total protein. For the intact protein isolate this concentration is too low to form a gel under conditions given. Other work also reports significant effects of partial hydrolysis on the aggregation and gelation of proteins (table 1). In most studies, the hydrolysate was studied *as-is*. In these systems, hydrolysis and aggregation of the protein are coupled. Hence, the amounts of intact protein and peptides are

interrelated. Only a few studies identified which peptides had specific contributions. These contributions were related to emulsifying properties rather than to gel properties. For instance, peptides (α_{s1}-CN[f1-23], β-LG[f15-20], β-LG[f41/43-60], β-LG[f1-8] and β-LG[f61-69/70+149-162]) were identified in isolated fractions as peptides that contributed to improved emulsifying properties (table 1). For many other peptides in the other hydrolysates it was not determined unambiguously which specific peptides play a role in these effects. These data show the potential of peptides as interaction mediators in heat-induced protein aggregation and gelation. Still, these studies only considered the properties of the hydrolysate (*as-is*), not the addition of the hydrolysate to intact protein solutions. The latter approach was studied for the addition of whey protein isolate hydrolysate to whey protein isolate solution.[6] It was shown that protein-peptide interactions (aggregation) can be induced at the expense of protein-protein interactions. In the same study it was shown that the protein hydrolysates also contain peptide fractions, which have no or limited tendency for self-aggregation or for interaction with intact whey proteins. The use of peptides to alter the rheological behaviour of dairy and plant protein systems has been predominantly focussed on peptide-peptide interaction, although some research has been devoted to protein-peptide interactions.[7, 43, 44]

It is not exactly known how protein-peptide interactions influence the heat-induced aggregation and gelation of proteins. Depending on the type of interaction between the peptides and the proteins several mechanisms are possible (figure 2). If there is no direct interaction between the peptides and the proteins, the peptides merely function as *inactive fillers*. If there is interaction between peptides and proteins, they may either induce clusters (*active fillers*) or reduce the reactivity of the electrostatic and hydrophobic groups in the unfolded proteins and thereby reduce the formation of large protein-protein aggregates (*shielding*). Here the interaction is assumed to be based on non-covalent interactions (such as electrostatic and

Table 1. Overview of hydrolysates/peptides on functional properties.

parental protein	Enzyme	peptides investigated	effect on functional properties	reference
WPI	BLP	12% WPI hydrolysate (DH 2%)	gel formation of hydrolysate at 40 °C	[42]
WPI	BLP	21% WPI hydrolysate (DH 2.2%)	gel formation of hydrolysate at 80 °C	[45]
WPI	Alcalase	WPI hydrolysate (DH 14%)	gelation and characterization of interactions	[46]
WPI	BLP	WPI hydrolysate (DH 3.2-6.8%)	aggregation in WPI and WPI hydrolysates	[47, 48]
WPI	Alcalase Prolyve Subtilisin Glutamyl endoprotease	WPI hydrolysate (DH 15-20%)	aggregation properties	[49]
β-lactoglobulin	BLP	7-20% β-LG hydrolysate	gel formation of all hydrolysates at 40 °C	[50]
β-lactoglobulin	Commercial trypsin and chymotrypsin	31 peptides	chymotryptic peptide [f15-20] enhances peptide aggregation	[51]
		33 peptides	chymotryptic peptide [f41/43-60], [f1-8] and [f61-69/70+149-162] decrease peptide solubility	
α-lactalbumin	BLP	1% α-La hydrolysate	stronger gel formation at 50 °C than β-LG equivalent	[52]
β-casein	Plasmin	βCN [f1-105/107]	improved emulsifying properties	[53]
α_{s1}-casein	Pepsin	α_{s1}-CN [f1-23]	improved emulsifying properties at lower pH	[54]
α_{s1}-casein	Pepsin	α_{s1}-CN [f1-23] + α_{s1}-CN [f154-199]]	synergistic effect on improved emulsifying properties	[55]
β-lactoglobulin	Trypsin	**F1**[a]**:** ([f84-91], [f1-14], [f125-135], [f15-20] [f142-148], [f92-100], [f25-40], [f21-40])	improved interfacial properties at pH 4 and 7	[56]
		F2: ([f92-100], [f61-69]=[f149-162], [f41-60], [f149-162])	improved interfacial properties at pH 4	
		F3: ([f149-162], [f41-60])	improved interfacial properties at pH 4 and 7	
		F4: (f61-69], [f61-70], [f61-69]=[f149-162] [f61-70]=[f149-162], [f41-60], [f149-162])	worse interfacial properties at pH 4 and 7	
		F5: (f61-69], [f61-70], [f61-69]=[f149-162] [f61-70]=[f149-162], [f41-60], [f149-162])	worse interfacial properties at pH 4 and 7	
		F6: (f61-69], [f61-70], [f61-69]=[f149-162] [f61-70]=[f149-162], [f41-60], [f149-162], [f76-83],)	equal interfacial properties at pH 4 and 7	

[a] F1-F6 = six fractions purified from the hydrolysate

hydrophobic interaction). The last option is that the peptides react with the proteins through the formation of covalent bonds (sulfhydryl-disulfide exchange reactions) and act as *protein-thiol-blockers*. Disulfide formation via sulfhydryl-disulfide exchange reactions can occur and are favoured at pH > 7.5[57] and elevated temperature.[58]

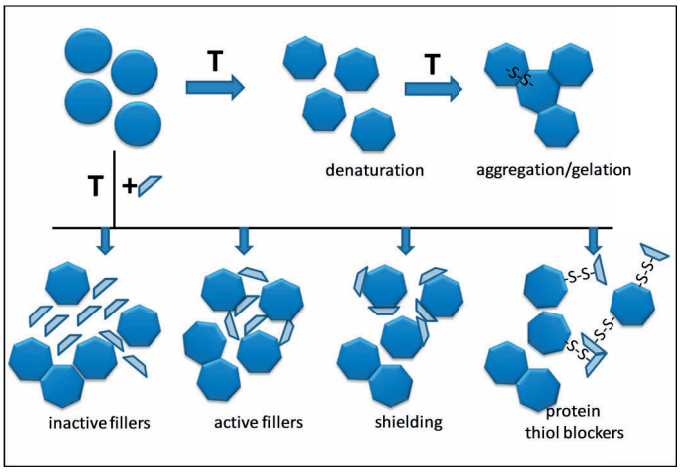

Figure 2. Possible mechanisms of protein-peptide interaction. Peptides can act as inactive fillers (no interaction), active fillers, shielding components of charged groups or as protein thiol-blockers.

To efficiently apply hydrolysates to adjust the heat-induced aggregation and gelation of proteins it is important to identify the mechanism(s) by which the peptides affect these properties and the physico-chemical properties of the peptides that are important in this respect.

Effects of protein-peptide interactions

As described above, it has been observed that gelation may occur during enzymatic protein hydrolysis. This is a direct result of the interactions between the different peptides formed during the hydrolysis. A number of studies have been performed to identify which peptides in a hydrolysate are involved in these aggregation processes.

Hydrolysis of whey protein isolate (WPI) with *Bacillus licheniformis* protease to DH 6.8% results in a turbid solution.[7] The hydrolysate was separated into two fractions by centrifugation at neutral pH, containing peptides, which have either low solubility or high solubility. Precipitation of WPI and its added hydrolysate was tested at a peptide concentration corresponding to 2-6 mM, based on the molecular masses of the peptides. The peptides β-lactoglobulin AB[f1-45], β-

lactoglobulin AB[f90-108] and α-lactalbumin [f50-113] were shown to form a maximal amount of protein-peptide aggregates at a molar peptide to protein ratio of 6. The peptides were also able to aggregate other proteins (e.g. β-casein and bovine serum albumin) than the parental one. A number of these aggregating peptides were also identified in other studies.[43, 50, 51] In addition, several other β-lactoglobulin-derived peptides have been reported to aggregate with intact proteins.[43, 50, 51, 59, 60] An overview of these peptides and their properties is given in table 2. The range of values for the hydrophobicity and charge of these peptides does not directly show a clear contribution of either factor to the binding of the peptides to the intact proteins.

In the above studies, it was shown that certain peptides have the tendency to aggregate with themselves, with other peptides or with intact proteins. In another study[61], the opposite was observed by showing that peptides formed during tryptic hydrolysis of soy β-conglycinin cause less peptide aggregation than glycinin-derived peptides. This was attributed to the lower susceptibility of amino acids in the hydrophobic region of β-conglycinin to hydrolysis by subtilisin Carlsberg. This might result in release of less hydrophobic peptides from β-conglycinin, with a lower tendency to aggregate. It was suggested that these peptides possess aggregation-preventing properties. Aggregation-preventing properties of peptides have also been reported for milk and soy-derived peptides in the inhibition of blood platelet aggregation.[62, 63] These peptides were reported to be small (<500 Da) and hydrophilic. Based on these findings it is expected that peptides can affect the self-association of proteins and/or of protein-based structure elements, the latter described by Saglem and co-workers.[64]

Table 2. Bioactive peptides that have been identified to interact with β-lactoglobulin.

parental protein	peptides investigated	Hydrophobicity kcal[kcal/res.]*	Net charge	pI	Reference
β-lactoglobulin	β-lg [f125-135]	9.4[0.85]	-4.0	3.5	[43, 65, 66]
	β-lg [f130-135]	4.7[0.78]	-2.0	3.8	[43]
	β-lg [f69-83]	25.3[1.70]	4.0	10.7	[43, 65, 66]
	β-lg [f146-149]	1.5[6.10]	1.1	11.0	[43]
	β-lg [f102-105]	10.3[2.80]	0.0	5.9	[43, 65, 66]
	β-lg [f142-148]	10.8[1.54]	1.1	11.0	[65, 66]
	β-lg [f41-60]	27.5[1.35]	-3.0	4.0	[65]
	β-lg [f92-100]	13.0[1.44]	-1.0	3.9	[65, 66]
α_{s1}-casein	αs1-CN [f23-34]	21.5[1.75]	0.0	6.9	[43]
whey protein	β-lg AB [f1-45]	53.6[1.19]	-2.0	4.2	[47]

* Total and average hydrophobicity was calculated according to method of Bigelow (1967).[67]

To obtain information about the interaction of peptides with proteins and the subsequent effects on heat-induced aggregation and gelation of proteins several techniques will be used in this thesis. These are explained in the next section.

Surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS)

Protein-peptide interactions have been studied by various techniques, such as isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR). ITC is commonly used to determine the binding energy and number of binding sites involved in the interaction of peptides (or other compounds) with intact proteins.[68, 69] To allow correct interpretation of the data, pure compounds are needed. Surface plasmon resonance (SPR) is a method based on changes in the refractive index of a crystal when species adsorb or bind to its surface or to any material (e.g., proteins) coated onto its surface.[70, 71] While these techniques can detect binding to intact proteins, they do not allow the identification of the compounds bound. This identification can be achieved by surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). This technique was introduced to simplify sample extraction and subsequent identification of proteins by MS.[72] Since then, SELDI has been extensively used

in medical research to discover biomarkers,[73-75] and also in food research, e.g. to detect the interaction of isoflavones with soy and whey proteins.[76]

The principle of SELDI is illustrated in figure 3. The chip surface contains either binding ligand (SiO2, quaternary ammonium groups, carboxylate groups, methylene groups, nitrilotriacetic acid groups) or it is preactivated with carbonyldiimidazole or epoxide groups. The preactivated group allows covalent binding of proteins to the surface via the free amino groups. The epoxide group can also bind free sulfhydryl groups.[77]

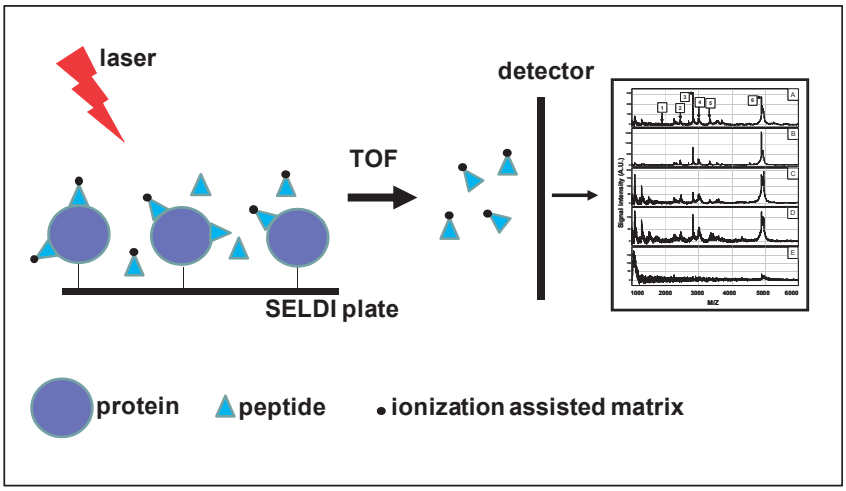

Figure 3. Principle of SELDI-TOF-MS.

After the proteins are covalently bound to the chip, the solution containing the molecules of interest (in our case a hydrolysate containing different peptides) can be brought in contact with the chip to allow binding to the protein. After this, the solution is removed and the plate is washed to remove all the non-bound molecules. Then, a MS matrix is added and the chip is entered into the TOF (time of flight) mass spectrometer. Upon ionization, the non-covalently linked molecules will be desorbed and analysed by MS.

Electrowetting

To study the heat-induced gelation of proteins, typically a Couette type rheometer is used. In such systems, usually volumes >1 mL are needed to perform reliable measurements. Since the critical gel concentration of whey proteins is 8% (w/v), more than 80 mg of protein is needed for each experiment. In this study, the effect of specific peptides on the gelation should be determined. Due to limited sample amount of these specific peptides, multiple experiments with such rheometers cannot be performed. To reduce the required sample volumes, an alternative rheological measurement was used: electrowetting (EW).

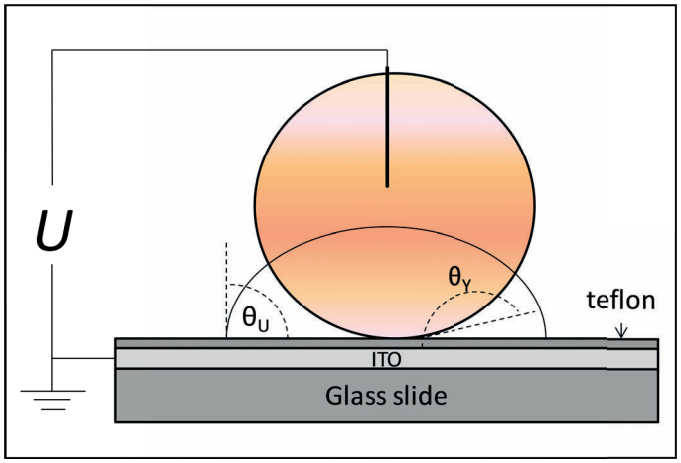

Figure 4. Experimental set-up of electrowetting (U = voltage).

In EW an aqueous (gelled) droplet (3 μL) is placed on a dielectric substrate. The substrate consists of a glass slide with a precoated Indium Tin Oxide (ITO) electrode layer and is covered with a layer of Teflon to make sure the liquid droplet does not spread on the substrate. The second electrode is positioned into the droplet.

When a voltage (U) is applied, it results in a change in the wetting angle (θ). The wetting angle is measured by a goniometer and is described by the electrowetting equation[78]:

$$\eta \equiv \frac{\varepsilon\varepsilon_0}{2d\gamma} \times U^2 = \cos(\theta) - \cos(\theta_Y) \quad (1)$$

This change of the wetting angle as a function of the applied voltage was first described for mercury. The thin insulating (Teflon) layer between the electrode and the droplet was later added to eliminate the problem of electrolysis.[79] This set-up is also known as electrowetting on dielectric substrate (EWOD), typically abbreviated to electrowetting (EW). The applicability of this technique to study protein gelation has been tested by determination of the gel strength, as complex modulus, of gelatin solutions at different temperatures.[80] It was shown that similar values were obtained with EW and rheometry with a Couette type geometry.

Enzymatic hydrolysis of proteins

The extent of hydrolysis of a protein hydrolysate is quantitatively described by the degree of hydrolysis (DH). This parameter is defined by the number of cleaved peptide bonds (*h*) as percentage of the total number of peptide bonds (h_{tot}) present in the parental protein molecule.[81] While the DH does not contain any concrete information about either the remaining amount of intact proteins, or the peptide composition of the hydrolysate it is the one parameter that is easy to determine. Therefore, this parameter is commonly used in industrial applications and research to characterize hydrolysates.

The type of peptides formed during hydrolysis (from a given substrate) depends on the enzyme used. It was previously found that during the hydrolysis of β-lactoglobulin with *Bacillus licheniformis* protease (BLP) aggregating peptides were formed. Therefore, this enzyme (glutamyl endopeptidase) is also used in this thesis. BLP is a Glu-specific protease, but is sometimes found to hydrolyse the NH_2-side of Asp residues, although with a 100-1,000 times lower affinity.[82] The enzyme exhibits an optimal activity at pH 7.5-8.0[82] at 50°C[83] and can be inactivated by lowering the pH to 2.[84] Next to BLP, another Glu-specific protease is used, *Staphylococcus aureus* V8 protease. The glutamyl endopeptidase V8 hydrolyses peptide bonds at the NH_2-side of Glu residues. It has been reported[85]

that V8 also cleaves at the NH_2-side of Asp residues, but to a 3,000 times lesser extent. The V8 enzyme exhibits an optimal activity at pH 7.8 at 37 °C[86, 87] and is inactivated by the protease inhibitor benzyloxycarbonyl-L-leucine-L-glutamic acid-chloromethyl ketone (Z-LE-CMK). In these ways, the hydrolysates do not have to be heated to inactivate the enzymes, thereby avoiding heat-induced changes in the peptide composition.

Aim of the study

It has been suggested that products with high protein contents can help to decrease food intake due to effects on the satiety in obese people, and to prevent loss of muscular mass in elderly people.[1] It was found that the sensory quality of products with relative high protein contents (e.g. high protein nutrition bars) decreased rapidly during storage.[88] To improve the sensory quality of such products, the gel properties need to be controlled. Since the conditions of these products are set, and a high protein content is needed, it was decided to study the effect of addition of protein hydrolysates on the textural properties of heat-induced gels. In this thesis the aim was to study the possible role of peptides as interaction mediators in (high) protein systems in relation to binding, aggregation and gelation of the intact protein present. Studying the binding of peptides to protein allows us to determine and identify which peptides are important in the protein-peptide interaction. Research on the subsequent heat-induced denaturation and aggregation of intact proteins in the presence of these specific peptides gains insight in the different types of interactions (covalent/non-covalent) and allows us to determine whether these interactions are specific or generic.

Outline of the thesis

In this chapter a brief overview is given about the current state of knowledge on protein-peptide interactions in relevant food protein systems. The binding of peptides, from a β-lactoglobulin hydrolysate made by the Glu-specific

Staphylococcus aureus V8 protease, to proteins was studied with SELDI-TOF-MS in chapter 2.

In chapter 3 the effect of binding peptides from a soluble peptide fraction of a β-lactoglobulin hydrolysate made by *Bacillus licheniformis* protease on the denaturation and aggregation of β-lactoglobulin was studied. The characteristics of the bound and unbound peptides were compared.

Chapter 4 describes the study on the binding of two peptides in particular to β-lactoglobulin. The effect on aggregation and gelation of β-lactoglobulin in the presence of these peptides was studied. Electro wetting was used to investigate the effect of the presence of these peptides on the gelation of β-lactoglobulin.

The observations for pure peptides in chapter 4 were further studied in a complex system in chapter 5. In this chapter the gelation properties of an industrial relevant protein system, whey protein isolate (WPI) in the presence of a WPI hydrolysate was studied.

Chapter 6 describes the influence of specific peptides on the formation of sulfurous volatiles from β-lactoglobulin at elevated temperature. A mechanism for the interaction of the peptides with the protein and the formation of sulfurous volatiles was proposed.

In chapter 7, the results obtained in this research are discussed.

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Chapter 2

SELDI-TOF-MS as a rapid tool to study food related protein-peptide interactions

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Abstract

The use of SELDI-TOF-MS was investigated as a rapid tool to detect peptides present in a crude protein hydrolysate, that are capable to bind to intact food proteins. A purified and well characterized β-lactoglobulin preparation was extensively hydrolyzed by the Glu-specific enzyme V8 from *Staphylococcus aureus*. Characterization of this hydrolysate by SELDI-TOF-MS and MALDI-TOF-MS resulted in sixteen identified peptides, which covered 98 % of the primary sequence of β-lactoglobulin. To identify peptides capable to bind non-covalently to intact proteins, the complete hydrolysate was applied to covalently bound ovalbumin, glycinin, β-lactoglobulin and β-casein on a SELDI ProteinChip PS-20. Six peptides (AB [f29-45], AB [f90-108], AB [f138-158], B [f63-89], AB [f1-45], AB [f135-162] bound to these four different proteins with decreasing affinity to glycinin>ovalbumin>β-lactoglobulin>β-casein. Peptides, which bound to these proteins were AB [f1-45] and AB [f135-158]. Using different concentrations of Triton X-100 (up to 2 %) as a washing step prior to MS detection, enabled a rapid distinction between the peptides bound with respect to protein binding capacity.

Introduction

In food systems the presence of peptides was found to change the functional properties of the proteins such as aggregation and gel properties. One hypothesis states that (some) peptides can bind specifically to proteins through non-covalent interactions.[1-5] Such interactions can dominate the physical and functional properties of the (aggregating) system, even when the peptide in question is only a minor component. To control the properties of mixed protein-peptide systems, it is important to identify which peptides interact and when such interactions occur. Hydrolysates are mixtures of numerous different peptides, so a first screening is necessary to select the most important peptides capable of binding to proteins. Protein-peptide interactions have been extensively studied by various techniques. Isothermal titration calorimetry (ITC) has been used to study the complex formation of proteins with a pure peptide at constant temperature.[6, 7] Surface plasmon resonance (SPR) is a method based on changes in the refractive index of a crystal when species adsorb to or bind to its surface or to any material (e.g., proteins) coated onto its surface.[8-10] Another technique to screen protein-peptide interaction is (multi-dimensional) liquid chromatography mass spectrometry (LC-MS) by which the (dissociated) protein-peptide complex can be separated on basis of different affinities to a column surface (LC). Subsequent analysis of the peptides separated by mass spectrometry (MS) reveals the identity of the peptides.[11, 12]
All the above techniques mentioned require isolation of either the pure peptides or isolation of the protein-peptide complex, which is laborious. Therefore these techniques are less suitable to screen large numbers of peptides for their ability to bind to proteins, at different conditions.

During the last decade surface-enhanced-laser-desorption-ionization-time-of flight-mass-spectrometry (SELDI-TOF-MS) has been used many times to study proteins and peptides in medical research.[13, 14] SELDI-TOF-MS has been used to a far lesser extent in food related research.[15] SELDI-TOF-MS is based on two different powerful techniques: affinity chromatography and mass spectrometry.[16] In the

first step the compounds in a mixture bind to a surface with a distinct chemical or biochemical affinity property. After removing the unbound compounds, the bound compounds are analysed by laser desorption/ionization mass spectrometry. The major feature of this technique is the high sensitivity (in comparison to e.g., MALDI-TOF-MS) and the ability of high-throughput screening of samples (in comparison to e.g. ITC).

In the present study SELDI-TOF-MS was used to rapidly identify binding of peptides present in a protein hydrolysate to different food related proteins, which were covalently bound to the surface of a ProteinChip PS-20. In addition to this, differences in binding strengths between the peptides identified were estimated from Triton X-100 washing experiments.

Materials and Methods

Chemicals and reagents

Fresh bovine milk for in-house isolation of pure β-lactoglobulin was provided by De Ossenkampen, Wageningen University, Wageningen, The Netherlands. ProteinChip PS20 and ProteinChip Gold were purchased from Biorad (Hercules, CA, USA). α-Cyano-hydroxycinnaminic acid (CHCA) and 2,5 dihydroxybenzoic acid (DHB) were obtained from Bruker Daltonics (Bremen, Germany). Acetonitrile was purchased from Biosolve (Valkenswaard, The Netherlands). Trifluoroacetic acid (TFA) and commercial β-lactoglobulin were obtained from Sigma (St. Louis, MI, USA). DEAE Sepharose CL6B was purchased from GE-Healthcare (Uppsala, Sweden). *Staphylococcus aureus* V8 enzyme was obtained from Roche Diagnostics (Almere, The Netherlands). The protease inhibitor benzyloxycarbonyl-L-leucine-L-glutamic acid-chloromethyl ketone (Z-LE-CMK) was purchased from Commonwealth Biotechnologies (Richmond, VA, USA). Ovalbumin[17], glycinin[18] and β-casein purified according to Leaver and Law (1992)[19] were present at the Laboratory of Food Chemistry of Wageningen University. All proteins have a purity of > 95% w/w (as determined by SDS-PAGE). All other reagents used were purchased from Merck (Darmstadt, Germany).

Isolation and purification of β-lactoglobulin from bovine milk

β-Lactoglobulin was isolated and purified according to de Jongh et al. (2001).[20] 20 L of fresh bovine milk yielded approximately 30 gram of pure β-lactoglobulin.

Characterization of β-lactoglobulin

SDS-PAGE was performed on a 10-15% gradient gel using the PhastSystem (GE Healthcare, Uppsala, Sweden). Samples (5 mg/mL) were diluted 1:2 in sample buffer consisting of 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 5% (w/v) SDS, 0.02% (w/v) bromophenol blue and 2.5% (v/v) β-mercaptoethanol. Prior to analysis the samples were heated 10 min at 96 °C. The gels were stained with an aqueous solution consisting of 0.1% (w/v) Coomassie brilliant blue R-250 in 10% (v/v) acetic acid solution and destained with an aqueous solution consisting of 30% (v/v) methanol and 10% (v/v) acetic acid solution. The gels were preserved in an aqueous solution consisting of 5% (v/v) glycerol and 10% (v/v) acetic acid.

Nitrogen content

The total nitrogen content was determined (in triplicate) by Dumas method[21] with a NA 2100 Protein nitrogen analyzer (CE Instruments, Milan, Italy). Typically 10 mg of freeze dried sample was weighed in aluminium foil cups. The combustion temperature was 900 °C, reduction heater temperature was 680 °C. The nitrogen-to-protein conversion factor N x 6.33 (based on the primary sequence of β-lactoglobulin) was used to calculate the protein content. L-methionine was used for the calibration curve.

Capillary Electrophoresis (CE)

Capillary electrophoresis was carried out on an ACE 5500 system (Beckman Instruments, Fullerton, USA). Samples were diluted in reduction buffer consisting of 6 M urea, 5 mM tri-sodium citrate and 4.5 mM DTT, pH 8.5 to a final concentration of 5 mg/mL and subsequently filtered over 0.45 µm filter (Schleicher & Schuell, Dassel, Germany). The separation was performed by a fused silica capillary at 25

kV and 45 °C. Detection was performed at 214 nm. The running buffer was 0.38 M sodium citrate, pH 3.0 containing 6 M urea and 0.05% (w/v) methylhydroxyethylcellulose (MHEC, Sigma, USA).

Reversed Phase High Performance Liquid Chromatography (RP-HPLC)

The HPLC equipment consisted of two M 6000A pumps (Waters Assoc.), an ISS-100 automatic sample injector (Perkin-Elmer), a Kratos Model 7836 UV detector and a Waters Type 680 automated gradient controller. A 250 mm x 4.6 mm I.D. HiPore RP-318 column (Bio-Rad Labs.). Separation was started with 69% of solvent A (2% (v/v) acetonitrile and 0.1% (v/v) TFA in water). A gradient was generated immediately after injection of the sample started from 31% of solvent B (90% (v/v) acetonitrile and 0.08% (v/v) TFA in water) to 43% B in 15 min, from 43% to 49% B in 32 min, from 49% to 73% B 49% in 5 min and held for 5 min subsequently, before returning to starting conditions in 3 min. The flow rate was 0.8 mL/min and detection took place at 214 nm.

Differential Scanning Calorimetry (DSC)

DSC was performed on a Micro-DSC III (Setaram, Caluire, France). Typically, 0.9 gram of a 30 mg/mL β-lactoglobulin sample in 50 mM sodium phosphate buffer, pH 5.8 was equilibrated for 10 min at 20 °C. The sample was subsequently heated from 20 to 120 °C at a heating rate of 1 °C/min and cooled down to 20 °C at a rate of 1 °C/min.

Hydrolysis of β-lactoglobulin

Enzymatic hydrolysis of β-lactoglobulin by *Staphylococcus aureus* V8 Glu specific enzyme was performed by the pH-stat method.[22] β-Lactoglobulin was dissolved in 5 mL milliQ water to a final concentration of 10 mg/mL. The pH was adjusted to pH 8.0 with 0.1 N NaOH, and the solution was equilibrated at 40 °C for 30 min. The enzyme was added in an E/S ratio of 1/50 (w/w) and hydrolysis was performed until 1) a maximum degree of hydrolysis (DH_{max}), or 2) a degree of hydrolysis of 6.8%

(DH 6.8%) for binding studies. During hydrolysis the pH was kept constant at pH 8.0 using 0.4 M NaOH. To reach the DH_{max} the hydrolysis was allowed to continue until the slope of NaOH consumption was lower than 0.05 µl/min. After hydrolysis the V8 enzyme reaction was inhibited by adding Z-LE-CMK in an enzyme:inhibitor ratio of 1:1 (w/w) and cooled on ice. This led to total inactivation of the enzyme V8 as determined by RP-HPLC (data not shown). Comparison of the DH values obtained from repeated experiments showed that the variation in DH was less than 10%.

Matrix Assisted Laser Desorption Ionization Time Of Flight Mass Spectrometry (MALDI-TOF-MS)

Hydrolysates with DH_{max} and DH 6.8% were desalted using the Omix Ziptip (Varian, Palo Alto, CA, USA). For this, the Ziptip was prewetted with 50% (v/v) acetonitrile and subsequently equilibrated/washed with 0.1% (v/v) TFA. Samples were eluted from the Ziptip with 50% (v/v) acetonitrile containing 0.1% (v/v) TFA. Before diluting the sample with the energy absorbing molecule (EAM) solution, 100 mg of the EAM (α-cyano hydroxycinnamic acid, CHCA) was washed three times in 1 mL acetonitrile and finally suspended in 1 mL 50% (v/v) milliQ water, 49.9 (v/v) acetonitrile and 0.1% (v/v) formic acid. The suspension was centrifuged (10 minutes at 21.000 * g at room temperature) and the supernatant was successively used to dilute 1 µL of the desalted sample 10 times in EAM. Two µL of this solution was applied to a gold plate. The samples were air-dried prior to analysis on an UltraFlex TOF (Bruker Daltonik GmbH, Bremen, Germany) in the positive mode using an acceleration voltage of 25 kV. Calibration was carried out using the peptide calibration standard II (Bruker Daltonik) containing the following components with $[M+H]^+$ average molecular weights between brackets: Bradykinin 1-7 (757.86); Angiotensin II (1047.19); Angiotensin I (1297.49); Substance P (1348.64); Bombesin (1620.86); Renin substrate (1760.03); ACTH clip 1-17 (2094.43); ATCH clip 18-39 (2466.68); Somatostatin 28 (3149.57).

Surface Enhanced Laser Desorption Ionization Time of Flight Mass Spectrometry (SELDI-TOF-MS)

SELDI-TOF-MS experiments were performed in duplicate on a ProteinChip SELDI System Enterprise Edition (BioRad Laboratories, Hercules, CA, USA). For the identification of peptides in the two hydrolysates, samples were diluted 10 times in the energy absorbing molecule EAM solution (α-cyano-hydroxycinnaminic acid, CHCA). From the diluted samples 2 µL was added to a ProteinChip Gold surface and air-dried prior to reading at a laser intensity of 1000 AU. The instrument settings used were: Detector sensitivity 9; detector voltage 2900 V; from position 20-80 each tenth position was read; 10 laser shots were collected per position. Calibration was carried out using Peptide Calibration Standard II (see previous section).

Covalent coupling of the proteins to the Proteinchip and binding of the peptides to the proteins was based on the suppliers' protocol (ProteinChip Applications Guide, BioRad Laboratories, Hercules, CA, USA). To this end, for covalent coupling of proteins (β-lactoglobulin, ovalbumin, β-casein and glycinin) to the epoxy moieties of the ProteinChip PS-20 via their amino groups, proteins were diluted in 0.1 M sodium carbonate buffer pH 8.2 to a final concentration of 1 mg/mL. From this protein solution 5 µL was applied to the pre-equilibrated with PBS ProteinChip PS-20 and placed onto a rack in a closed container filled partially with water to limit evaporation of the sample droplets and incubated overnight at 4 °C. After incubation, the excess of protein solution was removed and the residual free epoxy groups were blocked with 1 M ethanolamine at pH 8.0 for 30 minutes at room temperature. Next, the ProteinChip PS-20 surface was washed two times 10 minutes with PBS, pH 7.4 containing 0.5% (v/v) Triton X-100 and subsequently washed 10 minutes with PBS, pH 7.4. To confirm the binding of proteins to the ProteinChip PS-20, two times 0.5 µL freshly prepared 2,5 dihydroxybenzoic acid (DHB) (saturated solution in 50% (v/v) acetonitrile in water) was applied to the proteins covalently bound to the ProteinChip PS-20 prior to reading this at high laser intensity of 4,000 AU.

For binding of the peptides to the ProteinChip PS-20 bound protein, 5 µL of hydrolysate with DH 6.8% in PBS, pH 7.4 containing 0.1% (v/v) Triton X-100 was added to the ProteinChip PS-20 surface and incubated in a humidity chamber overnight at 4 °C. After incubation the ProteinChip PS-20 was washed three times 10 minutes in PBS, pH 7.4 containing 0.1% (v/v) Triton X-100.
For covalently bound β-lactoglobulin different washing conditions were used: After incubation the ProteinChip was washed 10 minutes with different concentrations (0.1-2.0% (v/v)) Triton X-100 or 8 M urea. Next, the ProteinChip PS-20 was washed 10 minutes in PBS, pH 7.4 and finally washed 10 minutes in milliQ water. The ProteinChip PS-20 was allowed to dry to the air and successively two times 0.5 µL of CHCA solution was added to the ProteinChip PS-20 surface and air-dried prior to reading at a laser intensity of 1,000 AU.

Results/Discussion

β-Lactoglobulin preparation

For this research β-lactoglobulin was purified to have a very pure and well characterized protein and hence, to obtain well defined fragments after hydrolysis of β-lactoglobulin. SDS-PAGE confirmed that the purified β-lactoglobulin was more than 99% pure. From capillary electrophoresis (CE) it was clear that there was no α-lactalbumin present and only 1% of the β-lactoglobulin was lactosylated (data not shown). The protein content (N x 6.33) of purified β-lactoglobulin determined by Dumas was 92% (w/w), the moisture content was 5% (w/w) and the ash content was 3% (w/w). RP-HPLC data showed that the purified β-lactoglobulin contained 72% of the genetic variant A and 28% of the genetic variant B. DSC experiments showed that the denaturation temperature (T_d) was 80.3 °C and the ΔH_{cal} was 241 kJ/mol, which is in agreement with literature.[23]

Composition of β-lactoglobulin hydrolysates

Extent of hydrolysis: Two different hydrolysates were prepared, one to the experimental maximum DH (DH_{max}), which enables identification of the

experimental smallest possible peptides and one to a DH 6.8%. The DH_{max} was found to be approximately 11%, indicating that beside specific cleavage after Glu also some cleavage after Asp took place.[24] Considering only Glu specificity the DH_{max} would be 8.6 % and including Asp specificity the DH_{max} would be 15%. For the binding studies of peptides to different proteins by SELDI TOF MS, β-lactoglobulin was hydrolyzed to DH 6.8%. At this point approximately 90% of the native β-lactoglobulin is degraded (as determined by HPLC), but there are still intermediate fragments present. Such intermediate fragments are still susceptible for further hydrolysis. It was shown by Creusot et al. that intermediate fragments obtained after a similar hydrolysis (to DH 6.8%) by another Glu specific enzyme (BLP) can bind to proteins.[25] Both enzymes have the same specificity for glutamate residues, but the decrease of intact β-lactoglobulin as function of the DH is different (data not shown). Although the V8 enzyme has a different mode of action than the BLP enzyme the choice to use DH 6.8% was based on the previous finding by Creusot et al.[26]

Hydrolysate composition: In the hydrolysate DH_{max} five and thirty-eight values for m/z were observed by MALDI-TOF-MS and SELDI-TOF-MS respectively. Table 1 shows sixteen peptides in the range of approximately 1,000-5,000 Dalton. These peptides are identified by comparing the observed m/z with the theoretical m/z. The sixteen identified peptides from table 1 cover about 98 % of the primary sequence of β-lactoglobulin (figure 1). The other observed, but not identified, m/z values can include peptides with reshuffled disulphide bridges[27] or peptides with modifications (i.e. lactosylation). Five of the peptides identified (AB [f29-45], B [f46-65], AB [f135-162], AB [f109-131], AB [f63-89]) missed one or more cleavages after glutamate and four peptides were a result of cleaving after Asp instead of Glu residue. Six peptides (AB [f135-158], AB [f138-158], AB [f135-162], AB [f90-108], AB [f12-45], AB [f1-45]) were also found by others and previously held to be responsible for initiating aggregation of β-lactoglobulin.[25, 28] The first mentioned authors considered peptide AB [f1-45], as the main aggregating peptide. Analyzing

the hydrolysate with DH 6.8% ten values for m/z were observed, which could be assigned to ten peptides already identified in the hydrolysate with DH_{max} (table 1).

Table 1. MS results from SELDI-TOF and MALDI-TOF experiments with peptides from hydrolysis of β-lactoglobulin with V8 protease (DH_{max}) on gold plate target.

observed mass (Da)	theoretical mass[a] (Da)	MC[b]	possible fragment	peptide sequence [c]	DH 6.8 (%) [d]	$H\Phi_{av}$ (kcal/res)[e]	Net charge at pH 7.4 [f]
929.5	929.5	0	AB [f56-62]	E-ILLQKWE	No	1.54	0.0
1025.3	1026.6	0	AB [f66-74]+Na	E-CAQKKIIAE	No	0.82	+0.9
1361.9	1361.7	0	AB [f34-45]	D-AQSAPLRVYVEE	Yes	1.04	-1.0
1474.0	1475.7	0	A [f115-127]	E-QSLVCQCLVRTPE	No	1.08	-0.2
1686.8	1687.0	0	AB [f75-89]	E-KTKIPAVFKIDALNE	Yes	1.45	+1.0
1903.2	1903.0	1	AB [f29-45]	D-ISLLDAQSAPLRVYVEE	Yes	1.25	-2.0
2308.8	2309.2	3	B [f46-65]	E-LKPTPEGDLEILLQKWENGE	Yes	1.21	-3.0
2335.2	2335.2	0	AB [f90-108]	E-NKVLVLDTDYKKYLLFCME	Yes	1.51	-0.1
2436.5	2436.3	0	AB [f138-158]	D-KALKALPMHIRLSFNPTQLEE	Yes	1.30	+1.0
2554.3	2555.1	3	B [f109-131]+Na	E-NSAEPEQSLACQCLVRTPEVDDE	No	0.79	-5.2
2826.4	2826.5	0	AB [f135-158]	E-KFDKALKALPMHIRLSFNPTQLEE	Yes	1.31	+1.0
2993.5	2993.6	2	B [f63-89]+Na	E-NGECAQKKIIAEKTKIPAVFKIDALNE	Yes	1.23	+0.9
3307.7	3307.7	1	AB [f135-162]	E-KFDKALKALPMHIRLSFNPTQLEEQCHI	Yes	1.27	+1.0
3607.9	3606.9	0	AB [f1-33] +Na+2MeO	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLD	No	1.21	-1.0
3695.7	3695.9	0	AB [f12-45]	D-IQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEE	No	1.25	-2.0
4895.5	4895.6	0	AB [f1-45]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEE	Yes	1.19	-2.0

[a] Theoretical mass derived from ExPASy, SIB, Switzerland ($[M+H]^+$, Cys in reduced form)
[b] MC = missed cleavage based on Glu specificity of V8 protease
[c] Peptide sequence starting with residue after which the V8 enzyme has cleaved
[d] Also present in hydrolysate with DH 6.8%
[e] Average hydrophobicity was calculated according to the method of Bigelow (1967) [29]
[f] The net charge was calculating according to the following formula:

$$Z = \sum_i N_i \frac{10^{pKa_i}}{10^{pH} + 10^{pKa_i}} - \sum_j N_j \frac{10^{pH}}{10^{pH} + 10^{pKa_j}}$$

where N_i are the number, and pKa_i the pKa values, of the N-terminus and the side chains of arginine, lysine, and histidine. The *j*-index belong to the C-terminus and the aspartic acid, glutamic acid, cysteine and tyrosine residues.

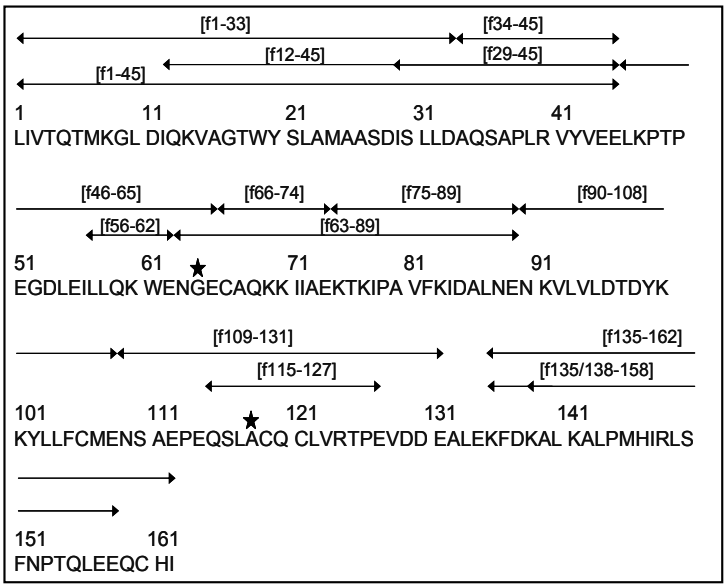

Figure 1. Primary sequence of β-lactoglobulin B. The arrows depict the observed fragments from hydrolysis by V8 protease. The asterisks depict the modifications for the genetic variant A: G64D and A118V.

Covalent binding of proteins to ProteinChip PS-20.

Ovalbumin, β-casein, glycinin and β-lactoglobulin were covalently bound to a ProteinChip PS-20 with epoxy moieties on the surface of the chip. To verify whether the proteins were bound to the ProteinChip surface high laser intensities were used to break the link of the protein to the surface. Below a laser intensity of 2,000 AU no significant peak intensities in the signal were observed (data not shown). At higher laser intensities (4,000 AU), the spectra resolved peaks at approximately 45,000 m/z (Fig. 2A), 24,000 m/z (Fig. 2B), 20,000 and 31,000 m/z (Fig. 2C) and at 18,000 m/z (Fig. 2D). These masses could be assigned to ovalbumin, β-casein, glycinin and β-lactoglobulin, respectively, and so confirms that all proteins were (covalently) bound to the ProteinChip PS-20. For β-lactoglobulin also the dimeric form was observed (37,000 m/z, Fig. 2D). To detect non-covalent protein-peptide interactions, lower laser intensities (1,000-1,500 AU) are used further on.

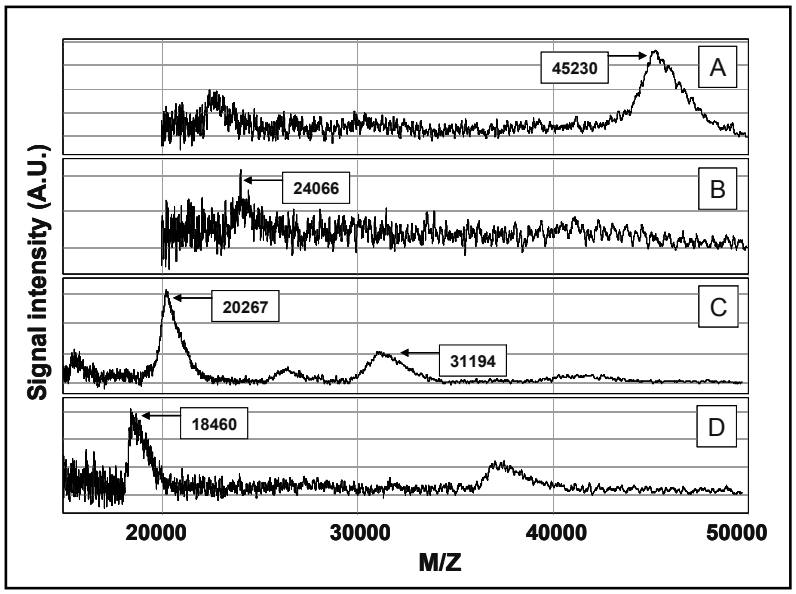

Figure 2. SELDI-TOF-MS spectra of **A.** ovalbumin, **B.** β-casein, **C.** glycinin and **D.** β-lactoglobulin covalently bound to Proteinchip PS20 and DHB as EAM. Laser intensity was 4,000 A.U. to break binding of protein to the ProteinChip surface.

Interaction of peptides to proteins

To screen the binding capability of specific peptides to proteins, four different proteins (ovalbumin, glycinin, β-lactoglobulin, β-casein) were covalently bound to separate spots on the ProteinChip PS-20. These proteins are derived from food related sources but differ in origin, composition and size, and allow to give also insight in the applicability of this method over a broad range of different food related proteins.

Figure 3 shows the spectra of peptides of the DH 6.8% hydrolysate bound to the four different proteins. The masses observed were all in the range of 1,000-5,000 m/z. Below 1,000 m/z the values were not reliable anymore due to machine limitations.

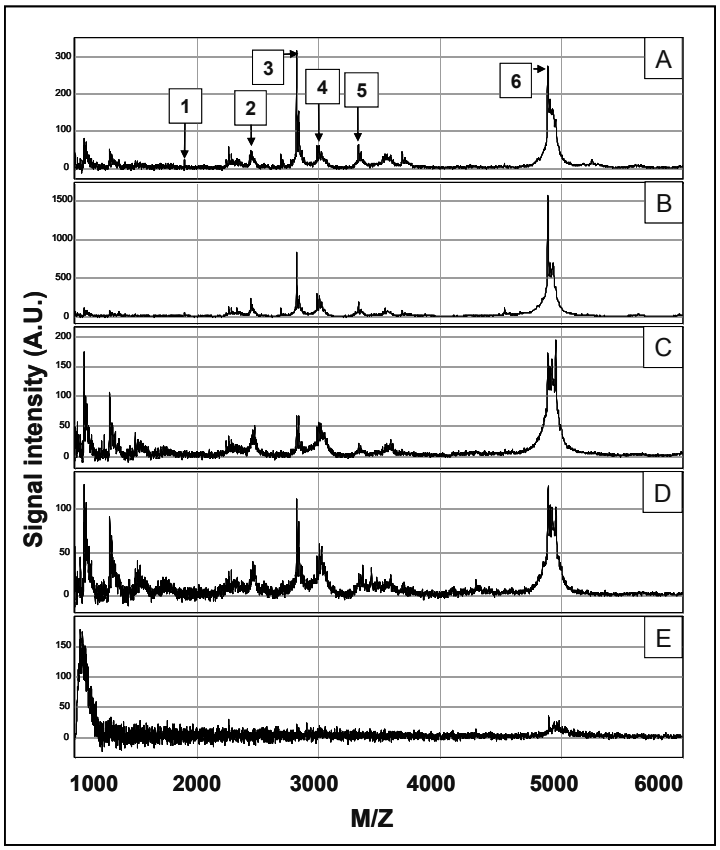

Figure 3. SELDI-TOF MS spectra of peptides bound to **A**. ovalbumin, **B.** glycinin, **C.** β-lactoglobulin, **D**. β-casein, **E.** no protein. The assigned fragments bound to ovalbumin (A) are 1. AB [f29-45]; 2**.** AB [f90-108]; 3. AB [f135-158]; 4. B [f63-89]; 5. AB [f135-162]; 6. AB [f1-45]. The source of the peptides was β-lactoglobulin hydrolyzed with V8 till DH = 6.8 %. The scale of the Y-axis of each spectrum is adjusted to the peak with maximum intensity.

No significant signals were observed for m/z values higher than 5,000 although these values were observed (but not identified) in the original DH 6.8% hydrolysate (no further data shown). Non-covalent binding of specific peptides present in the hydrolysate with DH 6.8% to the proteins on the SELDI support resulted in the identification of six masses. These masses were assigned to the following peptides: **1.** AB [f29-45]; **2.** AB [f90-108]; **3.** AB [f138-158]; **4.** B [f63-89]; **5.** AB [f135-162]; **6.** AB [f1-45]. The first fragment AB [f29-45] is part of the fragment AB [f1-45]. Binding of this fragment could imply that the C-terminal part of peptide AB

[f1-45] plays an important role in binding. The latter three peptides AB [f90-108]; AB [f135-158] and AB [f1-45] were also found by others[25, 28, 30] to have aggregating capacity as is discussed in the previous section. Interestingly, despite large differences in size, overall net charge and hydrophobicity between the four proteins (table 2) the same six peptides are found to bind.

Table 2. Properties of proteins used for the binding studies.

Protein	theoretical mass[a] (Da)	$H\Phi_{av}$ (kcal/res)[b]	Net charge at pH 7.4 [c]
β-lactoglobulin A/B	18367/18281	1.22/1.21	-9.5/-8.5
ovalbumin	42750	1.12	-12.4
β-casein	23583	1.36	-7.8
Glycinin subunit G1	52599	1.01	-6.6
G2	51393	0.98	-12.7
G3	51392	1.01	-9.6
G4	61200	0.94	-32.1
G5	55422	0.95	-15.1

[a] Theoretical mass derived from ExPASy, SIB, Switzerland ($[M+H]^+$, Cys in reduced form, except for two Cys residues involved in the disulphide bridge formation per Glycinin subunit).
[b] Average hydrophobicity was calculated according to the method of Bigelow(1967)[29]
[c] The net charge was calculating according to the following formula:

$$Z = \sum_i N_i \frac{10^{pKa_i}}{10^{pH} + 10^{pKa_i}} - \sum_j N_j \frac{10^{pH}}{10^{pH} + 10^{pKa_j}}$$

where N_i are the number, and pKa_i the pKa values, of the N-terminus and the side chains of arginine, lysine, and histidine. The *j*-index belong to the C-terminus and the aspartic acid, glutamic acid, cysteine and tyrosine residues.

This indicates that although there is selectivity (not all peptides bind) the binding is not specific (they bind to all four proteins). The signal intensity of the peptides bound to glycinin is 5-10 times higher than the signal intensity of the peptides bound to the other proteins, suggesting a higher affinity of the peptides for glycinin than for ovalbumin, β-lactoglobulin and β-casein consecutively. A lower negative net charge at pH 7.4 (more negative) and a lower average hydrophobicity for some glycinin subunits than for the other proteins suggests that both hydrophobic interactions, electrostatic interactions can play an important role in the binding mechanism of these peptides to the proteins. Another explanation for the differences in signal intensity of the bound peptides could be that the molar amount of protein bound the ProteinChip PS-20 was not equal for all four proteins. Further

experiments (e.g. protein staining or ellipsometry) could give an indication about the amount of bound proteins, but because of the small quantities to be determined new techniques would have to be developed, which was out of scope of this article. The above mentioned binding of specific peptides to the proteins was performed in a standard buffer containing 0.1% Triton X-100 to avoid a-specific binding of peptides to the ProteinChip PS-20 surface. To study the affinity of specific peptides to the protein, increasing concentrations of Triton X-100 were used to displace the bound peptides from the protein.

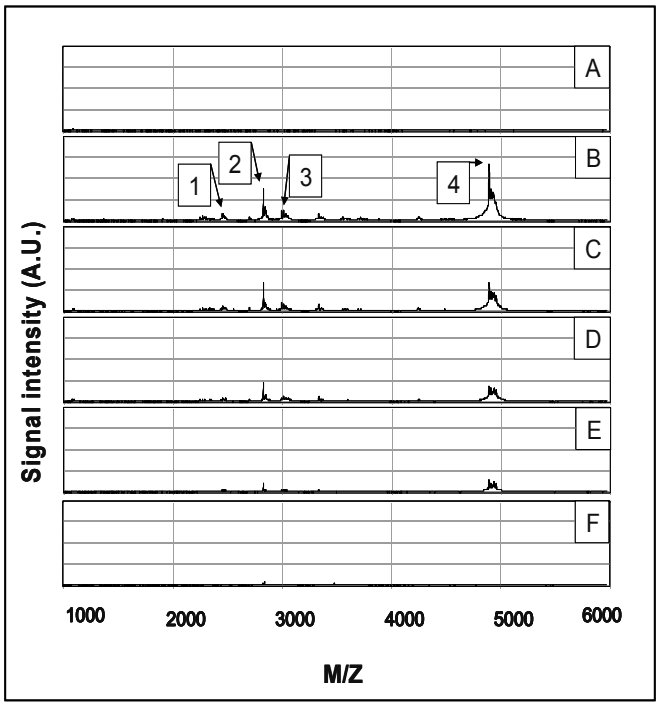

Figure 4. SELDI-TOF MS spectra of non-covalent binding peptides released β-lactoglobulin. **A.** no peptides added. Washing steps were performed with PBS containing **B.** 0.1%, **C.** 0.5%, **D.** 1.0%, **E.** 2.0% Triton X-100 or **F.** 8 M urea. The numbered peaks are identified as peptide 1. AB [f138-158]; 2**.** AB [f135-158]; 3. AB [f63-89]; 4. B [f1-45]. The Y-axis is re-scaled to a maximum of 2,000 A.U. for all spectra.

After incubation of the DH 6.8% hydrolysate with β-lactoglobulin covalently bound to the ProteinChip PS-20 surface, affinity of the peptides to the protein was investigated by applying different washing conditions (0.1-2% Triton X-100).

No signals were observed when no peptides were added, confirming that β-lactoglobulin covalently bound to the surface showed no peaks at a laser intensity of 1,500 AU (figure 4A).

Peptides were partially removed by increasing amounts of Triton X-100 (figures 4B-E). At the highest concentration Triton X-100 used still 20-35% of the peptides are bound to β-lactoglobulin. It is expected that higher concentrations of Triton X-100 are needed to remove more peptides from the protein, but based on curves in figure 5 it can be argued whether Triton X-100 has the capacity to remove all peptides bound to the protein. Therefore, an 8 M solution of urea was used for the complete displacement of all the peptides from the protein (figure 4F). This implies that the mechanism of the protein-peptide interaction is at least partially based on hydrophobicity. Differences in the degree of removal by Triton X-100 were found with at least four of the peptides assigned (figure 5).

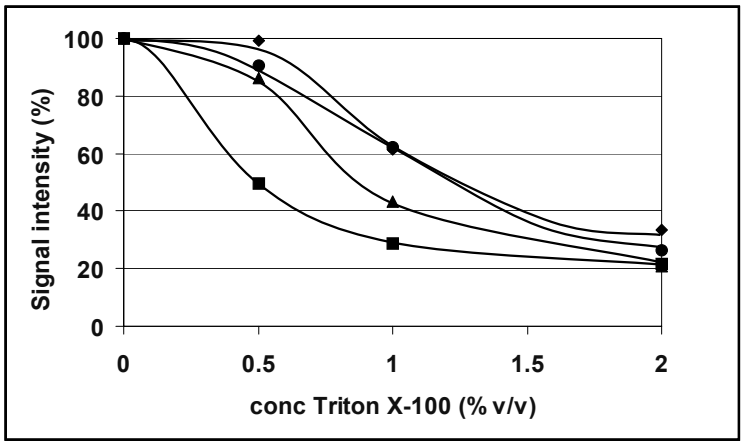

Figure 5. Removal of peptides from β-lactoglobulin covalently bound to a ProteinChip PS-20 by different concentrations of Triton X-100. Signal intensity of peptide AB [f135-158] (●), peptide AB[f1-45] (■), peptide AB[f63-89] (▲) and peptide AB [f138-158] (♦).

The figure clearly shows that peptide AB [f1-45] was removed at a lower Triton X-100 concentration than peptide AB [f63-89] and peptides AB [f135/138-158], respectively. Obviously, peptide AB [f1-45] has a lower affinity for β-lactoglobulin than the other three peptides. The average hydrophobicity, $H\Phi_{av}$, (table 1) of peptide AB [f1-45] (1.19 kcal/res) is lower than the $H\Phi_{av}$ of peptide AB [f63-89] (1.23 kcal/res), [f138-158] (1.30 kcal/res) and peptide AB [f135-158] (1.31 kcal/res). In addition, the net charge at pH 7.4 of peptide AB [1-45] (-2.0) is lower than the net charge of peptide AB [f63-89] (+1.0), AB [f138-158] (+1.1) and peptide AB [f135-158] (+1.1). These results suggest that the binding mechanism of peptide AB [1-45] to β-lactoglobulin is based both on hydrophobicity and electrostatic interaction, although peptide AB [f1-45] contains a larger conserved hydrophobic domain (12 residues) than the other three peptides (each containing a conserved hydrophobic domain of maximally 6 residues).

To study the electrostatic interaction between peptides and proteins, which was out of the scope of this article, the same SELDI-TOF-MS procedure can be performed using washing buffer containing salts to shield the charges on the protein and peptides.

Conclusion

In this research we have shown that SELDI-TOF-MS is a rapid tool to identify specific peptides from a heterogeneous protein hydrolysate capable of binding to food related proteins. The technique was demonstrated to work for four food related proteins, which indicates that SELDI-TOF-MS can be used for a broad variety of proteins and peptides which could interact with each other. Washing the bound peptides from β-lactoglobulin with increasing concentration of detergent provides an estimate for binding affinity. A similar approach can be followed by changing the solvent quality using urea or salt. More detailed quantification of the interaction energies and binding constants by isothermal titration calorimetry is part of current research. These findings show that SELDI-TOF-MS is a suitable and

rapid tool to screen binding of peptides to a variety of proteins and to make a first step in identifying these peptides.

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Chapter 3

The characteristics and effects of specific peptides on heat-induced aggregation of β-lactoglobulin

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Abstract

A bovine β-lactoglobulin hydrolysate, obtained by the hydrolysis by the Glu-specific enzyme *Bacillus licheniformis* protease (BLP), was fractionated at pH 7.0 into a soluble and an insoluble fraction and characterized by LC-MS. From the 26 peptides identified in the soluble fraction, five peptides (A[f97-112]=[f115-128], AB[f1-45], AB[f135-157], AB[f135-158] and AB[f138-162]) bound to β-lactoglobulin at room temperature. After heating of β-lactoglobulin in the presence of peptides, eight peptides were identified in the pellet formed, three of them belonging to the previously mentioned peptides. Principle component analysis revealed that the binding at room temperature (to β-lactoglobulin) was related to the total hydrophobicity and the total charge of the peptides. The binding to the unfolded protein could not be attributed to distinct properties of the peptides. The presence of the peptides caused a 50% decrease in denaturation enthalpy (from 148 ± 3 kJ/mole for the protein alone to 74 ± 2 kJ/mole in the presence of peptides), while no change in secondary structure or denaturation temperature was observed. At temperatures < 85 °C, the addition of peptides resulted in a 30-40% increase of precipitated β-lactoglobulin. At pH < 6 no differences in the amount of aggregated β-lactoglobulin were observed, which indicates the lack of binding of peptides to β-lactoglobulin at those pH values as was also observed by SELDI-TOF-MS. Although only a few peptides were found to participate in aggregation, suggesting specificity, principal component analysis was unable to identify specific properties responsible for this.

Introduction

The heat-induced denaturation and aggregation of intact whey proteins have been widely studied. Both can occur during processing of food products and affect the product properties such as foam, emulsion and gel properties.[1] The types and sizes of the aggregates formed during heat treatment are of major importance for the texture of protein gels.[2-5] For intact protein systems the aggregation can be affected by system conditions (e.g. pH, T), but was also found to be affected by the addition of protein hydrolysates.

The aggregation itself is typically described by two successive steps, namely:

$$N \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} D \qquad \text{Denaturation} \qquad (1)$$

$$D + A_x \xrightarrow{k_2} A_{x+1} \qquad \text{Aggregation} \qquad (2)$$

The transition of proteins from the native (N) to the denatured (D) state is considered to be a reversible first-order reaction. The subsequent aggregation reaction is an irreversible bimolecular reaction. Since unfolded monomeric proteins can react to monomers and to aggregates (A) of different sizes the overall reaction does not follow simple reaction order (0, 1, or 2) kinetics. For this reason a general reaction order model has been used to describe the rate at which unfolded monomers react to aggregates. The equation for this general reaction is:

$$C_{N,\,t} = C_{N,0} - k_n\, C_{N,0}{}^n \qquad (3)$$

The aggregation kinetics is described by the concentration of native proteins as function of time ($C_{N,\,t}$), using the reaction order (n) and reaction rate (k). It was found that system conditions (pH, ionic strength) play an important role in the aggregation rate of β-lactoglobulin[6], but less in the case of ovalbumin.[7] Changes in these conditions can affect the types of aggregates formed as well as the resulting gel properties.

Besides system conditions, protein aggregation is also affected by the presence of other proteins or peptides. It has been shown that (heat-induced) gels of protein hydrolysates are either stronger or weaker than those of the parental protein, depending on the enzyme used.[8, 9]

The growth of whey protein aggregates was found to be inhibited by the presence of κ-casein and Na-caseinate.[10, 11] The role of peptides has been illustrated by several papers. The addition of a total hydrolysate of whey protein isolate (WPI) to β-lactoglobulin resulted in increased aggregation.[12] This hydrolysate was later fractionated, yielding one peptide fraction with mainly hydrophobic and one with highly negatively charged peptides. Both fractions increased the denaturation temperature of β-lactoglobulin at neutral conditions.[13] Tryptic hydrolysates of Na-caseinate were demonstrated to increase the size of β-lactoglobulin aggregates, but to lead to dissociation of Na-caseinate micelles.[14] To understand the effects of hydrolysates on the aggregation of proteins it is important to know whether specific peptides have particular contributions on the effects observed. In a study on the addition of self-aggregating peptides of WPI hydrolysate made by the Glu-specific enzyme *Bacillus licheniformis* protease (BLP) the peptides β-lactoglobulin AB[f1-45], AB[f135-158] and AB[f90-108] were identified as the main peptides present in the aggregated fraction of WPI/peptide mixture.[15, 16] These observations all indicate the significant influences that peptides can have on the aggregation behavior of proteins. The mechanisms by which the peptides in these hydrolysates interact with the protein and lead to different aggregates and gels are not known in detail. These mechanisms could involve non-specific interactions or of the binding of specific peptides to the intact or denatured proteins. To understand the effects of peptides on heat-induced aggregation of intact proteins in the present article first specific protein-peptide interactions were determined. Next, the effects of these peptides on the denaturation and heat-induced aggregation of β-lactoglobulin were studied. Using principal component analysis the characteristics of bound and non-bound peptides were compared. For this study a soluble peptide fraction formed after hydrolysis of β-lactoglobulin by BLP was used.

Materials and Methods

Chemicals and reagents

β-Lactoglobulin was isolated from fresh bovine milk, purified and characterized as described previously.[17] β-Casein was purified according to Leaver & Law (1992).[18] ProteinChip PS20 was purchased from Biorad (Hercules, CA, USA). α-Cyano-hydroxycinnaminic acid (CHCA) was obtained from Bruker Daltonics (Bremen, Germany). *Bacillus licheniformis* Protease (BLP) (product name NS-46007, E.C. 3.4.21.19) described to be specific for Glu-Xaa peptide bonds and to a lesser extent for Asp-Xaa peptide bonds[19], was kindly provided by Novozymes A/S (Bagsvaerd, Denmark). The activity of the enzyme is 0.7 AU/mL as given by the manufacturer.

Hydrolysis of β-lactoglobulin and fractionation of peptides

β-Lactoglobulin was dissolved in milliQ water to a final protein concentration of 10 mg/mL. The pH was adjusted to pH 8.0 with 0.1 N NaOH and the solution (500 mL) was kept at 40 °C for 30 min. The enzyme was added in an E/S ratio of 1/500 (v/v). During hydrolysis the pH was kept constant at pH 8.0 using 0.4 M NaOH. The hydrolysis was performed until a degree of hydrolysis of 6.8% ($DH_{6.8}$) was reached as determined from the added amount of NaOH. At this DH value the intact β-lactoglobulin level is as low as possible and at the same time a broad range of peptides is present. After hydrolysis, the pH was adjusted to 2.0 with 5 M HCl. After 30 min the hydrolysate was set to pH 7.0 by 5 M NaOH. This resulted in total inactivation of BLP (data not shown). After 30 min the hydrolysate was separated by centrifugation (20 min; 21,000 *g;* 10 °C) into a pellet and supernatant fraction based on previous work.[7] The peptides in the supernatant fraction were used for further experiments. For compositional analysis the total hydrolysate and the supernatant fractions were diluted 1:1 (v/v) with 8 M urea. The pellet fraction was re-dissolved in a final concentration of 4 M urea to the same final volume as the total hydrolysate and the supernatant fraction.

Nitrogen content

Total nitrogen contents of the total and fractionated hydrolysate were determined by Dumas method AOAC[20] using a Flash EA 1112 NC analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Typically, 10 mg of freeze-dried sample was weighed in aluminium foil cups. The nitrogen-to-protein conversion factor N x 6.33 (based on the primary sequences of β-lactoglobulin A and B (72% and 28% (w/w) abundance in the sample, respectively)[17] was used to calculate the protein content. L-Methionine was used for the calibration curve.

Heating experiments

Stock solutions of 50 mg/mL of β-lactoglobulin and 5 mg/mL of peptides in milliQ water were each adjusted to pH 7.0 with 0.1 M HCl for further experiments. This concentration was chosen in order to be able to form aggregates, but at the same time to prevent formation of gels (at > 80 mg/ml). These stock solutions were mixed to yield final concentrations of 5 mg/mL of β-lactoglobulin and 2.5 mg/mL of peptides. A solution of 5 mg/mL of β-lactoglobulin in 20 mM NaCl (to correct for the increase in ionic strength during hydrolysis and inactivation of the enzyme) and a solution of 2.5 mg/mL of peptides in milliQ water were used as references. With these conditions four sets of experiments were performed.

1- T= 75, 80, 85, 90 °C (120 min, pH 7)
2- t= 0, 30, 60, 120, 240 min (80 °C, pH 7)
3- pH 3, 4, 5, 6, 7, 8 (120 min at T=T_d (pH 3-4 at 88 °C, pH 5-8 at 80 °C).

For a fourth sample set the stock solution of β-lactoglobulin was diluted to yield final concentrations between 2.5 and 25 mg/mL and the peptide stock solution to a final concentration of 2.5 mg/mL. This set was heated at 80 °C for 120 min.

Upon heating all protein molecules unfold. Upon cooling (20 °C) part of the molecules refold to a native-like state. These native-like proteins do not precipitate at pH 4.7.[21] Therefore, the amount of native-like β-lactoglobulin was determined by the method of de Wit (1990).[22] Samples were adjusted to pH 4.7 ± 0.1 with 0.1 M HCl and subsequently centrifuged (10 min; 21,000 g; 10 °C), after which the

concentration of native-like β-lactoglobulin in the supernatant was determined by size exclusion chromatography.

In addition the composition of a heat-induced pellet was determined for three samples: 25 mg/mL of β-lactoglobulin with and without 2.5 mg/mL of peptides and 2.5 mg/mL of peptides alone. These samples were heated at 80°C for 120 min at pH 7.0. After heating the samples were cooled down and centrifuged without adjusting the pH (pH 7.0; 10 min; 21,000 g; 10 °C). The pellets were washed twice with MilliQ water, re-solubilized in 8 M urea to the original volume, and analyzed by reversed phase ultra performance liquid chromatography.

Size exclusion chromatography (SEC)

Samples were analyzed on an Äkta purifier (GE Healthcare, Uppsala, Sweden). A Superdex 75 column with a bed volume of 23.5 mL was equilibrated with 20 mM TRIS-HCl buffer, pH 7.2 containing 150 mM NaCl at a flow rate of 0.8 mL/min. Next, 50 µL of sample was injected onto the column. Detection took place at 214 nm. The column was calibrated using the proteins BSA, ovalbumin, chymotrypsinogen and ribonuclease with a mass of 67000, 42750, 20200, 15700 Da, respectively. The amount of native-like β-lactoglobulin was determined by the peak area at the time interval of 11-13 min. The peak area of the native-like fraction β-lactoglobulin divided by the peak area of unheated β-lactoglobulin was defined as C_t/C_0. These values were used to calculate the reaction order of denaturation/aggregation using equation 3.

Reversed Phase Ultra Performance Liquid Chromatography (RP-UPLC)

Samples were analyzed on a Waters Acquity UPLC system (Waters, Milford, MA, USA) using an Acquity UPLC BEH300 C18 column (Waters, 2.1 x 150 mm, 1.7 µm particle size). The eluents were 1% (v/v) acetonitrile (ACN) with 0.030% (v/v) trifluoroacetic acid in milliQ water (eluent A) and 100% (v/v) ACN with 0.030% (v/v) trifluoroacetic acid (eluent B). All samples were diluted to a final concentration of 1 mg/mL in eluent A prior to injection (4 µL injection volume). The column was

thermostated at 40 °C. The flow rate was 250 µL/min and detection took place from 200-400 nm (PDA detector). The elution profile was: 0 - 2 min, linear gradient from 3% - 10% (v/v); 2 - 6 min, linear gradient from 10% - 22% (v/v) B; 6 - 14 min, linear gradient from 22% - 35% B; 14 -18 min, linear gradient from 35% - 100% (v/v) B; 18- 20 min, isocratic on 100% (v/v) B, 20-22 min, linear gradient from 100% - 3% (v/v) B; 22-26 min, isocratic on 3% (v/v) B.

Electron spray ionization mass spectrometry (ESI-MS)

Electrospray ionization was performed on a Synapt High Definition Mass Spectrometer (Waters), a hybrid quadrupole and orthogonal acceleration time of flight (oa-TOF) instrument. Samples from UPLC were directly infused to the standard electrospray (z-spray) source at infusion rates between 3 and 5 µL/min. The capillary of the ESI source was operated at a voltage of 3 kV, with the source operating in positive ion mode. The sample cone was operated at 40 V, required to avoid gas-phase unfolding and to preserve non-covalent interactions. The trap T-wave collisional cell which is located just before the drift tube contained argon gas at a pressure of 2.5×10^{-2} mbar. The oa-TOF-MS was operated over the scanning range of *m/z* 500–3,500 at a pressure of 1.8×10^{-6} mbar. Data acquisition was performed with MassLynx v4.1 and peak assignment was based on comparison of the samples with an in silico digestion of β-lactoglobulin by a Glu-specific enzyme using the program BiopharmaLynx v2.1 (Waters).

Calculation of molar concentration of peptides

The molar concentrations of all peptides present in the total hydrolysate (table 1), as well as in the supernatant and pellet were calculated from the UV peak areas in the RP-UPLC chromatograms using the following equation:

$$C = 1 * 10^{6} \left(\frac{A}{\varepsilon_{214} \ell V_{inj} k_{\text{cell}}} \right) f \qquad (4)$$

In which *C* (μM) is the concentration of the peptide, *A* (AU min) is the RP-UPLC peak area of the absorbance at 214 nm. The path length of the UV cell, ℓ, provided by the manufacturer, is 1 cm. The injection volume V_{inj} was 4 μL and the flow rate was f = 250 μL/min. The cell constant, k_{cell}, (0.64) was determined by calibration with solutions of β-casein at known concentrations under the same experimental conditions. The molar extinction coefficients ε_{214} of the individual peptides was calculated according to Kuipers and Gruppen (2007).[23] Using these values the concentration of each peptide in the total hydrolysate, supernatant fraction (% in sup) and the re-dissolved pellet fraction (% in pel) was determined. This was used to calculate the relative proportion of each peptide in the supernatant fraction and the total hydrolysate (tables 1 and 3).

Dynamic Light Scattering (DLS)

Dynamic light scattering measurements were performed using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) equipped with a 4 mW HeNe laser beam with a wavelength of 633 nm, at a scattering angle of 173 °. The intensity of scattered light (I_s) and effective hydrodynamic diameter ($d_{h,eff}$) of solutions of single and mixed solutions of β-lactoglobulin and peptides were monitored during heating. The aggregation was followed for β-lactoglobulin at four concentrations (5, 10, 20 and 25 mg/mL) in the presence of peptides (5 mg/mL). Solutions of pure β-lactoglobulin (5-20 mg/mL) in 20 mM NaCl and a solution of peptides in milliQ (5 mg/mL) were used as controls. The pH of all solutions was adjusted to pH 7.0 with 0.1 M NaOH. For the experiments, the initial temperature was set to 20 °C. Typically, 50 μL of sample was covered with 50 μL paraffin oil to prevent water evaporation from the sample and analyzed for 5 min. Subsequently, the sample was removed from the apparatus and the temperature was set to 80 °C. When the Zetasizer Nano had reached the temperature set the sample was placed back into the Zetasizer Nano and analyzed for at least 25 min. The $d_{h,eff}$ reported are diameters corresponding to the dominant peak of the distribution analysis, as performed by the Malvern DTS software, version 6.01.

Surface Enhanced Laser Desorption Ionization Time Of Flight Mass Spectrometry (SELDI-TOF-MS)

SELDI-TOF-MS experiments were performed as described previously.[17] β-Lactoglobulin was covalently bound to a ProteinChip PS-20. The binding of peptides was studied at pH 2.8, 4.7, 7.1 and 9.3. To this end, the peptides were dissolved in phosphate buffered saline solution (PBS) containing 0.1% (v/v) Triton X-100 and the pH was adjusted by 0.1 M HCl and 0.1 M NaOH to the previously mentioned values. Aliquots (5 µL) were added to the ProteinChip PS-20 surface and incubated in a humidity chamber overnight at 4 °C. After incubation, the ProteinChip PS-20 was washed three times for 10 minutes in PBS, pH 7.4 containing 0.1% (v/v) Triton X-100. The ProteinChip PS-20 was washed 10 minutes with PBS, pH 7.4 and finally washed 10 minutes in milliQ water. The ProteinChip PS-20 was allowed to dry to air after which 0.5 µL of CHCA solution was added to the ProteinChip PS-20 surface and air-dried (two times) prior to reading at a SELDI-TOF-MS laser intensity of 1,000 AU.

Differential Scanning Calorimetry

The unfolding of β-lactoglobulin in the presence or absence of peptides was studied using a MicroCal VP DSC (GE Healthcare, Amersham, UK) with a 0.5 mL cell. Typically, 25 mg/mL of β-lactoglobulin in aqueous solution with or without 5 mg/mL of peptides was adjusted to pH 7.0 with 0.1 M HCl and analyzed within a temperature interval of 20-100 °C at a scan rate of 1 °C/min. As a reference, 30 mg/mL of β-lactoglobulin in aqueous solution containing 20 mM NaCl was used. The denaturation temperature (T_d) was defined as the temperature with the maximum heat capacity. From the area under the denaturation peak the enthalpy of unfolding (ΔH in kJ/mole) was obtained.

Circular Dichroism Spectroscopy

Far-UV and near-UV circular dichroism experiments were carried out as described previously.[24] Typically, 5 mg/mL of β-lactoglobulin in aqueous solution containing

0.25 mg/mL of peptides was adjusted to pH 7.0 with 0.1 M NaOH. Samples were incubated overnight at 4 °C. For far-UV experiments the samples were diluted 50 times in MQ water prior to the CD experiments and spectra were recorded from 195-260 nm at 20 and 95 °C (total unfolding). To determine the propensities of the secondary structure content of β-lactoglobulin in the presence of peptides, the spectra of β-lactoglobulin and peptides alone were also recorded in the same concentrations used is in the β-lactoglobulin/peptide mixture. The spectrum of the peptides was subtracted from that of the β-lactoglobulin/peptide mixture to reveal the β-lactoglobulin spectrum as it is in the presence of peptides. The spectra were analyzed for secondary structure elements using the standard CDNN program.[25] The experimental spectra were compared with 33 reference spectra from structurally well-characterized globular proteins. For near-UV spectra the samples were diluted 20 times in MQ water and spectra were recorded from 250-350 nm at 20 and 95 °C. The peptides did not give a signal in near-UV CD, therefore, the spectrum of β-lactoglobulin+peptide was not corrected.

Characteristics of peptides

All peptides were described with respect to the asymmetry and heterogeneity of the distribution of charge and hydrophobicity. To quantify both parameters routines were programmed in Matlab. The asymmetry was calculated by weighting the charge of each amino acid with the distance to the center of the peptide (expressed in the number of amino acids), divided by the summation of absolute weighted charges as expressed in equation 5a and 5b.

$$asym^{+} = \frac{\sum iZ_i}{\sum iABS(Z_i))} \qquad (5a) \qquad asym^{-} = \frac{\sum iZ_i}{\sum iABS(Z_i))} \qquad (5b)$$

In which *i* is the relative location to the center of the peptide and Z_i is the charge of the amino acid at location *i.* The additional charges of the N- and C-terminal groups were also taken into account. This calculation was performed separately for positively and negatively charged residues as well as for the net charge. These values go from 0 (all charges centered on the middle of the sequence) to 1. The

methodology resembles that of dipole moments. The resulting value is a measure of the polarity of the peptide and expresses a vector pointing from the length center to the charge center of a peptide. To verify whether large patches of clustered charges are present in the sequence another parameter was used. This parameter, the 'degree of blockiness' (DB) is a quantitative parameter that describes the extent to which the (+/-) charges are evenly distributed in blocks along the sequence. A block is a consecutive sequence of one or more similarly charged amino acids (either + or -). The number of blocks (B) as well as the total number of charged residues ($n^{+/-}$) is determined from the sequence. The DB is then calculated as:

$$DB^{+} = \frac{n^{+} - B}{n^{+} - 1} \qquad (6a) \qquad DB^{-} = \frac{n^{-} - B}{n^{-} - 1} \qquad (6b)$$

If all charged residues are separated, the number of blocks equals the total number of charged residues and DB = 0. In the other extreme, when all charged residues are in one block, DB = 1.

Similar quantitative parameters were used for the hydrophobic asymmetry ($asym^{\Phi}$) and DB for hydrophobicity (DB^{Φ}), using the hydrophobicity values of the hydrophobic residues. The results of principal component analysis were plotted in biplots. In addition, a cluster analysis was performed using the *K-means* routine (with no set number of clusters). This routine divides the samples in clusters to minimize the point-to-centroid distance for points in each cluster.

Results

Hydrolysis and characterization of fractionated hydrolysate

The β-lactoglobulin-BLP hydrolysate (DH = 6.8%) was separated at pH 7.0 into a supernatant and pellet fraction. From the nitrogen balance it was calculated that 119.3 mg of total hydrolysate was divided over 67.9 mg (56.9%) in the supernatant and 45.6 mg (38.2%) of the pellet fraction. The peptide compositions of the total hydrolysate, the supernatant fraction and the pellet fraction were analyzed by RP-UPLC (figure 1).

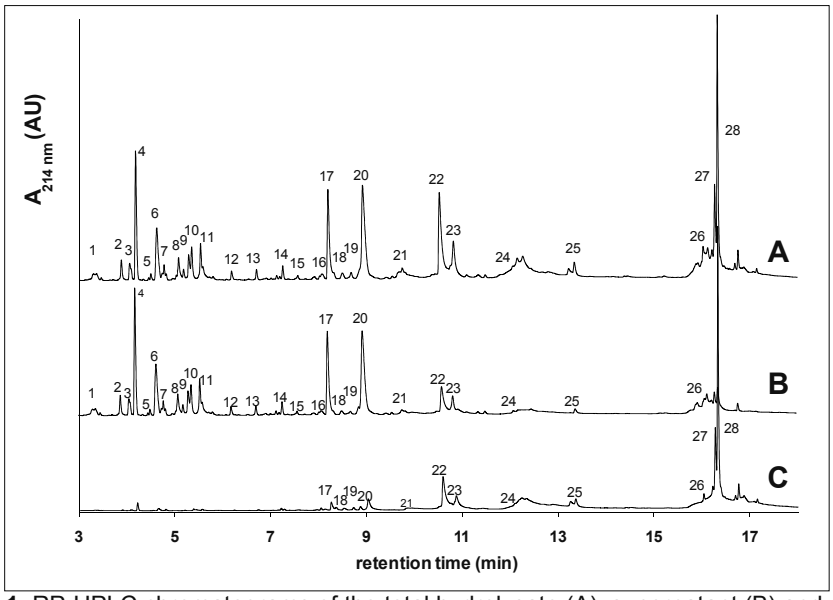

Figure 1. RP-UPLC chromatograms of the total hydrolysate (A), supernatant (B) and re-dissolved pellet (C). The peaks assigned are listed in table 1. The y-axis is re-scaled to a maximum of 0.75 AU for all spectra.

The sum of the peak areas of the peptides in the soluble and insoluble fraction is equal to the sum of the peak areas in the total hydrolysate, confirming that the supernatant and pellet fractions represent the complete hydrolysate. In the total hydrolysate 28 peptides were identified by mass and annotated (table 1). Of these, 17 peptides (1-17) were each on average 93% (based on molar amounts) present in the supernatant fraction. These peptides have shorter retention times (2-13 min) than peptides 18-28 (13-27 min) that appear predominantly in the pellet fraction (on average 61%). The proportion of peptides in the supernatant decreases with increasing retention time (table 1). The only exceptions to this trend are peptides 20 and 21. They are more abundant in the supernatant than in the pellet despite their relatively high retention times.

Table 1. Peptides identified in the total hydrolysate and subfractions

Peak no. in fig. 1	Observed mass (Da)	Theoretical mass[a] (Da)	Possible fragment	Peptide sequence[b]	Proportion in Supernatant (%)	$H\Phi_{tot}$/ [HΦav] (kcal)[c]	Net charge at pH 7.0[d]	Rel. concentration in total hydrolysate (% (mol/mol))
1	432.2	432.2	AB [f52-55]	E-GDLE	100	2.4[0.6]	-2.0	4.7
2	683.4	683.4	AB [f46-51]	E-LKPTPE	97	9.6[1.6]	0.0	3.4
3	876.3	876.5	B [f63-67]=[f159-161]	E-NGECA=QCH	95	0.8[0.1]	-0.9	3.0
4	589.3	589.3	AB [f59-62]	L-QKWE	95	4.5[1.1]	0.0	6.1
5	984.5	984.5	B [f106-108]=[f115-119]	E-CME=QSLAC	91	4.5[0.6]	-1.0	1.0
6	966.5	966.5	AB [f45-53]	E-ELKPTPEGD (-H_2O)	95	9.6[1.1]	-2.0	9.8
7	789.3	789.4	AB [f128-134]	E--VDDEALE	89	4.9[0.7]	-4.0	3.3
8	1499.8	1499.8	AB [f66-74]= [f159-162]	E-CAQKKIIAE=QCHI	100	13.6[1.0]	+1.1	2.5
9	979.3	979.4	B [f63-68]=[f158-160]	E-NGECAQ=EQC	100	0.8[0.1]	-2.0	4.9
10	1800.0	1800.0	B [f63-74]=[f159-162]	E-NGEQACKKIIAE=QCHI	93	16.8[0.9]	+0.1	2.4
11	1482.7	1482.7	AB [f12-24]	D-IQKVAGTWYSLAM (Met ox)	98	17.7[1.4]	+1.0	2.8
12	978.5	979.4	AB [f131-138]	D--EALEKFDK	100	8.8[1.1]	-1.0	1.1
13	485.3	485.3	AB [f56-59]	E--ILLQ	92	7.8[1.9]	0.0	4.9
14	1360.7	1360.7	AB [f34-45]	D-AQSAPLPVYVEE	92	15.4[1.3]	-2.0	0.8
15	948.5	948.5	AB [f82-89]	V-FKIDALNE	77	10.3[1.3]	-1.0	0.6
16	1258.8	1258.8	AB [f75-85]	E-KTKIPAVFKID	77	18.6[1.7]	+2.0	0.8
17	928.5	928.6	AB [f56-62]	E--ILLQKWE	88	12.3[1.8]	0.0	5.6
18	3572.6	3572.8	A [f97-112]=[f115-128]	D-TDYKKYLLFCMENSAE=EQSLVCQCLVRTPE	65	22.8[1.0]	-2.0	0.4
19	2916.5	2917.5	AB [f138-162]	D-KALKALPMHIRLSFNPTQLEEQCHI	67	31.4[1.3]	+1.1	0.3
20	1686.0	1686.0	AB [f75-89]	E-KTKIPAVFKIDALNE	89	21.7[1.4]	+1.0	16.4
21	1557.9	1557.9	AB [f76-89]	K-TKIPAVFKIDALNE	100	20.2[1.3]	0.0	4.0
22	2825.5	2825.6	AB [f135-158]	E--KFDKALKALPMHIRLSFNPTQLEE	41	31.6[1.3]	+1.1	6.0
23	2696.5	2696.6	AB [f135-157]	E--KFDKALKALPMHIRLSFNPTQLE	50	31.6[1.4]	+2.1	3.2
24	2814.2	2814.4	AB [f105-129]	L--FCMENSAEPEQSLACQCLVRTPEVD	19	23.1[0.9]	-4.1	4.2
25	2553.2	2553.4	AB [f90-110]	E--NKVLVLDTDYKKYLLFCMENS	23	28.6[1.4]	0.0	0.9
26	3696.9	3696.0	AB [f12-45]	D-IQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEE	49	40.4[1.2]	0.0	1.2
27	4006.1	4006.2	AB [f1-38]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAP	22	44.2[1.2]	-1.0	1.7
28	4894.7	4895.6	AB [f1-45]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEE	12	53.6[1.2]	-2.0	3.9

[a] Theoretical mass derived from ExPASy, SIB, Switzerland ($[M+H]^+$).
[b] Peptide sequence starting with residue after which the BLP enzyme has cleaved. The "=" sign represents the disulfide bond.
[c] Total hydrophobicity was calculated according to the method of Bigelow 1967. Average hydrophobicity (H_{av}) = total hydrophobicity (Ht_{ot}) / total # residues in the peptide. The cysteine's in the disulphide bonds are not taken into account.
[d] The net charged was calculating according to the following formula:

$$Z = \sum_i N_i \frac{10^{pKa_i}}{10^{pH} + 10^{pKa_i}} - \sum_j N_j \frac{10^{pH}}{10^{pH} + 10^{pKa_j}}$$

where N_i are the number, and pKa_i the pKa values, of the N-terminus and the side chains of Arginine, Lysine, and Histidine. The *j*-index belong to the C-terminus and the Aspartic Acid, Glutamic Acid, Cysteine, Tyrosine amino acids. The disulfide bond was not taken into account for calculating the net charge.

These two peptides are relatively small (15 and 14 amino acids, respectively) and are more similar to the ‘supernatant’ peptides (average size: 9 ± 3 amino acids), than to the ’pellet‘ peptides (average size: 29 ± 7 amino acids).

Binding of specific peptides

To understand the effects that peptides may have on the aggregation kinetics of proteins it is important to first determine whether these peptides have a specific interaction with the proteins. The binding of peptides from the soluble peptide fraction to β-lactoglobulin was investigated at room temperature by SELDI-TOF-MS at four different pH values (figure 2). At pH 2.8 there is no significant binding of peptides to β-lactoglobulin as there is no difference between the mass spectra of the blank and the sample. At higher pH values peptides start to bind and at pH 7 five peptides were found to bind to β-lactoglobulin. They were identified as peptides A[f97-112]=[f115-128], AB[f1-45], AB[f135-157], AB[f135-158] and AB[f138-162]. The peptide AB[f1-45] appears mainly in the pellet fraction, but the small amount present in the supernatant fraction (table 1) binds to β-lactoglobulin. Interestingly, the peptides AB[f135-157] and AB[f135-158] still bind β-lactoglobulin at pH 9.3.

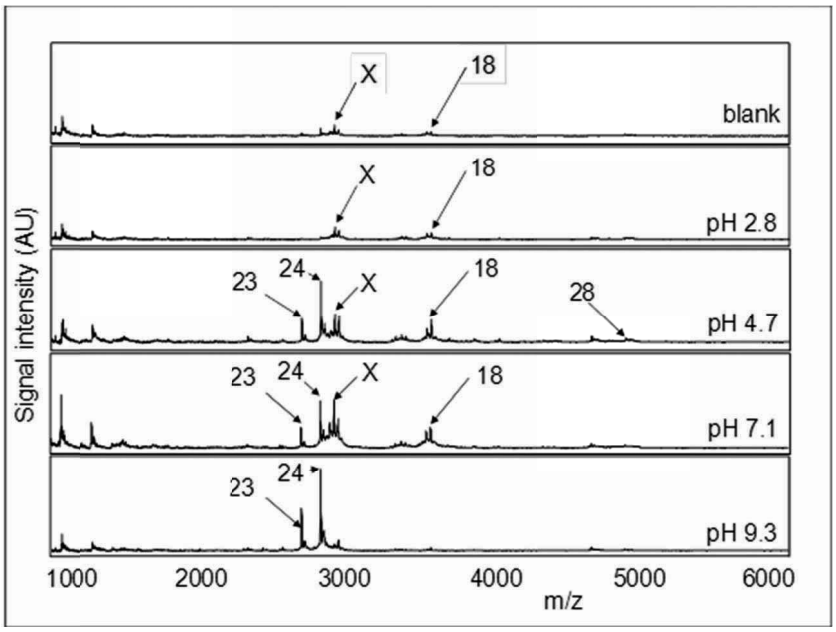

Figure 2. SELDI-TOF-MS spectra of binding of the peptides bound to β-lactoglobulin at different pH values. The assigned fragments bound to β-lactoglobulin are 23. AB[f135-157]; 24 AB[f135-158]; X (not observed in LC-MS). AB[f138-162]; 18. A[f97-112]=[f115-128]; 28. AB[f1-45]. The y-axis is re-scaled to a maximum of 1,000 AU for all spectra.

Effect of peptides on aggregation of β-lactoglobulin.

Identification of peptides in pellet of aggregated β-lactoglobulin

β-Lactoglobulin was heated (80 °C, 120 min, pH 7.0) in the absence and presence of peptides. A quantitative analysis of the heat-induced pellet showed that both the amounts of aggregated β-lactoglobulin and peptides are higher in the mixed system than in either the individual protein or the individual peptide solutions (figure 3).

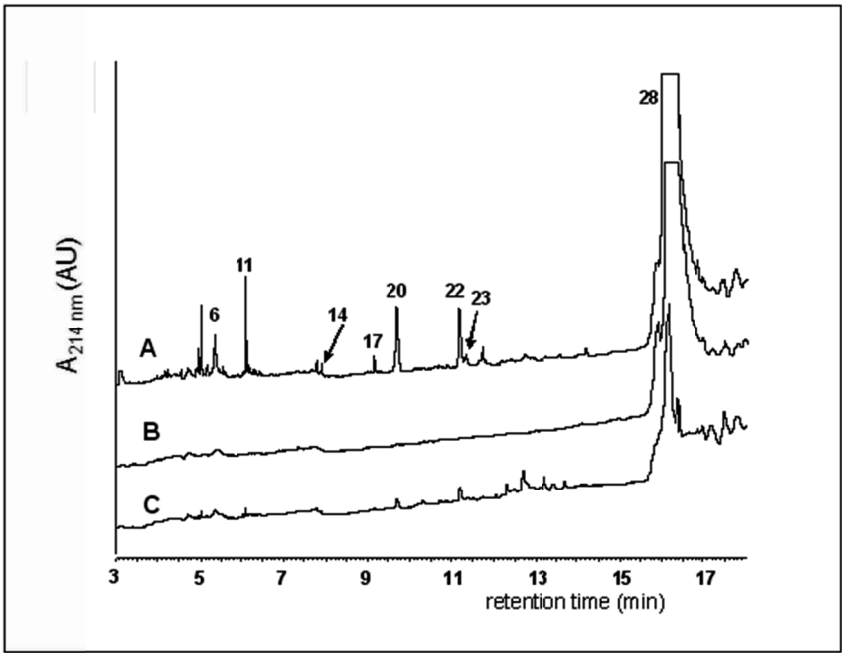

Figure 3. RP-UPLC chromatograms of the pellet formed after precipitation at pH 4.7 of heated and acid precipitated β-lactoglobulin + peptides (A), β-lactoglobulin (B) and peptides (C). MS based assignment of the peaks: 6. [f45-53]; 11. [f12-24]; 14. [f34-45]; 17. [f56-62]; 20. [f75-89]; 22. [f135-158]; 23. [f135-157]; 28. β-lactoglobulin + [f1-45]. The y-axis is re-scaled to a maximum of 0.05 AU.

From the 26 peptides present in the supernatant fraction eight were identified in the heat-induced pellet: Peaks 6. AB[f45-53]; 11. AB[f12-24]; 14. AB[f34-45]; 17. AB[f56-62]; 20. AB[f75-89]; 22. AB[f135-158]; 23. AB[f135-157]; 28. β-lactoglobulin + AB[f1-45].

Effect of peptides on the extent of heat-induced aggregation of β-lactoglobulin.

The remaining amount of native-like β-lactoglobulin as a result of heating at different temperatures for 120 min at pH 7.0 (C_t) was determined by size-exclusion chromatography and divided by the amount of unheated β-lactoglobulin (C_0) (figure 4). For pure β-lactoglobulin the amount of aggregated material increases with the temperature. This is due to the

increased initial reaction rate of denaturation/aggregation at higher temperatures.[27, 28]

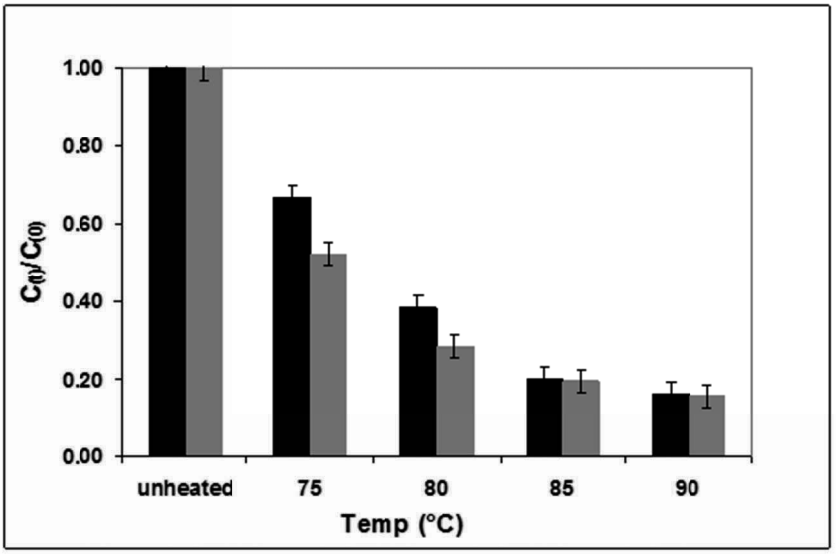

Figure 4. Relative amount of native-like β-lactoglobulin (C_t/C_0) in the absence (black) and presence (grey) of peptides after pH 4.7 precipitation (to remove aggregated β-lactoglobulin) as function of heating temperature for 120 min at pH 7.0.

The remaining amount of native-like β-lactoglobulin comprises soluble non-native monomers.[21] In the presence of peptides an additional aggregation of β-lactoglobulin (30-40%) is observed at 75-80 °C. At temperatures higher than 85 °C this effect becomes less pronounced. Since the pure β-lactoglobulin already showed more than 80% aggregation, the addition of peptides has no additional effect at these time-temperature combinations (120 min at T> 85 °C). Therefore, subsequent experiments were performed at 80 °C. At this temperature the presence of peptides resulted in approximately 30% more aggregated β-lactoglobulin in comparison to β-lactoglobulin without peptides (figure 5). This effect is most clear after 30 min heating.

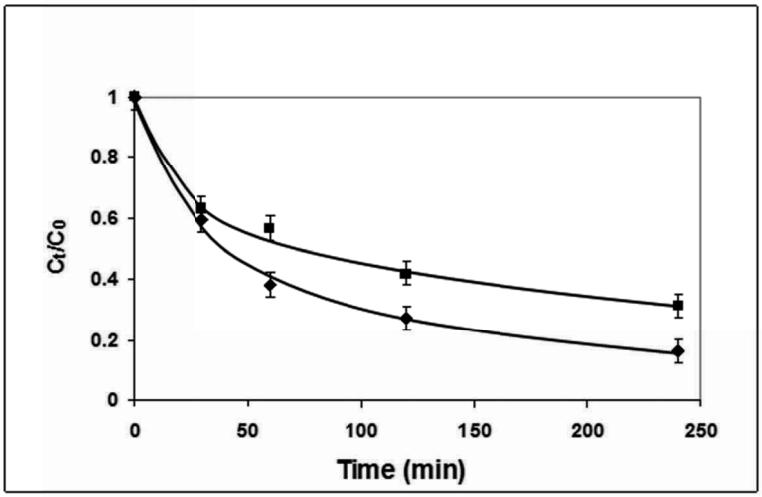

Figure 5. Relative amount of native-like β-lactoglobulin (C_t/C_0) in the absence (squares) and presence (diamonds) of peptides after pH 4.7 precipitation (to remove aggregated β-lactoglobulin) as function of heating time at 80°C, pH 7.0.

To investigate whether the presence of peptides influences the initial reaction rate of denaturating/aggregating β-lactoglobulin, different concentrations of β-lactoglobulin with and without peptides were heated. The apparent initial reaction rate (k) and the reaction order (n) for the denaturation/aggregation of a β-lactoglobulin solution in the presence of peptides do not differ significantly from β-lactoglobulin alone (table 2). The reaction order (n) was calculated to be 1.3 and 1.2 ± 0.2 for the β-lactoglobulin alone and β-lactoglobulin+peptide mixture, respectively.

Table 2. Initial reaction rate (k) of heat-induced aggregation of different concentrations β-lactoglobulin in the absence and presence of 2.5 mg/mL peptides for 120 min at 80 °C.

	k (s^{-1})	
	No peptides	**2.5 mg/mL peptides**
2.5 mg/mL β-LG	$1.9*10^{-4}$	$2.4*10^{-4}$
5.0 mg/mL β-LG	$2.7*10^{-4}$	$2.9*10^{-4}$
12.5 mg/mL β-LG	$3.5*10^{-4}$	$3.6*10^{-4}$
25.0 mg/mL β-LG	$4.5*10^{-4}$	$4.3*10^{-4}$

In addition, light scattering experiments were performed to describe the aggregates that were being formed. For this, the light scattering was

monitored during heating of pure protein solution, peptide solution and their mixtures (figure 6). At 20 °C the size (effective hydrodynamic diameter, $d_{H,eff}$) of β-lactoglobulin in the absence of peptides is 4.6 nm (no further data given). The signal intensity for all β-lactoglobulin concentrations in the presence of peptides increases but only a small increase in size (6.0 nm, no further data given) was observed. Upon increasing the temperature to 80 °C (indicated by T↑ in figure 6) the scattering intensity decreases initially due to the dissociation of the β-lactoglobulin dimers into monomers.

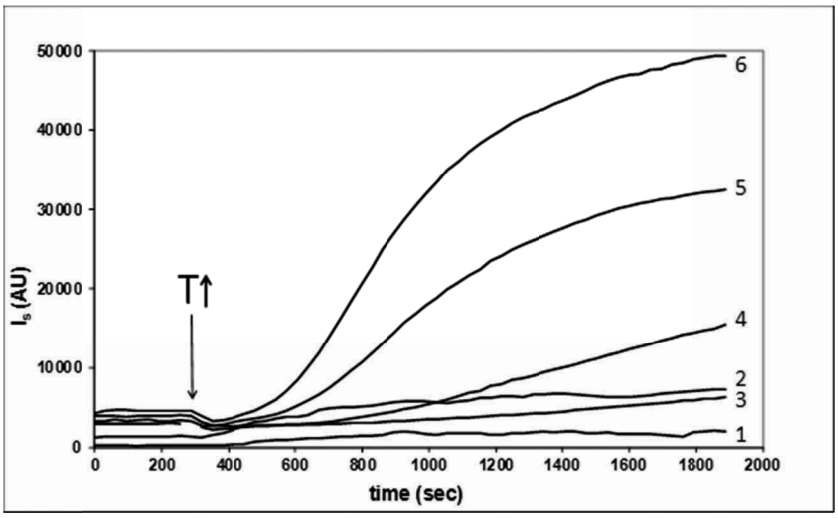

Figure 6. Intensity of scattered light versus time. 1: average of four β-lactoglobulin concentrations (5, 10, 20 and 25 mg/mL), 2: 5 mg/mL peptides from soluble fraction, 3-6: 5, 10, 20 and 25 mg/mL β-lactoglobulin in the presence of 5 mg/mL peptides. The arrow indicates the time where the temperature was changed from 20 to 80 °C.

For β-lactoglobulin alone no significant increase in the light scattering intensity upon heating was observed (<5 kAU). In contrast to the pure solutions, the mixtures showed a significant increase of the light scattering intensity (until 49 kAU) upon heating and the increase was proportional to the ratio β-lactoglobulin to peptides. The difference between pure and mixed systems clearly shows the effect of peptides on the aggregation of β-lactoglobulin.

In addition to light scattering intensity, the size of the aggregates was determined from the dominant peak in a distribution analysis of the particle

sizes. At 20 °C, the dominant contribution to the intensity of scattered light is still from the dimers and monomers. After increasing the temperature to 80 °C, the aggregates dominate the contribution to the light scattering intensity. For β-lactoglobulin alone, it was not possible to establish reliable values for the hydrodynamic radii. For β-lactoglobulin + peptide mixture the size of the aggregates was 31-32 nm. The aggregate size does not change strongly with heating time. This indicates that the aggregation process is one in which more and more aggregates of a well-defined average size are formed. The above described results show that in the presence of the peptides, aggregates formed at a higher rate/extent than in the absence of peptides, while the initial rate constant and reaction order of the aggregation is not different from β-lactoglobulin alone.

Effects of pH

To study the effects of pH on the peptide-induced aggregation of β-lactoglobulin, samples were heated at $T=T_d$ at different pH values. The denaturation temperature of β-lactoglobulin at pH < 4 is around 88.5 °C, while at pH > 4 it is 79.9 °C.[8] These denaturation temperatures were confirmed by our own DSC measurements (data not shown).

At pH 3-5 there are no clear differences between the aggregation in the presence and absence of peptides (figure 7). The high amount of aggregation (low C_t/C_0) is, besides heating effect, due to the iso-electric point of β-lactoglobulin (pI = 4.9)[29]. At pH values > 6 the amounts of aggregated β-lactoglobulin in the presence of peptides is 2-2.5 times higher than without peptides. At pH > 6 the protein has a negative net charge, while some peptides that were shown to bind to β-lactoglobulin at room temperature, such as AB[f135-158/157], have a positive net charge (table 1). The effect of pH on the aggregation follows the effect of pH on the binding of peptides to β-lactoglobulin at room temperature, as determined by SELDI-TOF-MS.

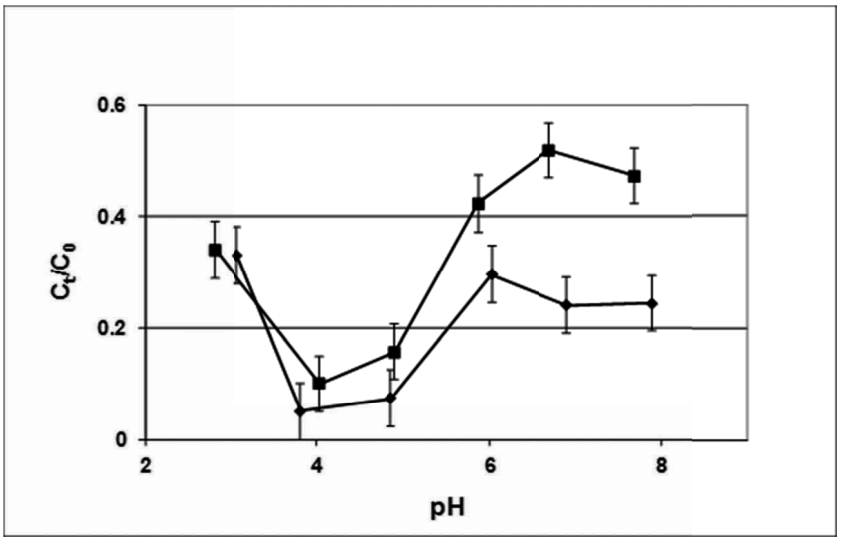

Figure 7. Relative amount of native-like β-lactoglobulin in the absence (square) and presence (diamonds) of peptides as function of pH, after heating for 120 min at T_d.

Effect on the denaturation temperature and conformation of β-lactoglobulin

DSC experiments show that the denaturation temperature of β-lactoglobulin is decreased only by 0.4 °C in the presence of peptides (data not shown). This indicates that the peptides do not have a major effect on the denaturation temperature. Nevertheless, the presence of the peptides reduced the enthalpy of unfolding (ΔH) of β-lactoglobulin by a factor two. This change in unfolding enthalpy is not due to changes in secondary structure as determined by far-UV CD (figure 8A). The estimated secondary structure of β-lactoglobulin in the presence of peptides, after subtraction of the peptide spectrum (predominantly random coil), consisted of 16% α-helix, 34% β-sheet, 34% random coil and 16% of β-turn structure and did not differ significantly from that of β-lactoglobulin alone: 17% α-helix, 32% β-sheet, 34% random coil (R.C.) and 16% β-turn, respectively (see inset figure 8A).

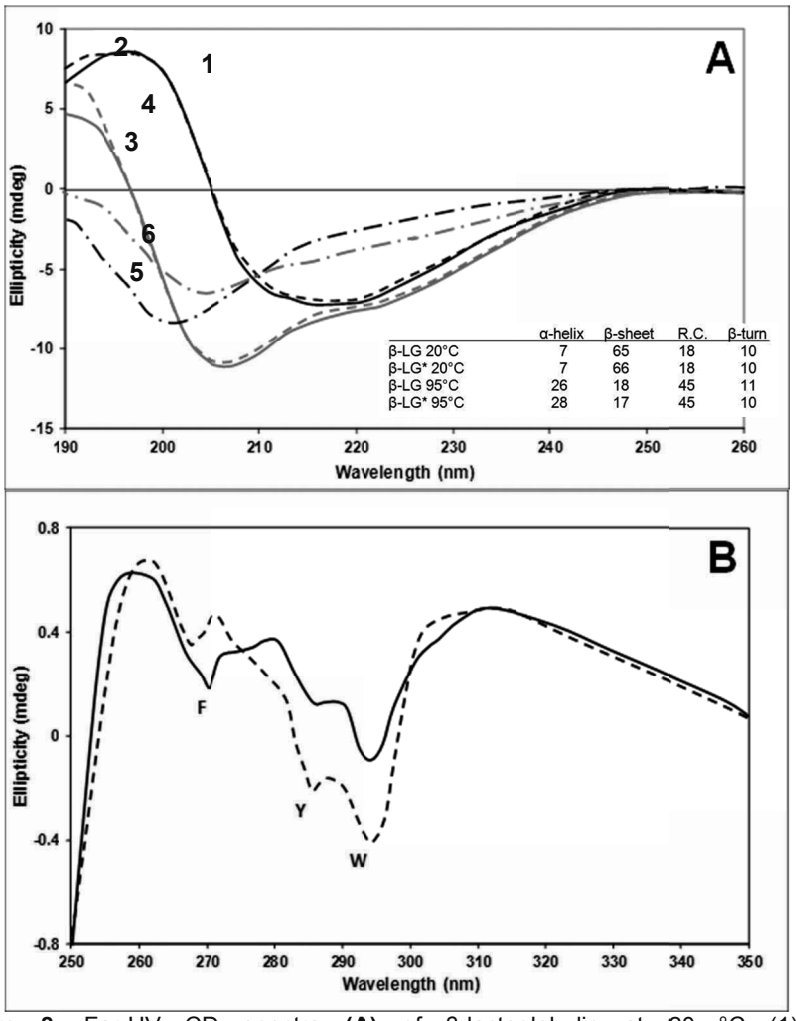

Figure 8. Far-UV CD spectra **(A)** of β-lactoglobulin at 20 °C (1); β-lactoglobulin+peptides at 20 °C (2); β-lactoglobulin at 95 °C (3); β-lactoglobulin+peptides at 95 °C (4); peptides alone at 20 °C (5) and 95°C (6). Inset: Propensities of secondary structures elements of β-lactoglobulin in the absence (β-LG) and presence (β-LG*) of peptides. Near-UV CD spectra **(B)** of β-lactoglobulin in the absence (solid line) and presence (dashed line) of peptides at 20 °C.

The values are in fairly good agreement with literature.[30-32] At 95 °C (maximum unfolding) the propensities of β-turn structure and random coil were increased at the expense of β-sheet structure for both situations (see inset figure 8A). However, the propensities of α-helix structure at this temperature remains almost the same (16-18%) and is in agreement with

literature.[32] Figure 8B shows the near-UV CD spectrum (250-350 nm). Ellipticities in this region originate mainly from phenylalanine, tryptophan and tyrosine. Since the peptides did not show any intensity in the near-UV spectra, changes in the spectra of β-lactoglobulin in the presence of peptides can be interpreted as changes in the tertiary structure of the protein. The ellipticity of the phenylalanine residues at 270 nm in the presence of peptides has decreased in intensity and moved towards lower wavelength by 2-3 nm indicating that the local environment of this residue changes to a more polar character.[33] The presence of peptides increased the ellipticities of the residues tryptophan and tyrosine of β-lactoglobulin at 295 and 285 nm. This suggests that the residues in the presence of peptides have less mobility, which could be attributed by the binding of (some) peptides in the proximity of these residues. At 90°C for β-lactoglobulin with and without peptides no ellipticity was observed indicating that no significant tertiary structure was present (no further data shown).

Discussion

Shared characteristics of β-lactoglobulin binding peptides

Using the known sequences of the peptides in the hydrolysate, a principal component analysis (PCA) of their properties was performed. The first aim was to identify whether certain groups of peptides shared specific characteristics. The second aim was to see if these shared characteristics (if present) could be related to properties of these peptides, such as solubility or the binding to non-heated or heated β-lactoglobulin. For the first aim, PCA plots were made based on either the total number of, or the distribution (asymmetry and degree of blockiness) of charge and hydrophobicity (figure 9A-C respectively). The solubility of peptides (expressed as the proportion present in the supernatant, table 1) correlates inversely with both the length and the total hydrophobicity (figure 9A). There is also a correlation between the length and the total amount of positive and negative charges. These correlations were also observed in direct plots of length versus either of these parameters (R^2>0.65, not shown).

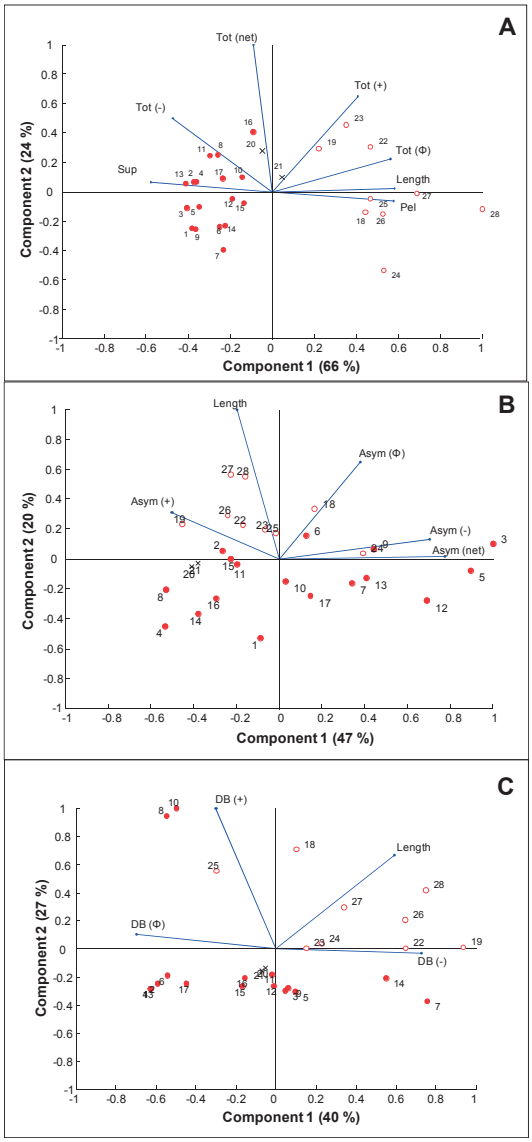

Figure 9: Biplots for the principal component analysis of the 28 peptides identified in the hydrolysate using the total values (A), asymmetry (B) or degree of blockiness (C) values for the charge and hydrophobicity, peptides 1-17(●), 18,19,22-28(○) and 20-21 (✕).

This indicates that amino acids are distributed rather homogeneously over the primary sequences of the peptides. As a result there are no peptides with very an unique (e.g. very high positive charge) composition. This is

confirmed by the fact that the asymmetry and degree of blockiness (of charge and hydrophobicity) are typically perpendicular to the contributions of concentration in the pellet and length. This means that the distribution of the peptides over pellet and supernatant does not correlate with the charge distributions. A *K-means* clustering routine showed that the peptides could be differentiated in two groups. Group 1 contained the peptides 1-17 + 21, 22. These peptides have a high solubility (as seen from their high proportion in the supernatant). Group two contained peptides 18,19 + 23-28, which have a lower solubility. For both groups of peptides, the average values of all properties were calculated (and their corresponding standard deviations, table 3). There is a significant difference ($p<0.05$) between these two groups with respect to the solubilities, lengths and total hydrophobicities of the constituent peptides.

Protein-peptide interactions at room temperature.

Based on the above and previous work[17], the supernatant fraction was chosen for studies on the interaction of peptides in this fraction with intact β-lactoglobulin.

The peptides (A[97-112]=[115-128], AB[f1-45], AB[f135-157], AB[f135-158] and AB[f138-162]) were able to bind to β-lactoglobulin at room temperature. Peptides AB[f135-157/158] have been reported previously to bind to β-lactoglobulin.[15-17, 34] Binding of peptides AB[135-157] and AB[135-158] at pH 9.3 can be explained by the negative net charge (-14.7) of β-lactoglobulin, and the attractive positive net charge of +1.5 and +0.5 of the peptides, respectively. The other peptides present possess a repulsive negative net charge at this pH. It has further been shown[17] that the affinity of AB[f135-158] for β-lactoglobulin in the presence of increasing amounts of surfactant Triton X-100 is higher than those of other peptides, e.g. AB[f1-45]. These observations indicate that the binding of peptides AB[f135-157] and AB[f135-158] to β-lactoglobulin is based on electrostatic binding, via positive charges on the peptides.

Table 3. Characteristics of different populations of peptides (average ± standard deviations) identified based on their properties (solubility, binding at room temperature, binding during heating).

	% in sup[a]	% in pel[b]	Length[c]	Tot (net)[d]	Tot (+)[e]	Tot (-)[f]	Asym (net)[g]
Supernatant fraction	***91.2 ± 9.3***	***8.1 ± 9.2***	***9.3 ± 3.7***	-0.5 ± 1.3	2.1 ± 1.0	-2.6 ± 0.9	0.6 ± 0.5
pellet fraction	***38.7 ± 20.5***	***60.3 ±20.5***	***29.4 ± 8.0***	-0.9 ± 1.9	3.7 ± 1.0	-4.6 ± 1.2	0.5 ± 0.3
Non-binding	80.3 ± 26.3	19.0 ± 26.2	12.8 ± 8.0	-1.2 ± 1.0	***2.2 ± 1.0***	-3.0 ± 1.2	0.6 ± 0.4
Binding	47.0 ± 22.3	52.0 ± 22.3	29.4 ± 9.1	-1.6 ± 0.5	***4.4 ± 0.5***	-4.4 ±1.5	0.6 ± 0.3
Non-aggregating	77.6 ± 30.6	21.6 ± 30.4	13.3 ± 10.7	-1.1 ± 1.3	2.0 ± 0.9	-3.1 ± 1.3	0.5 ± 0.4
Aggregating	70.7 ± 28.4	28.5 ± 28.3	17.3 ± 11.2	0.1 ± 1.6	3.2 ± 1.4	3.1 ± 1.4	0.7 ± 0.2

	Asym (+)[h]	Asym (-)[i]	DB (+)[j]	DB (-)[k]	Tot (Φ)[l]	Asym (Φ)[m]	DB (Φ)[n]
Supernatant fraction	0.4 ± 0.7	0.6 ± 0.4	0.1 ± 0.3	0.1 ± 0.3	***3.0 ± 1.9***	0.6 ± 0.4	0.5 ± 0.3
pellet fraction	0.5 ± 0.7	0.4 ± 0.4	0.1 ± 0.2	0.3 ± 0.4	***11.1 ± 3.4***	0.1 ± 0.1	0.3 ± 0.2
Non-binding	0.6 ± 0.5	0.6 ± 0.4	0.1 ± 0.3	0.1 ± 0.3	***4.4 ± 3.7***	0.5 ± 0.4	0.5 ± 0.3
Binding	0.6 ± 0.4	0.4 ± 0.4	0.1 ± 0.2	0.5 ± 0.4	***11.5 ± 3.5***	0.1 ± 0.1	0.3 ± 0.12
Non-aggregating	0.6 ± 0.5	0.5 ± 0.4	0.2 ± 0.4	0.1 ± 0.2	4.3 ± 4.4	0.5 ± 0.4	0.5 ± 0.4
Aggregating	0.7 ± 0.4	0.6 ± 0.4	0.0 ± 0.0	0.2 ± 0.4	7.0 ± 4.6	0.4 ± 0.4	0.4 ± 0.3

* Marked areas indicate those properties where significant differences were observed.
[a] percentage in supernatant; [b] percentage in pellet; [c] length of peptide; [d] total net charge; [e] total positively charged residues; [f] total negatively charged residues; [g] asymmetry of net charged residues; [h] asymmetry of positively charged residue; [I] asymmetry of negatively charged residue; [j] degree of blockiness of positively charged residues; [k] degree of blockiness of negatively charged residues; [l] total hydrophobicity; [m] asymmetry of hydrophobic residues; [n] degree of blockiness of hydrophobic residues

Calculation of the average characteristics (table 3) shows two clear differences between peptides that bind and those that do not: The total hydrophobicity and the total positive charge are both higher for the bound peptides than for the non-bound peptides. This confirms the hypothesis that the opposite charge between protein and (areas of the) peptide determines the binding, which has been reported previously.[35]

Protein-peptide interaction at elevated temperature.

In the acid-precipitated pellet of heat-induced aggregated β-lactoglobulin eight peptides were identified (figure 3). Two of these peptides (AB[135-157] and AB[135-158]) also bind to β-lactoglobulin at room temperature (figure 2). In addition, six new peptides were identified that did not bind to the non-heated protein. Since only 6 of the 26 peptides are observed in the heat-induced pellet, the binding is likely due to specific interactions. As they did not bind to the non-heated protein, their binding is probably related to the unfolding of β-lactoglobulin. The concentrations of these peptides in the pellet were quantified by re-dissolving the pellet in the original volume (table 4).

Table 4. Concentrations of peptides in the peptide supernatant fraction and pellet of heat-induced β-lactoglobulin.

		Conc. of peptides (μM)	
Peak no. table 1	**Peptide**	**In supernatant fraction**	**In pellet[a)]**
6	AB [f45-53]	66.1	46.2 [0.7][b)]
11	AB [f12-24]	21.4	19.1 [0.9]
14	AB [f34-45]	5.9	3.7 [0.6]
17	AB [f56-62]	33.8	3.9 [0.1]
20	AB [f75-89]	94.1	47.3 [0.5]
22	AB [f135-158]	15.8	17.3 [1.1]
23	AB [f135-157]	12.4	2.5 [0.2]
28	AB [f1-45]	5.0	n.q [-]

n.q. = not quantified, because peptide elutes at same time as β-lactoglobulin
[a)] = the pellet was re-dissolved in 8 M urea in the original volume
[b)] = ratio of peptides in the pellet and supernatant fraction

It was calculated that the peptides AB[f45-53], AB[f12-24], AB[f34-45] are for > 70% present in the heat-induced pellet. Despite the fact that only a few peptides are found in the pellet, an analysis of their properties (table 3) does not indicate at

any significant difference from the other 20 peptides. Also the principal component analysis (figure 8) does not show any clear clustering of these peptides, confirming the absence of clear, unique shared properties.

Effect of peptides on the aggregation and conformation of β-lactoglobulin.

Heating of β-lactoglobulin not only showed the presence of peptides, but also an increased amount of aggregated β-lactoglobulin. This effect occurred only at prolonged heating times (figure 5). The presence of peptides also affects the size of the aggregates that are formed. At 20 °C the size of β-lactoglobulin increases from 4.6 nm to 6.0 nm in the presence of peptides, indicating binding of peptides to β-lactoglobulin. After heating at 80 °C, β-lactoglobulin clearly forms aggregates in the presence of peptides, while in the absence of peptides no such aggregation is observed. The decrease of native-like β-lactoglobulin in the absence of peptides (figure 4) is due to unfolding of the protein which is detectable only after precipitation at pH 4.7.

Surprisingly, we observed a factor of two reduction in the enthalpy of unfolding (ΔH) of β-lactoglobulin due to the presence of peptides. This could suggest two different mechanisms happening; 1) the protein is already partially unfolded due to the binding of peptides, 2) binding of peptides upon unfolding of β-lactoglobulin causes an endothermic reaction and consequently, decreases the exothermic effect of unfolding of β-lactoglobulin. Only during the second mechanism the peptides will most probably interact with heat-induced exposed hydrophobic regions, but will not initially induce unfolding.

Binding of the peptides did not have any effect on the secondary structure of β-lactoglobulin before and after heating (figure 8A). On the other hand, the presence of peptides changed the local environment of the Trp and Tyr residues (figure 8B) to less polar. This could indicate that some peptides bind in the proximity of these residues and hence, affect the denaturation/aggregation. The binding of these peptides prior to unfolding is not expected to result in a change of ΔH. The ΔH value typically depends on the difference in exposure of buried amino acids to the

solvent upon heating. If peptides are already bound to the protein prior to unfolding, there should be no contribution to ΔH.

Based on the above and the observation that more peptides bind to the unfolded protein than to the protein at room temperature, it seems that the decrease in ΔH should be attributed to the endothermic effect of binding of extra peptides during the unfolding of β-lactoglobulin. Further studies with pure (synthesized) peptides will be carried out to investigate the relevance of binding of these peptides to proteins in relation to aggregation and gelation of protein systems.

Conclusion

Only part of the peptides present in the experiments bind to β-lactoglobulin, either at room temperature or upon unfolding. For these sets of peptides no unique shared properties could be found, although at room temperature binding can be explained by electrostatic interactions. The presence of the peptides caused a 50% decrease in denaturation enthalpy, while no change in secondary structure or denaturation temperature was observed.

Binding of peptides to β-lactoglobulin increased the amount of aggregated β-lactoglobulin at pH > 6. Hence, it is evident that the addition of specific peptides can alter the aggregation properties.

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Chapter 4

Protein-peptide interaction: Study of heat-induced aggregation and gelation of β-lactoglobulin in the presence of two peptides from its own hydrolysate

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H.A. Kosters, P.A. Wierenga, R. de Vries, H. Gruppen. Protein-peptide interaction: Study of heat-induced aggregation and gelation of β-lactoglobulin in the presence of two peptides from its own hydrolysate.

Abstract

Two peptides, [f135-158] and [f135-162]-SH, were used to study the binding of the peptides, as well as the subsequent effects on aggregation and gelation of intact β-lactoglobulin. The interaction between peptides and protein was observed in the solubility behaviour of the intact protein at different pH values. The peptides showed minimal solubility between pH 7 and 9. For the protein-peptide mixture the solubility was minimal between pH 5 and 9. Using SELDI-TOF-MS, it was found that [f135-158] bound equally to β-lactoglobulin, ovalbumin and lysozyme, indicating that the binding mechanism does not depend to specific molecular properties, or even the net charge of the protein. The mass intensity observed for peptide [f135-162]-SH decreased in the order ovalbumin>β-lactoglobulin> lysozyme. The number of free sulfhydryl groups in the proteins decreases in the same order, indicating an additional contribution of covalent interactions. While heating of either pure protein or pure peptide solutions did not result in aggregation, the mixtures did show heat-induced aggregation. Furthermore, the aggregation in the presence of [f135-162]-SH is 10 times higher than in the presence of [f135-158], which shows the additional influence of the free sulfhydryl group. A clear difference between these two peptides was also observed in the gel strength of heat-induced gels. The replacement of additional β-lactoglobulin by [f135-158] resulted in a decrease in gel strength, while replacement by peptide [f135-162]-SH increased the gel strength.

Introduction

In several studies it has been shown that the aggregation and gelation behavior of proteins is affected by enzymatic hydrolysis. In all cases significant changes in the rheological properties and the appearance of gels were found.[1-4] Depending on the enzyme used, the degree of hydrolysis and the conditions of gelation either an increased or a decreased gel strength was observed. Control over gel properties can thus be obtained by controlling the hydrolysate properties. It was further observed that under some conditions aggregation and gelation started during hydrolysis.[1, 5, 6] This shows that the peptides formed can associate to form aggregates. If the peptides can interact with each other, they might also interact with intact protein. From these studies the idea was formed that the addition of hydrolysates to intact protein solutions could be used to change the heat-induced aggregation and gelation of intact protein.

Previous studies indeed showed that aggregation of intact WPI was induced by the addition of whey protein isolate (WPI) hydrolysate.[7] Similarly, the presence of a soluble fraction of a β-lactoglobulin hydrolysate increased the amount of intact β-lactoglobulin aggregates formed upon heating.[8] At the same time, the denaturation enthalpy of the intact β-lactoglobulin was decreased by 50% due to the presence of this hydrolysate fraction.[8] This further supported the idea that the peptides affected the heat-induced aggregation and gelation of proteins by binding to the protein. In the same study, the binding of peptides from the soluble β-lactoglobulin hydrolysate to intact β-lactoglobulin was studied by SELDI-TOF-MS. It was shown that five from the twenty-eight peptides detected in the hydrolysate bound to the intact protein by non-covalent interactions.[9] Consequently, it was hypothesized that the binding of certain specific peptides to the intact proteins determines the observed effects on (heat-induced) aggregation and gelation. In the present study, two of the β-lactoglobulin derived peptides that were found to bind to β-lactoglobulin, [f135-158] and [f135-162]-SH, are used to study effects on aggregation and gelation of intact β-lactoglobulin. The peptide β-lactoglobulin [f135-158] was selected because it was previously found to act as an protein

aggregating peptide during β-lactoglobulin hydrolysis by BLP.[7, 10] Furthermore, it was found to bind to β-lactoglobulin and to be present as one of the dominant peptides in a heat induced pellet of a mixture of β-lactoglobulin and its soluble BLP hydrolysate.[9] The other peptide, β-lactoglobulin [f135-162]-SH, is chosen since it is highly similar to the first peptide, but it contains one free cysteine residue and allows to distinguish the additional effect of sulfhydryl exchange reactions. The effects of these peptides on the rheological properties of heat-induced gels are studied using the electrowetting technique.[11] In this technique, smaller sample volumes are needed as compared to traditional Couette rheometry (3 μL and 1,000 μL respectively).

Material and Methods

β-Lactoglobulin was isolated from fresh bovine milk, purified and characterized as described previously.[9] Lysozyme and L-cysteine were obtained from Sigma (St. Louis, MO, USA), and ovalbumin was purified as described previously.[12] Three peptides based on sequences [f135-158], [f135-162]-SH and [f159-162]-SH from β-lactoglobulin were chemically synthesized by Biomatik (Wilmington, DE, USA). The main characteristics of these peptides are given in table 1. The purity of each peptide was found to be >95%, based on the RP-UPLC peak area at 214 nm compared to the total area (data not shown). Silicon oil SIL 180 (Thermo Fischer Scientific, Waltham, MA, USA) was filtered over a 0.22 μm filter (Schleicher & Schuell, Dassel, Germany) before use. α-Cyano-hydroxycinnaminic acid (CHCA) was obtained from Bruker Daltonics (Bremen, Germany). All other chemicals were of analytical grade.

Blocking of reactive sulfhydryl group

The reactive sulfhydryl group in peptide [f135-162]-SH was blocked by addition of 1.5 mL of a 10mM solution of *N*-ethylmaleimide (NEM) in milliQ water to 1.5 mL of a 20 mg/mL (6mM) peptide [f135-162]-SH solution.[13] The mixture was adjusted to pH 7.0 with 0.1 M NaOH and incubated 3 hours at room temperature. After

incubation the excess of NEM was removed by dialysis using a Float-A-Lyzer G2 membrane with a cut-off of 500-1000 Dalton (Spectrum Laboratories, Rancho Dominquez, CA, USA) overnight at 4 °C and subsequently freeze dried. Blocking of the free sulfhydryl group was confirmed by the Ellman's reagent assay as described previously.[12] The modified peptide [f135-162]-SH is further denoted as [f135-162]-SX.

Table 1. Characteristics of the proteins and peptides used in this study

Name	Exp. Mass (Da)	Theor.Mass (Da)	$H\Phi_{tot}/[H\Phi_{av}]$** (kcal/[kcal*res^{-1}])	Net charge at pH 7.0	*pI*	# SS/ [SH]
[f135-158]	2827.0	2827.3	31.6[1.31]	+1.1	8.5	0[0]
[f135-162]-SH	3308.0	3308.9	35.5[1.27]	+1.1	8.5	0[1]
[f135-162]-SX	3433.0	3434.0	unknown	+1.1	8.5	0[0]
[f159-162]-SH	499.6	499.3	4.0[0.99]	0	7.2	0[1]
β-Lactoglobulin	n.d.*	18306	196[1.21]	-8.3	5.2	2[1]
Ovalbumin	n.d.*	42750	431[1.12]	-11.6	5.2	1[4]
Lysozyme	n.d.*	14313	127[0.98]	+7.7	9.3	4[0]

* not determined
** Total and average hydrophobicity was calculated according to method of Bigelow (1967).[14]

Solubility profiles

The solubility of β-lactoglobulin in the presence and absence of peptides [f135-158] and [f135-162]-SH were studied at pH 3-11. The peptides were dissolved in water at 5.0 mg/mL at pH 3 in the presence or absence of 5.0 mg/mL β-lactoglobulin. The pH was adjusted by addition of 0.5 M NaOH to 1 mL of the solution in a quartz cuvette, after which the pH was measured. The transmission of the sample mixtures at 600 nm was used as a measure for the solubility and corrected for the dilution due to the amount of NaOH added.

Circular Dichroism Spectroscopy

Far-UV circular dichroism experiments were carried out as described previously.[12] Typically, 1.0 mg/mL of peptide was dissolved in 50 mM sodium phosphate buffer, pH 7.0. For far-UV experiments the samples were diluted 10 times in the same buffer prior to the CD experiments and spectra were recorded from 195-260 nm at 20 and 90 °C (total unfolding). The spectra were analysed for

secondary structure elements using the standard CDNN program[15], using 33 reference spectra from structurally well-characterized globular proteins.

Surface Enhanced Laser Desorption Ionization Time Of Flight Mass Spectrometry

SELDI-TOF-MS experiments were performed as described earlier.[9] β-Lactoglobulin, ovalbumin and lysozyme were covalently bound to separate spots on the ProteinChip PS-20. A fourth spot was left empty (but blocked with ethanolamine) to determine the non-specific binding of peptides to the ProteinChip. Experiments were carried out in duplicate. The peptides were dissolved to a final concentration of 5.0 mg/mL in phosphate buffered saline solution (PBS buffer) containing 0.1% (v/v) Triton X-100 (to reduce non-specific binding), and the pH was adjusted to pH 7.4 by adding 0.1 M HCl, or 0.1 M NaOH. Aliquots (5 µL) of this solution were added to the ProteinChip PS-20 surface and incubated in a humidity chamber overnight at 4 °C. After incubation, the ProteinChip PS-20 was subsequently washed three times by incubation for 10 minutes in an excess of PBS buffer, pH 7.4 containing 0.1% (v/v) Triton X-100. The ProteinChip PS-20 was washed in excess PBS buffer, pH 7.4 and in milliQ water prior to being air-dried. Before the MS experiment, 0.5 µL of CHCA solution was added, air-dried and repeated again. The ProteinChip PS-20 was read at a laser intensity of 1,000 AU.

Dynamic Light Scattering (DLS)

Dynamic light scattering measurements were performed using a Zetasizer Nano ZS (Malvern Instruments, Malvern, U.K.) equipped with a 4 mW HeNe laser beam with a wavelength of 633 nm, at a scattering angle of 173°. The intensity of scattered light (I_s) and the hydrodynamic diameter (d_h) of the monomers, peptides and aggregates were measured for a solution of 5.0 mg/mL β-lactoglobulin in 50 mM sodium phosphate buffer, pH 7.0 containing 0, 0.1, 0.5, 1.0, 2.5 and 5.0 mg/mL peptides. These concentrations correspond to molar protein:peptide ratios, which are denoted in table 2. Typically, 50 µL of sample was covered with 50 µL paraffin oil to prevent water evaporation from the sample and analyzed for 130 or

300 seconds at 20 °C. Subsequently, the sample was removed from the Zetasizer Nano and the temperature was set to 80 °C. When the Zetasizer Nano had reached the temperature set the sample was placed back into the Zetasizer Nano and analyzed for at least 25 min.

Table 2. Calculated molar protein/peptide ratio's based on 5.0 mg/mL β-lactoglobulin solution and different concentrations peptide solution.

Concentration peptide mg/mL	Molar ratio protein:peptide		
	[f135-158]	[f135-162]-SH	[f135-162]-SX
0	0	0	0
0.1	10:1	10:1	10:1
0.5	10:7	10:6	10:5
1.0	10:13	10:11	10:11
2.5	10:32	10:28	10:27
5.0	10:66	10:56	10:53

Surface tension measurements

The interfacial tension at 20 ºC between 50 mM sodium phosphate buffer, pH 7.0 and the silicone oil was determined using the Automated Drop Tensiometer (ADT; I.T. Concept, Longessaigne, France). A pendant droplet of 7 µL containing the buffer was formed at the tip of a syringe needle placed in a cuvette containing the silicon oil. The surface tension, γ [mN/m] was calculated by the software of IT Concept.

Electrowetting (EW)

Preparation of the dielectric support

Dielectric supports were prepared as described previously.[11] A glass slide with a conductive indium tin oxide (ITO) layer was cleaned with milliQ water, 70% (v/v) ethanol and n-heptan, consecutively for 15 min in an ultrasonic bath for each step. Subsequently, the glass slide was dip-coated into a 6% (v/v) amorphous Teflon AF (1600) in perfluorinated solvent FC 75 (DuPont, Wilmington, DE, USA), retracted from this solvent at a speed of 15 cm/min and dried for 10 min at 110 °C in vacuum. The dip-coat and drying step were repeated once and finally the support

was dried first at for 10 min 160 °C and then at for 30 min 340 °C (again in vacuum).

Preparation of gelled droplets

For the first experiments stock solutions of 120 mg/mL β-lactoglobulin in 50 mM sodium phosphate buffer, pH 7.0 were prepared in the presence or absence of 2.5 mg/mL peptide or 2.5 mg/mL β-lactoglobulin in the case of the blank. The stock solution was further diluted to reach concentrations ranging from 4 - 120 mg/mL β-lactoglobulin solution in the same buffer solution. In this way the total concentration was varied, while the protein/peptide mass ratio of 48:1 is constant. For the second experiment the peptide concentration was varied. Therefore, a β-lactoglobulin solution in 50 mM sodium phosphate buffer, pH 7.0 was mixed with the peptide solution to final peptide concentrations ranging from 0-50 mg/mL in 100 mg/mL β-lactoglobulin solution. For the blank, β-lactoglobulin was added to the β-lactoglobulin solution in the same concentrations.

Samples were degassed for 10 min. From each solution several gelled droplets were made by adding a droplet of 3 µL to a Teflon vial containing 1 mL silicone oil. Samples were heated for 45 min in a preheated oven at 80 °C. After heating, the samples were allowed to cool down for 15 min. at room temperature. The droplets were transferred from the Teflon vial onto the dielectric support, which was already placed in a glass container containing 10 mL of silicone oil at room temperature.

Micro-rheology

The complex modulus of heated protein solutions was determined using an electrowetting (EW) setup (figure 1).[16] An electrode (Wolfram wire sharpened by electrolysis) was inserted in the top of the droplet. An oscillating voltage was then applied over the electrode in the droplet and the electrode in the dielectric support. The AC voltage (sine wave, frequency f_c = 1 kHz) was generated by a function generator (Hewlett Packard 33120A, Palo Alto, CA, USA). The peak-peak voltage was varied between 0 and 30 V (yielding a root mean square voltage between 0 and 21.21 V) using amplitude modulation with a sinusoidal wave with a frequency of 10 mHz, The contact angle ($cos\ \theta$) as function of applied voltage (U) was

monitored by an optical contact angle measuring system G10 (Krüss, Hamburg, Germany) and calculated by Drop Shape Analysis (DSA) software, version 1.90.0.14 (Krüss). Each droplet was measured during two time periods (200 s) of the amplitude modulation, at two different places on the support. In this way, at least four sets of data were obtained for each sample.

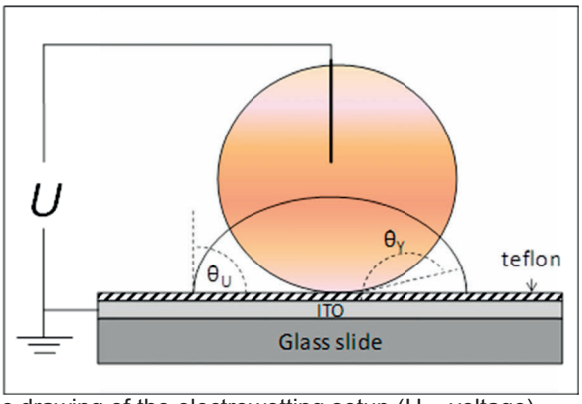

Figure 1. Schematic drawing of the electrowetting setup (U = voltage).

Calculations

To determine the elastic modulus of the gelled droplets first the thickness of the Teflon layer on the dielectric support was determined. For this determination, the change in contact angle (θ [Rad]) of a 50 mM sodium phosphate buffer droplet, pH 7.0 was measured as a function of the applied voltage (U [V]). The layer thickness was inferred from the classic electrowetting equation[17]:

$$\eta \equiv \frac{\varepsilon\varepsilon_0}{2d\gamma} \times U^2 = \cos(\theta) - \cos(\theta_Y) \quad (1)$$

Where η is the dimensionless electrowetting number, θ is the measured contact angle in the presence of the electric field, and θ_Y is Young's angle (contact angle in the absence of the electric field). Furthermore, ε=2.1 is the relative dielectric permittivity of the Teflon layer, ε_0 the dielectric permittivity of vacuum, d the Teflon layer thickness [m], and γ the interfacial tension [N/m] of the buffer solution in

silicone oil at 20 °C, that was determined independently and found to be equal to $1.34*10^{-2}$ N/m.

Electrowetting of non-heated protein droplets, and non-gelled heated protein droplets can also be analyzed with the traditional electrowetting equation, Eq. (1). For a known thickness *d* of the Teflon layer such an analysis then gives the surface tension of the protein-coated oil-buffer interface. For gelled droplets, an extension (Eq. (2)) of the electrowetting equation has been worked out by Banpurkar[11]:

$$\eta = \cos(\theta) - \cos(\theta_Y) + \frac{8GR_0}{3\gamma\pi} \times H(\cos(\theta), \cos(\theta_Y)) \quad (2)$$

With

$$H(X,Y) = \left(\frac{4^{1\backslash 6}(3+X)(1+X)^{3/2}}{(1-X)^{4/3}(2+X)^{1/6}} \right) \times \left(1 - \frac{(1-X)(2+X)^{1/2}(1+Y)^{3/2}}{(1-Y)(2+Y)^{1/2}(1+X)^{3/2}} \right) \quad (3)$$

To determine the elastic shear modulus of gelled droplets at higher concentrations, a value for the surface tension was estimated from low concentration electrowetting data (concentrations < 4% w/v). The surface tension value found from the electrowetting data is independent of protein/peptide concentration for concentrations <4% (w/v). Hence, it is assumed that this same value can also be used for analyzing data at higher protein/peptide concentrations. This leaves the complex modulus G as the only adjustable parameter when using Eq. (3) to fit data at higher protein/peptide concentrations. It has been shown that the results obtained for the elastic modulus with this technique are comparable to those obtained with traditional bulk rheology.[11]

Results and Discussion

Secondary structure of peptides

The relative amounts of secondary structure elements in the model peptides as determined by circular dichroism are listed in table 3. The peptides contain 46-47% β-sheet structure and 48-49% random coil at 20°C. α-Helix and β-turn structures are almost negligible (2-4%). These values are in fairly good agreement with literature about the presence of secondary structure in a mixture of related

peptides[8], but in contrast to the secondary structure of this sequence within the intact β-lactoglobulin molecule. Estimation of the amounts of structure elements by the software program RasMol (v2.7.5, RasWin Molecular Graphics, Bernstein & Sons, Bellport, NY, USA) reveals that the amino acid sequence 135-158/162 within β-lactoglobulin consists of about 30% α-helix and 15% β-sheet structure. Heating of the peptide solution at 80°C for 10 min. does not change the composition of secondary structure significantly.

Table 3. Relative amount of secondary structure elements (% ± 5 %) of the peptides at 20°C and 80°C

	[f135-158]		**[f135-162]-SH**		**[f135-162]-SX**	
	20°C	**80°C**	**20°C**	**80°C**	**20°C**	**80°C**
α-Helix	2	3	2	3	2	3
β-Sheet	46	46	47	48	46	48
β-Turn	3	5	3	4	3	4
Random Coil	49	46	48	45	49	45

Binding of peptides at room temperature.

The solubilities of [f135-158] and [f135-162]-SH were characterized as a function of pH, in the presence or absence of β-lactoglobulin. Visual turbidity was observed for both β-lactoglobulin-peptide mixtures, while for β-lactoglobulin alone no visual turbidity was observed. For both peptides a minimum solubility is observed around pH 8.5 (figure 2), which corresponds with the isoelectric point of the peptides. At pH < 7 the peptides [f135-158] and [f135-162]-SH are soluble, as indicated by the high transmission at 600 nm. For the protein-peptide mixture the solubility of both peptides is decreased over a wide range (pH 5.0-9.5), indicating association between the peptides and the protein.

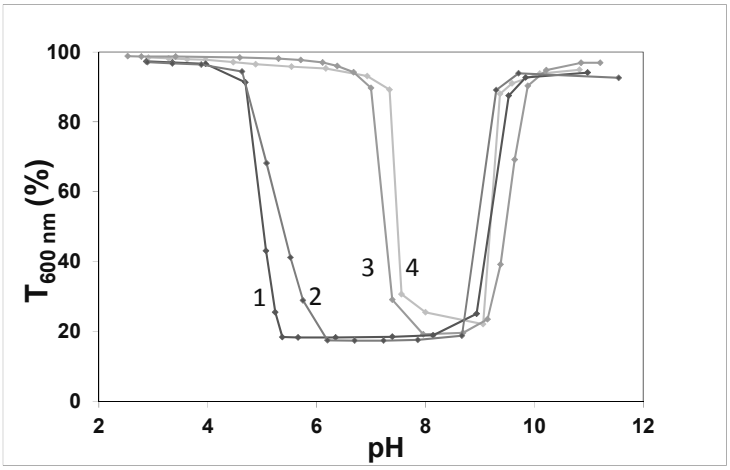

Figure 2. Transmission at 600 nm of (1) β-lactoglobulin + peptide [f135-158], (2) β-lactoglobulin + peptide [f135-162]-SH, (3) peptide [f135-158] and (4) [f135-162]-SH. Concentration of all components in solution was 5.0 mg/mL in MQ water.

The protein-peptide association was further determined by SELDI-TOF-MS for the proteins β-lactoglobulin, ovalbumin and lysozyme. The peptides did not show non-specific binding to the SELDI chip under the conditions used in the experiment. The peptides did bind to β-lactoglobulin, ovalbumin, and lysozyme at room temperature (figure 3). Peptide [f135-158] (m/z = 2826) bound equally to the three different proteins, as indicated by the similar signal intensity (figure 3A). In these mass spectra, two masses for this peptide are found. The second mass (m/z = 2826 + 16) is due to the oxidation of the methionine residue. The oxidation of methionine is a common modification of methionine containing peptides.[18] For peptide [f135-162]-SH larger differences were found in the signal intensity after binding to different proteins. The signal of the peptide bound to lysozyme (figure 3B) was approximately 10 times higher than the signal from the peptides bound to ovalbumin and β-lactoglobulin. The results show that both peptides bind to proteins at room temperature. The binding to β-lactoglobulin was expected based on the decreased solubility at pH 5-7 observed for mixtures of native β-lactoglobulin and peptides at room temperature. The fact that the peptides bind similarly to ovalbumin as to β-lactoglobulin shows that the binding does not depend on specific

properties of the protein, such as the retinol binding site of β-lactoglobulin. Rather, both proteins are negatively charged while both peptides are positively charged (+1.0 at pH 7.0).[8] This suggests that the binding is due to electrostatic attraction.

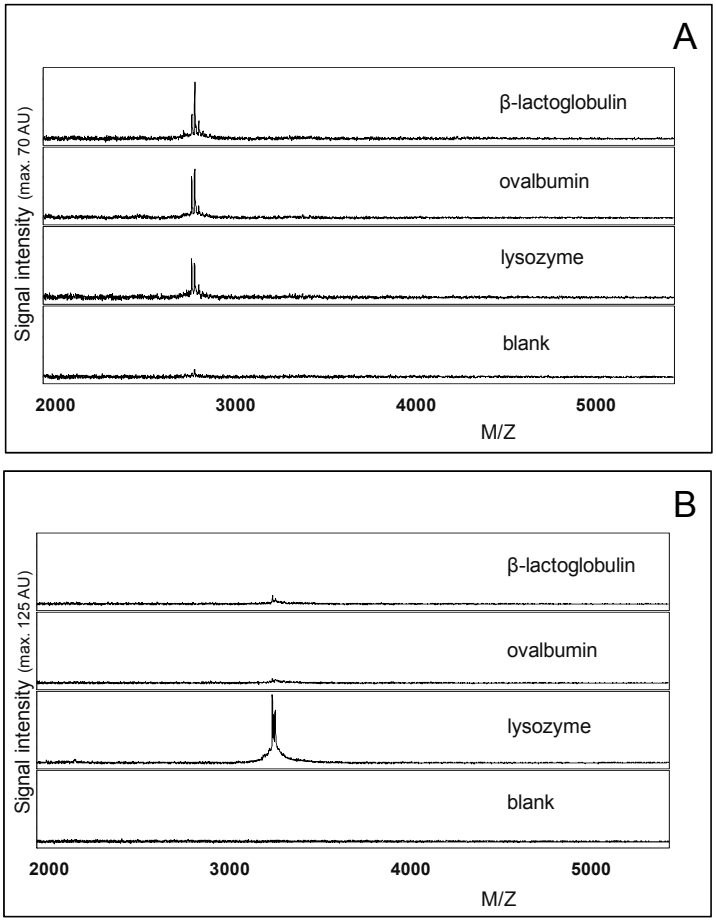

Figure 3. SELDI-TOF-MS spectra of peptide [f135-158] **(A)** and peptide [f135-162]-SH **(B)** bound to β-lactoglobulin, ovalbumin and lysozyme at pH 7.4.

However, [f135-158] binds equally well to lysozyme while the net charge of lysozyme at this pH is also positive (+8.0). This shows that other properties of the peptide such as the total hydrophobicity (31.6 kcal for both peptides)[8] may be important. It is expected that, based on the similarity of the peptide sequence, both peptides show similar binding behavior. Indeed, the mass intensity of [f135-162]-

SH when bound to lysozyme is quite similar to that for [f135-158], but is lower for binding to β-lactoglobulin, and even lower for ovalbumin. The signal intensity decreases with the number of free sulfhydryl groups in the proteins, showing that the protein is bound to the SELDI chip by the amino groups and not by free SH groups as has been suggested.[19] Lysozyme contains four disulfide (SS) bonds, but no free sulfhydryl groups (SH), β-lactoglobulin has 2 SS-bonds and one free SH, ovalbumin has 1 SS-bond and 4 free SH groups. This leads to the hypothesis that under these conditions (i.e. room temperature, pH 7.0) the free sulfhydryl group of the peptide could react to a free sulfhydryl group of the protein. The formation of this covalent link between protein and peptide would be formed after binding of the peptide to the protein and prevent later desorption during ionization, resulting in decreased signal intensity.

Effect of binding peptides on heat-induced aggregation of β-lactoglobulin.

The heat-induced aggregation of β-lactoglobulin in the presence or absence of peptides was studied by dynamic light scattering experiments (figure 4 and table 4). Prior to heating, the light scattering intensity is measured at 20 °C For the β-lactoglobulin/peptides mixtures a high light scattering intensity is observed at 20 °C, resulting from the fact that the peptides are not completely soluble under these conditions, leading to complexation (data not shown). As the peptide concentration was increased, again more light scattering intensity at 20 °C is observed. For mixed solutions of β-lactoglobulin and [f135-162]-SH, a low light scattering intensity at 20 °C is observed at low concentrations of the peptide (0.1, 0.2 and 0.5 mg/mL) but increased at peptide concentration of 1.0, 2.5 and 5.0 mg/mL again indicating complexation (data not shown).

Upon heating at 80°C, the light scattering intensity of all samples initially decreased to values below 5,000 AU within 250 seconds of heating showing that the complexes formed at room temperature dissociate at 80 °C (data not shown). After 250 seconds, in the absence of peptides, β-lactoglobulin has a low light scattering intensity (figure 4).

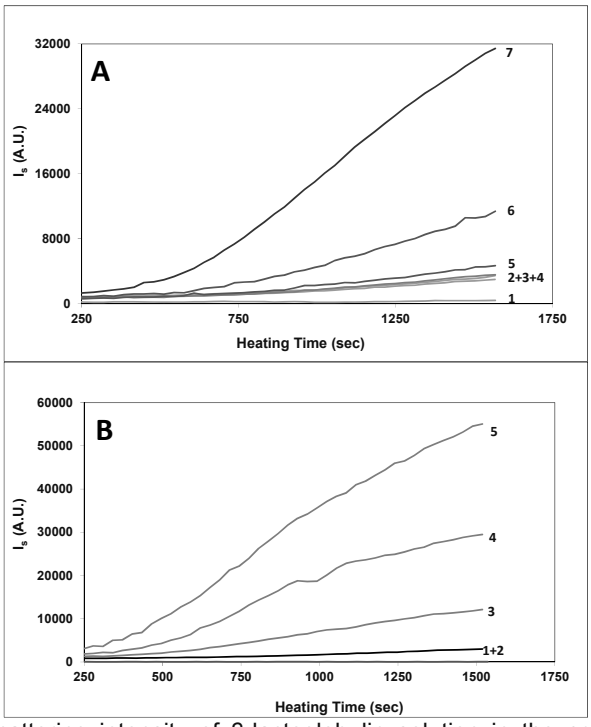

Figure 4. Light scattering intensity of β-lactoglobulin solution in the presence of peptide [f135-158] **(A)** and peptide [f135-162]-SH **(B)** during heating at 80°C. The curves represent (1) only 5.0 mg/mL peptide solution; (2) 5.0 mg/mL β-lactoglobulin; mixtures of 5.0 mg/mL β-lactoglobulin + (3) 0.1 mg/mL; (4) 0.5 mg/mL; (5) 1.0 mg/mL; (6) 2.5 mg/mL and (7) 5.0 mg/mL peptide solution. All components were dissolved in 50 mM sodium phosphate buffer, pH 7.0.

Under the experimental conditions (80 °C, pH 7.0), heating did not lead to a significant increase of the light scattering intensity of the peptide solutions. In the presence of β-lactoglobulin, the complexation with peptide [f135-158] is also reduced (figure 4A), as evidenced by the low scattering intensity of mixed solutions at low peptide concentrations (0.1, 0.5 mg/mL). After prolonged heating times, the light scattering intensity increases again, from 4,500 AU at 1.0 mg/mL peptides to a maximum value of 32,000 AU at 5.0 mg/mL peptide. These concentrations of peptides (1.0, 2.5 and 5.0 mg/mL) correspond to protein:peptide molar ratios of 1:1.3, 3.3, and 6.5, respectively. Since at lower concentrations no significant effect of the presence of [f135-158] was observed, this indicates that aggregation of β-

lactoglobulin is induced by binding of at least one peptide molecule per protein molecule. For peptide [f135-158], the hydrodynamic diameter (d_h) of protein/peptide aggregates range from 14-15 nm for the lowest concentration peptide (0.1 mg/mL) up to 22 nm for the highest concentration (5.0 mg/mL) peptide present (table 4). The same experiments were performed with peptide [f135-162]-SH, that deviates mostly from [f135-158] by the presence of the free sulfhydryl group (figure 4B).

Table 4. Hydrodynamic diameter (d_h) of β-lactoglobulin (β-LG) in the presence of different concentrations peptide [f135-158] or [f135-162]-SH after heating for 25 min. at 80 °C.

Sample	d_h (nm)
5.0 mg/mL β-LG	14
5.0 mg/mL [f135-158]	2
5.0 mg/mL β-LG + 0.1 mg/mL [f135-158]	15
5.0 mg/mL β-LG + 0.5 mg/mL [f135-158]	14
5.0 mg/mL β-LG + 1.0 mg/mL [f135-158]	16
5.0 mg/mL β-LG + 2.0 mg/mL [f135-158]	18
5.0 mg/mL β-LG + 5.0 mg/mL [f135-158]	22
5.0 mg/mL [135-162]-SH	3
5.0 mg/mL β-LG + 0.1 mg/mL [f135-162]-SH	35
5.0 mg/mL β-LG + 0.5 mg/mL [f135-162]-SH	43
5.0 mg/mL β-LG + 1.0 mg/mL [f135-162]-SH	51
5.0 mg/mL β-LG + 2.0 mg/mL [f135-162]-SH	75
5.0 mg/mL β-LG + 5.0 mg/mL [f135-162]-SH	>400

After heating, the presence of peptide [f135-162]-SH results in increased aggregation, reaching light scattering intensities of 12.0 at 0.1 mg/mL peptide up to 55,000 AU at 1.0 mg/mL peptide. At higher concentrations the peptide does not dissolve anymore. Consequently, the light scattering intensities measured do not give reliable results and are omitted from figure 4B. The hydrodynamic diameter of these aggregates are also higher for [135-162]-SH than for [f135-158] and range from 35 nm at 0.1 mg/mL peptide to >400 nm at the highest concentration (5.0 mg/mL) peptide [f135-162]-SH. From the comparison with [f135-158] and [f135-162]-SH, it seems that the presence of the free sulfhydryl group has a very significant effect on the aggregation of mixed protein and peptide solutions both at

room temperature and after heating. To confirm that the differences are due to the presence of the free sulfhydryl group, aggregation of β-lactoglobulin in the presence of [f135-162]-SX (5.0 mg/mL) was determined. In this peptide the free sulfhydryl group was blocked by NEM. The light scattering intensity upon heating reached values similar to those obtained with peptide [f135-158] (data not shown). The results show that peptides affect the amount and size of β-lactoglobulin aggregates formed during heating by non-covalent interaction ([f135-158] and [f135-162]-SX), but even more by covalent interactions through sulfhydryl reactivity ([f135-162]-SH). The importance of the free sulfhydryl group was further tested by the addition of equimolar amounts (based on SH in peptide [f135-162]-SH) of the peptide [f159-162]-SH to β-lactoglobulin. The light scattering intensity in the presence of peptide [f159-162]-SH was higher than in the case of β-lactoglobulin with peptide [f135-162]-SH (data not shown). This indicates that the rest of the peptide inhibits the formation of aggregates, which is mainly caused by the presence of the free sulfhydryl group.

Peptide induced gelation of β-lactoglobulin

The complex moduli (G) of heated solutions of β-lactoglobulin in the presence or absence of peptides were determined using the electrowetting technique. For this, first the interfacial tension of the heated droplets needs to be determined. At protein concentrations until 85 mg/mL the slope of $\Delta cos(\theta)$ versus U^2 was constant (figure 5A), indicating that no gelation occurred. This corresponds to earlier reported values for the minimal gel concentration of β-lactoglobulin under similar conditions.[20]

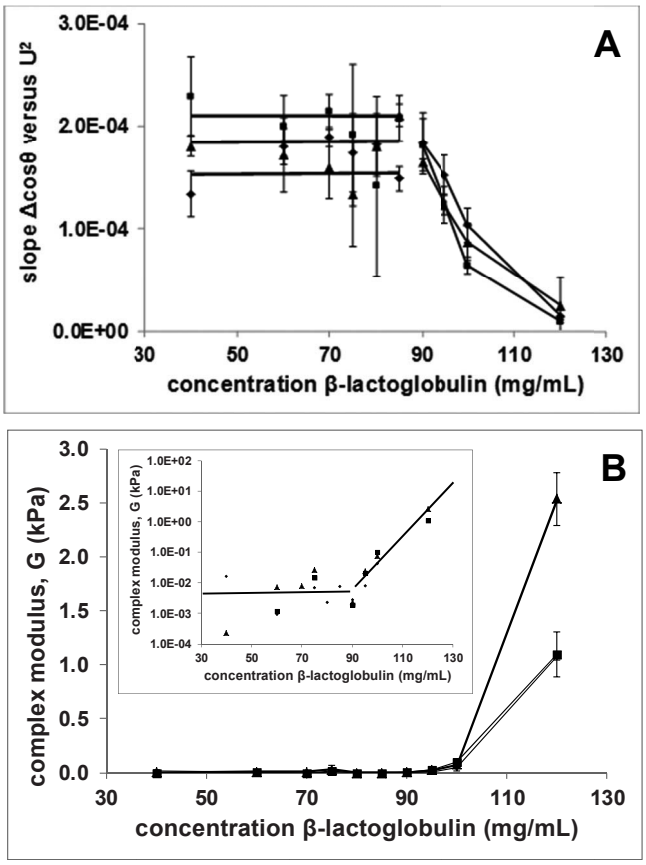

Figure 5A. The slope of *cos(θ)* versus U^2 plotted against the concentration β-lactoglobulin to calculate the interfacial tension [*γ*] (horizontal lines). **Figure 5B**. Complex modulus of heat-induced gels of β-lactoglobulin (♦) in the presence of peptide [f135-158] (■), peptide [f135-162]-SH (▲) 50 mM sodium phosphate buffer, pH 7.0. The mass ratio protein:peptide is 48:1.Inset: Y-axis in log-scale to show minimal gel concentration.

Therefore, the interfacial tension (*γ*, mN/m) for the heated droplets was calculated as the average value obtained at these concentrations and were $1.5*10^{-4}$, $2.1*10^{-4}$ and $1.8*10^{-4}$ mN/m for β-lactoglobulin (β-LG), β-LG + peptide [f135-158] and β-LG+peptide [f135-162]-SH, respectively. At concentrations > 90 mg/mL, gelation occurs, resulting in an additional contribution of the elastic modulus (*G*) to $\Delta\cos(\theta)$. For β-lactoglobulin, the calculated *G* increases to a value of 1.07 ± 0.03 kPa at a concentration of 120 mg/mL (figure 5B). These values agree with reported values

for β-lactoglobulin under the same conditions as determined by traditional rheometry.[21, 22]

The presence of peptides does not shift the minimum gel concentration dramatically (≈ 90 mg/mL, inset figure 5B). However, for peptide [f135-162]-SH the gel strength at the highest concentration is significantly higher (2.59 ± 0.24 kPa) than β-lactoglobulin alone, or in the presence of [f135-158] (1.10 ± 0.21 kPa). This effect was more clear when the gel strength was compared at constant concentration (100 mg/mL) β-lactoglobulin and increasing concentrations of peptides (figure 6). To correct for the increase in total proteinaceous material, a reference curve is included where additional β-lactoglobulin was added instead of peptides. At the highest concentration (120 mg/mL + 50 mg/mL β-lactoglobulin) the value was 42 ± 11 kPa. This is in agreement with previous studies on heat induced β-lactoglobulin gelation under the same conditions.[22] Although Banpurkar[11] found in electrowetting experiments a working range for G of 0.03-3 kPa (with gelatin gels), it is now legitimate to state that the electrowetting method can be used to determine the elastic modulus of protein gels until a value of 40 kPa, as is indicated by the dotted line in Figure 6.

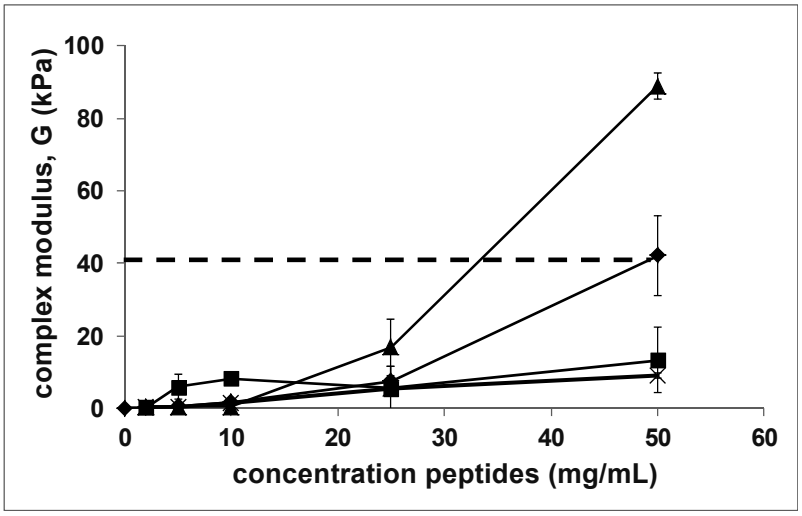

Figure 6. Complex modulus of heat-induced gel of 100 mg/mL β-lactoglobulin in the presence of different concentrations peptide [f135-158] (■), peptide [f135-162]-SH (▲), peptide [f135-162]-SX (**X**) or extra β-lactoglobulin (♦) in 50 mM sodium phosphate buffer, pH 7.0. The dashed line indicates the working range (0.01-40 kPa).

The complex modulus G of β-lactoglobulin gel in the presence of the sulfhydryl containing peptide ([f135-162]-SH) is significant higher than for β-lactoglobulin alone and increases to a final value of 89 ± 1 kPa for the highest concentration peptide [f135-162]-SH added (50 mg/mL). For peptide [f135-158], the gel strength at each concentration investigated is lower than the reference of β-lactoglobulin alone. This indicates that at constant concentration of proteinaceous material the presence of this peptide effectively decreases the gel strength, while at constant β-lactoglobulin concentration the presence of this peptide has no significant effect. Still, the aggregation behavior was found to be affected. It may be that the macroscopic gel properties are affected, but that these changes are not reflected in the G as measured in these experiments. Peptide [f135-162]-SX, in which the sulfhydryl group is blocked, shows similar behavior as peptide [f135-158]. The gel strength is lower than for β-lactoglobulin alone (9.2 ± 0.7 and 42 ± 11 kPa, resp.) and is almost equal to that for peptide [f135-158] (14 ± 1 kPa). The increase in gel strength after heat-induced gelation of β-lactoglobulin due to the availability of

additional sulfhydryl groups has also been demonstrated by others.[23, 24] These results clearly show the importance of the free sulfhydryl group in the gelation mechanism and gel strength of β-lactoglobulin. Moreover, the gel strength of β-lactoglobulin can be directed towards both lower and higher values.

Conclusion

The binding of peptides to the protein results in increased amounts of heat-induced aggregates of the protein. In a concentrated system these differences in aggregation behaviour lead to other gel properties and can be directed by the type of peptide. The sulfhydryl group in the peptide plays an important role in the amount of protein aggregates formed and increases the gel strength of a concentrated protein system.

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Chapter 5

Towards application of a specific whey protein isolate (WPI) hydrolysate to control the aggregation and gelation of WPI

Abstract

Whey protein isolate (WPI) was hydrolyzed by the Glu-specific enzyme *Bacillus licheniformis* protease (BLP) to a degrees of hydrolysis (DH) of 6.8% and 9.2%. Four cysteine-containing peptides (A/B[f159-162]; A/B[f90-108]; B[f115-127] and B[f63-89]) were identified in the soluble fraction of the WPI hydrolysates. In the presence of this hydrolysate fraction (2.5 mg/mL DH 9.2%) the amount of aggregates upon heating of WPI (80°C, pH 7.0) increased by a factor of 5 compared the WPI solution without added hydrolysate. When the free sulfhydryl groups in this hydrolysate were blocked by NEM, no increase in the amount of aggregates was observed. For rheological experiments, the soluble peptide fraction of the cysteine-containing hydrolysates (75 mg/mL) were added to a WPI solution (150 mg/mL) and heated at 80°C (pH 7.0). The storage modulus (G') was 1.4 and 1.8 times higher than a heated 225 mg/mL WPI solution, for DH 6.8%SP and DH 9.2%SP, respectively. Blocking of the free sulfhydryl groups in the DH 6.8% SP hydrolysate decreased the storage modulus to almost the same values as for 150 mg/mL WPI solution (16 times lower than the 225 mg/mL WPI solution). The addition of L-cysteine to 150 mg/mL WPI increased the gel strength 1.5 times compared to 150 mg/mL WPI, but was not as effective as the addition of equimolar amounts of cysteine-containing hydrolysates (5 times). These results show that the gel properties of protein systems can be significantly affected by the addition of peptides. The presence of sulfhydryl groups and the peptide composition are important factors that determine the final effect on the gel properties.

Introduction

It has been suggested that foods with high protein contents (more than twice the amount of proteins compared to related products) can induce satiety more than foods high in fat and carbohydrate content and hence can prevent over-eating.[1-3] Several studies have been reported on the properties of such high protein systems.[4-7] Typically, these systems suffer from a lack of storage stability and negative sensory properties, like tough, rubbery and dry mouth feel.[8, 9] To better control these properties, it is important to understand how the gel strength of high protein foods can be controlled. It has been shown in several studies that the gel properties of partially hydrolysed proteins are different than those of the intact parental proteins.[10-12] Therefore, the addition of protein hydrolysates to intact proteins seems a way to change gel properties under constant system conditions. Previous studies[13, 14] showed that partial hydrolysis of whey protein isolate (WPI) proteins by *Bacillus licheniformis* protease at 40-50°C led to gel formation. Other workers[11, 15] applied an additional heat step of 80°C after hydrolysis, which resulted in a 10 times stiffer gels compared to the parental proteins. In these studies the aggregation and gelation of the protein was studied in the hydrolysate. Hence, the amounts of intact proteins and peptides were coupled. Therefore, no conclusive information was obtained about the exact contribution from the peptides.

To determine the contribution of peptides on protein aggregation, peptides from a hydrolysate of β-lactoglobulin were added to solutions of intact protein in our previous work.[16] In that study, it was shown that certain peptides in the hydrolysate showed specific binding to the intact protein. From these peptides, two ([f135-158] and [135-162]-SH) were selected in a later study to test the effect of their binding to the gelation of protein.[17] In the presence of [f135-162]-SH heating of β-lactoglobulin resulted in increased aggregation and a higher gel strength, than heating in the presence of [f135-158]; a similar peptide but without a free sulfhydryl group. This showed that the presence of a reactive sulfhydryl group in a small

peptide sequence that can bind to intact protein may be an efficient tool in controlling the gel properties of heat-induced proteins.

To test the extent to whether the effects observed with pure peptides in a pure protein system are also valid in a complex, industrial relevant, systems, the role of free sulfhydryl groups present in a protein hydrolysate added to a whey protein isolate solution on the heat-induced gel properties were studied.

Materials and Methods

A commercial whey protein isolate (WPI) powder was used for the experiments (BiPRO, Davisco Foods International Inc., Le Sueur, MN, USA). According to the manufacturer, it consists of 74.0% (w/w) β-lactoglobulin (β-LG), 12.5% (w/w) α-lactalbumin (α-La), 5.5% (w/w) bovine serum albumin and 5.5% (w/w) immunoglobulins. The protein content of the powder on dry weight was 97.9% (w/w). The enzyme used was the Glu-specific endoproteinase *Bacillus licheniformis* protease, BLP (Novozymes, Nordisk, Denmark). The activity of the enzyme is 0.7 AU/mL as given by the manufacturer. All chemicals were of analytical grade and purchased from Sigma (Sigma Chemical Co., St. Louis, MO) or Merck (Darmstadt, Germany). All water used was of MilliQ (MQ) quality.

Hydrolysis of WPI

WPI was dissolved in 1L MQ water to a final concentration of 5 mg/mL and stirred overnight at 4 °C. The pH of the WPI solution was adjusted to pH 8.0 with 0.1 N NaOH and equilibrated at 40 °C. BLP (undiluted) was added in an enzyme/substrate ratio of 1/200 (v/v). Hydrolysis was performed by the pH-STAT method. The pH of the mixture was maintained at pH 8.0 by addition of 5 M NaOH. The hydrolysis was stopped at the desired DH (6.8% or 9.2%) by adjusting the pH to 2 with a 6 M HCl solution and incubating for 2 hours at 20 °C. To calculate the degree of hydrolysis, the following equation was used:

$$DH = B \times N_b \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{h_{tot}} \times 100\% \quad (1)$$

With B the volume of NaOH added; N_b is the molarity of NaOH (5 M); α is the average degree of dissociation of the α-NH_2 groups (in this case 1/α = 1.20). MP is the mass of the WPI protein in grams which was present in the sample; h_{tot} is the total number of peptide bonds in WPI substrate (8.8 meqv/g).[18] After enzyme inactivation at pH 2 the pH was brought back to pH 7.0 with 5 M NaOH. The hydrolysates (DH 6.8% and DH 9.2%) were centrifuged (20 min., 21,000*g, 10 °C) to obtain the soluble peptide fractions, further referred to as DH 6.8%SP and DH 9.2%SP. These were freeze-dried and stored at -20 °C.

Determination of degree of hydrolysis

To determine if the desired degrees of hydrolysis were reached, the amounts of free NH_2 groups in the total hydrolysate and the intact protein were determined by ortho-phtaldialdehyde (OPA) method as described before.[19] The DH was calculated from eq. 2:

$$DH = \frac{\#NH_2(\text{hydrolysate}) - \#NH_2(\text{intact})}{\#\text{total peptide bonds}} \times 100\% \quad (2)$$

All measurements were performed in duplicate.

Blocking of free sulfhydryl group

The hydrolysates DH 6.8%SP and DH 9.2%SP were dissolved in 5 mg/mL in phosphate buffered saline (PBS) solution, pH 7.0. A stock solution of 300 mM *N*-ethylmaleimide (NEM) in water was prepared. Subsequently, this stock solution was added to the hydrolysate to a final concentration of 3 mM. The samples were incubated for 2 hours at room temperature. After incubation, the sample solutions (both with and without NEM-treatment) were dialyzed by a Mini dialysis kit, 1 kDa cut-off, 2 mL (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) overnight against 20 mM sodium phosphate buffer, pH 7.0 at 4 °C to remove the excess of NEM and other salts. After dialysis, all sample solutions were freeze-dried. The blocked hydrolysate is referred to as DH 6.8%SP-SX and DH 9.2%SP-SX.

Determination of number of free sulfhydryl groups

The amount of free sulfhydryl groups (-SH) in the hydrolysate before and after blocking with NEM were determined by reaction with Ellman's reagent 5,5'-dithiobis(2-nitrobenzoic acid (DTNB) as described previously.[20] All measurements were performed in duplicate.

Detection of cysteine containing peptides

To detect the cysteine-containing peptides in the hydrolysates, the free sulfhydryl groups in the peptides were allowed to react with DTNB to form a peptide-TNB adduct, which is detectable at 412 nm. For this, samples of 2.0 mg/mL WPI solution in 0.1 M Tris buffer (pH 8.0) were mixed with 0.2% (w/v) DTNB solution in 0.1 M Tris buffer hydrolysate DH 6.8%SP and DH 9.2%SP and incubated for at least 24 hours at ambient temperature. The peptides with free cysteine groups are labeled by the DTNB, resulting in a mass increase of 198.5 Da, and increased absorbance at 412 nm.[21]

Samples were analyzed on an Accela RP-UPLC system (Thermo Scientific, San Jose, CA, USA). Samples (10 µL) were injected on an Acquity UPLC BEH C18 column (2.1 x 150 mm, 1.7 µm particle size) with an Acquity UPLC BEH C18 Vanguard pre-column (2.1 x 5 mm, 1.7 µm particle size; Waters). 5% (v/v) Acetonitrile (ACN) with 0.1% (v/v) formic acid in milliQ water (eluent A) and 80% (v/v) ACN with 0.085% (v/v) formic acid in milliQ water (eluent B) were used as eluents. The flow rate was 300 µL/min, and detection took place at 214 nm and 412 nm. The following elution profile was used: 0 - 1 min, eluent A; 1 -2 min linear gradient from 0% - 10% (v/v) B; 2 - 24 min, linear gradient from 10% - 60% B; 24 – 26 min, linear gradient from 60% - 100% (v/v) B; 26 – 29 min, isocratic on 100% (v/v) B, 29 – 30 min, linear gradient from 100% - 0% (v/v) B; 30-32 min, isocratic on 100% (v/v) A.

Electron spray ionization mass spectrometry (ESI-MS)

Mass spectrometric data were obtained by analyzing samples on a LTQ-XL (Thermo Scientific, San Jose, CA, USA) equipped with an ESI probe coupled to the RP-UPLC system. Helium was used as sheath gas and nitrogen as auxiliary gas. Data were collected over an m/z-range of 100–2000. The settings were optimized using 'Tune plus' (Xcalibur 2.07, Thermo Scientific) via automatic tuning. The system was tuned with angiotensin I in PI mode. In the PI mode, the ion transfer tube temperature was 250 °C and the source voltage 4.2 kV. Data acquisition and reprocessing were performed with Xcalibur 2.07 (Thermo Scientific).

Dynamic light scattering experiments (DLS)

The light scattering intensity (I_s) was measured using a Nanosizer (Malvern Instruments, Malvern, U.K.). 2.5 mg/mL WPI solution in water, 2.5 mg/mL hydrolysate DH 9.2%SP solution in water and a mixture of WPI and hydrolysate in the ratio 1:1 (v/v) were prepared. Prior to analysis, the samples were filtered using a 0.65 µm eppendorf filter and 45 µL of the filtrate was transferred into a Quartz cuvette. The samples were covered with 50 µL of paraffin oil to prevent evaporation. First, the light scattering signal was measured at 20 °C for 10 minutes (20 measurements of 30 seconds), to determine the initial state of the samples. Next, the cuvette was removed from the Nanosizer and the temperature was set at 80 °C. After the apparatus reached the set temperature the samples were placed back and the signal was measured at 80 °C for 25 minutes (50 measurements of 30 seconds). Measurements were performed at least in duplicate.

Rheological experiments

Rheological experiments were measured using a rheometer (Anton Paar, Physica MCR 501) equipped with a Couette geometry (Measuring Bob B-CC 10/Q1/TITANIUM, and Measuring cup C-CC10/T200/TITANIUM). Stock solutions of WPI (300 mg/mL) and hydrolysates DH 6.8%SP SH/SX and DH 9.2%SP SH/SX (150 mg/mL) were prepared and stirred for 24 hours at 4 °C. Samples were

prepared by mixing the stock solutions in 1:1 ratio to obtain final WPI/hydrolysate concentrations of 150 mg/mL and 75 mg/mL, respectively. Next, the pH of the peptide solution was adjusted to pH 7.0 with 6.25 M trifluoroacetic acid. This solution was incubated for 24 hours at 4 °C. The measurement consisted of four steps. First, the sample was heated from 20 °C to 80 °C with a rate of 3 °C/min. The sample was kept at this temperature for 60 min. to form a gel. Next, the sample was cooled down until 20 °C with the same rate of 3 °C/min. The storage (G') and loss (G") moduli were determined when the sample reached 20 °C and consisted of a strain sweep, in which oscillatory deformations were applied of increasing magnitude (0.01-10,000%), at a constant frequency of 1 Hz.[22] Measurements were performed at least in duplicate.

Results and Discussion

Hydrolysis of WPI and characterization of the hydrolysate

The DH of the WPI hydrolysate DH 6.8% determined by the OPA assay was in agreement with the DH (6.8%) aimed for by pH stat method (table 1). The peptide composition in the hydrolysates was analysed by RP-UPLC (figure 1), where the separation is based on the combination of length of peptides and absolute hydrophobicity.[23]

Table 1. The degree of hydrolysis by pH stat and OPA method and the amount of free SH groups in the presence and absence of SDS.

Sample	DH (%) by pH stat	DH (%) by OPA	-SDS mol SH/mol prot.	+SDS mol SH/mol prot.
WPI	0	0	0.50	0.60
WPI DH 6.8% SH	6.8	6.8	0.60	0.60
WPI DH 6.8% SX	6.8	6.8	0.02	n.d.[a]
WPI DH 9.2% SH	9.2	9.0	0.50	0.30
WPI DH 9.2% SX	9.2	9.0	0.08	n.d.[a]

[a] n.d. = not determined

To detect the cysteine-containing peptides in the hydrolysates, the free sulfhydryl groups in the peptides were allowed to react with DTNB to form a peptide-TNB adduct, which is detectable at 412 nm. The non-treated hydrolysate showed no peak intensities at 412 nm (Figure 1C), as expected. After modification by DTNB, three extra peaks were observed in the UPLC chromatograms at 214 nm compared to the chromatogram of the non-modified peptides (figure 1 D and 1B, respectively).

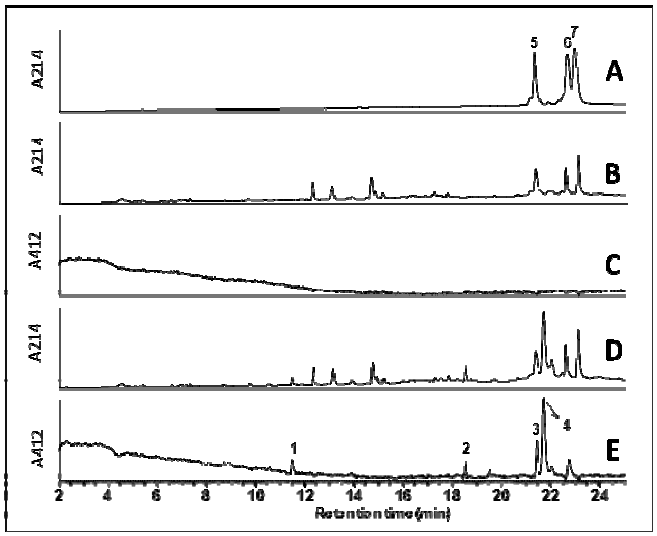

Figure 1. RP-UPLC chromatogram of the detection of WPI (A), WPI DH6.8% hydrolysate at 214 nm (B) and 412 nm (C) and WPI DH6.8% hydrolysate treated with DTNB at 214 nm (D) and 412 nm (E). Identified peptides are labelled as 1. peptide A/B[f159-162]; 2. A/B[f90-108]; 3. B[f115-127] + α-lactalbumin; 4. B[f63-89]; 5. α-lactalbumin; 6. β-lactoglobulin B; 7 β-lactoglobulin A. The y-as was scaled to 600,000 AU and 1,000 AU for the absorbance at 214 and 412 nm, respectively.

Further analysis of the DTNB-treated hydrolysate at 412 nm (Figure 1E) showed 9 peaks of which 6 peaks could be identified by MS (table 2) as 1. peptide A/B[f159-162]; 2. A/B[f90-108]; 3. B[f115-127]; 4. B[f63-89]; 5.α-lactalbumin; 6. β-lactoglobulin B; 7. β-lactoglobulin. The same peptides were found and identified in

the hydrolysate DH9.2% (data not shown). These results clearly show the presence of cysteine-containing peptides in the hydrolysate.

Table 2. Overview of cysteine-containing fragments from WPI found by LC-MS.

Peak nr	cys-peptide fragments in WPI	theoretical mass +TNB (Da)	observed mass +TNB (Da)
1	A/B[f159-162]	698.7	697.3
2	A/B[f90-108]	2533.4	2534.1
3	A[f115-127]+Na	1668.2	1667.0
4	B[f63-89]	3169.8	3168.0

Heat-induced aggregation

Heating of a WPI solution (2.5 mg/mL) resulted in a light scattering intensity of 3.5 kAU (Fig. 2). The presence of SH containing hydrolysate (DH 9.2%SP-SH) induced more aggregation, resulting in an increase of the light scattering intensity to a maximum intensity of 18 kAU. At longer heating times (>1700 s) the aggregates formed start to precipitate, resulting in a decrease in LSI. When the sulfhydryl groups are blocked, (DH9.2%SP-SX) no significant difference in the light scattering intensity in the absence or presence of hydrolysate is observed. In a previous work (chapter 4), the effect of peptide [f135-162]-SH on the aggregation of a single protein was observed Now, it has been shown that this effect is still significant when a hydrolysate (containing SH peptides) is added to a mixed protein (WPI) solution. In both cases an increase in the amount of aggregated protein is observed. In another study[24], the addition of free cysteine to WPI in a molar ratio (Cys/WPI) of 0.25 resulted, after storage of 35 days at 45 °C, in an increase in aggregation of WPI due to the increased thiol-disulfide interchange reaction. At a Cys/WPI molar ratio of 0.05 a decrease in WPI aggregation was observed. Apparently, the aggregation and hence, gelation of WPI can be directed by adjusting the WPI/cysteine ratio. Based on the results in this study we speculate that this accounts also for a WPI/cysteine-containing peptide system.

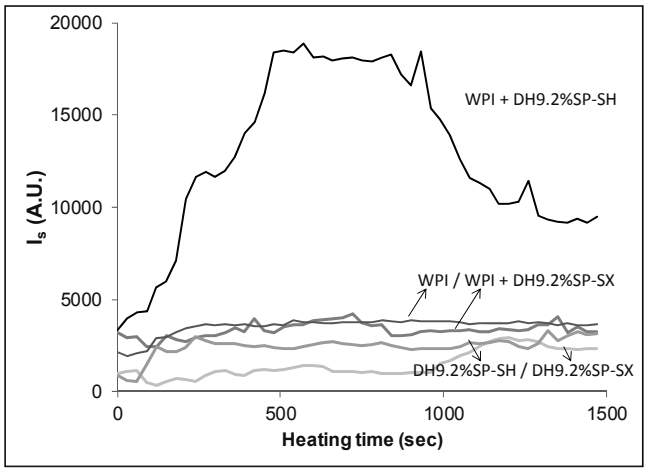

Figure 2. Light scattering of 2.5 mg/mL WPI, 2.5 mg/mL hydrolysate DH9.2% (+/- SH blocking) and WPI + hydrolysate DH9.2% (+/- SH blocking) in 1:1 (w/w) ratio in MQ-water, heated at 80°C, pH 7.0.

Heat-induced gelation

Heating of WPI solutions at 80 °C, pH 7.0 resulted in translucent and elastic gels, both at 150 and 225 mg/mL concentration (Fig 3A). In the presence of 75 mg/mL hydrolysate (DH6.8%SP-SH, or DH9.2%SP-SH) the WPI gel (150 mg/mL) becomes opaque (Fig 3B). The translucency gels are typically related to the formation of fine-stranded structures. These are formed under conditions of electrostatic repulsion between the proteins (away from the pI, or low ionic strength). Under conditions of reduced electrostatic repulsion, e.g. around the pI, or at higher ionic strength, milk-white gels are formed with a particulate structure.[25, 26] It has been hypothesized that this is due to the decreased aggregation rate.[27] The presence of the peptides results in opaque gels, which indicates that the peptides increase the rate of aggregation. This was indeed found in previous studies.[16]

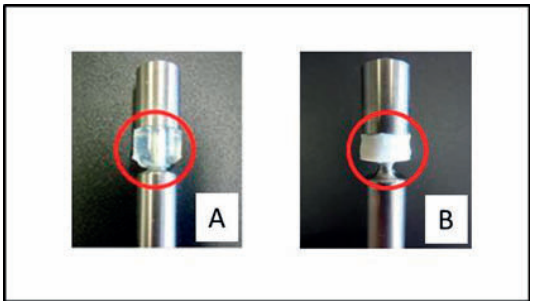

Figure 3. Gel formation of 225 mg/mL WPI solution (A) and 150 WPI + 75 mg/mL hydrolysate DH6.8%SP solution (B), heated at 80°C, pH 7.0.

In addition to the visual appearance, also the rheological properties were affected by the presence of peptides. Heating of a 150 mg/mL WPI solution resulted in a loss modulus (G'') of 0.21 ± 0.04 kPa and a storage modulus (G') of 1.13 ± 0.03 kPa (Fig. 4), which is in agreement with literature.[28, 29] Increasing the concentration of WPI with 75 mg/mL to 225 mg/mL resulted in an increase of G'' to 5.13 ± 0.73 kPa and G' to 42,9 ± 0.4 kPa. When 75 mg/mL DH 6.8%SP-SH was added to the WPI, both parameters reached even higher (1.4 times) values (G'' of 9.21 ± 2.14 kPa and G' of 58.4 ± 11.2 kPa). When the DH 6.8%SP-SX was added, where the sulfhydryl groups were blocked with NEM, G'' and G' had values of 0.50 ± 0.28 kPa and 2.73 ± 1.51 kPa, respectively. These values are similar to that of the 150 mg/m WPI solution itself, showing that, while the concentration of proteinaceous material was increased with 50%, the gel properties remained almost the same.

Comparing the gels made in the presence of the two sulfhydryl containing hydrolysates (DH 6.8%SP-SH and DH9.2%SP-SH) showed higher values for both G" and G' (20.4 ± 3.09 kPa and 75.5 ± 11.3 kPa respectively) for the sample with higher degree of hydrolysis. Blocking of the free sulfhydryl group in this hydrolysate decreased the gel strength, as was also observed for DH6.8%SP-SX, but not to the same extent. The effect of the SH groups was further investigated by the addition of free L-cysteine in equimolar (0.5 mg/mL) and 10 times (5 mg/mL) the molar equivalent of the SH groups present (table 1) in 7.5 mg/mL hydrolysates (Figure 4).

The addition of 0.5 mg/mL L-cysteine to 150 mg/mL WPI resulted in a 1.5 times increase of G' to 1.88 ± 0.30 kPa compared to 150 mg/mL WPI without L-cysteine (1.13 ± 0.03 kPa). Increasing the L-cysteine concentration 10 times led to an 12 times increase of G' (13.9 ± 1.39 kPa). Clearly, this shows that adding L-cysteine increases the gel strength of WPI, but not to such extent as the hydrolysates DH 6.8%SP-SH and DH 9.2%SP-SH (58.4 and 75.5 kPa, respectively) did.

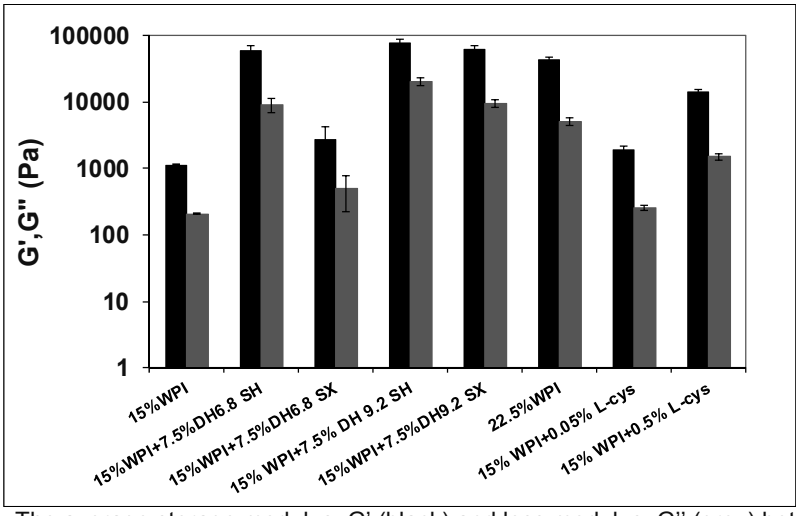

Figure 4. The average storage modulus, G' (black) and loss modulus, G'' (grey) between 0.01-1% strain of WPI solutions in the presence/absence of hydrolysate DH6.8%SP and DH9.2%SP (+/- SH blocking) and L-cysteine after heating at 80°C for 60 min at pH 7.0.

The ability of hydrolysate DH6.8%SP-SX to reduce the gel strength is in accordance with previous research.[30, 31] The observation that DH9.2%SP SX did not decrease the gel strength to a value comparable to 150 mg/mL WPI gel indicates that these peptides act more on shielding charges as DH6.8%SP SX does. However, the free sulfhydryl groups in both hydrolysates overcome the electrostatic repulsion and apparently interact with the disulfides bridges in the protein molecule via the thiol-disulfide interchange reaction. These short-range interactions contribute to the formation of the gel network and result in gels with increased gel strength.

Conclusion

The presence of cysteine-containing peptides in a WPI/hydrolysate mixture dominates the influence of peptides on the heat-induced aggregation of the parental protein WPI.. The gel strength of heat-induced WPI gels containing a hydrolysate with cysteine-containing peptides is the same as in intact protein systems with equal amounts of nitrogen ("protein"). Hydrolysates without the presence of the cysteine-containing peptides lower the gel strength. The use of cysteine-containing peptides is more effective in controlling the gel strength than L-cysteine and allows controlling the structure hardness during preparation of high protein systems.

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Chapter 6

Disulfide-containing peptides reduce the presence of sulfurous volatiles formed during heating of β-lactoglobulin solutions

Abstract

The formation of sulfurous volatiles from proteins (i.e. dihydrogen sulfide (H_2S) and methanethiol (MeSH)) is typically studied by heating in dry state, in the presence of reducing sugars. In this study, it is shown that even heating of solutions of the pure amino acids L-cysteine and L-methionine, in the absence of other compounds, results in the formation of H_2S and MeSH, respectively. Heating of 1.6 mM β-lactoglobulin (2 hours at 80°C, pH 7.0), results in the formation of 1.7 µM H_2S and 0.4 µM MeSH. The formation of these compounds was completely diminished by the addition of 0.4 mM L-cystine or 0.6 mM dipyridyl disulfide (PDS) during heating. Moreover, the addition of 4 mM L-cystine, to a solution of 0.74 µM H_2S and 0.26 µM MeSH also decreased the detected amount of sulfurous volatiles. The same effect was obtained when 1 mM soluble peptides fraction, obtained from β-lactoglobulin hydrolysis by *Bacillus licheniformis* protease (BLP), was used. This effect of reducing the detectable amount of H_2S and MeSH is not only due to the presence of disulphide bridges in the peptides, since after alkylation of the soluble peptides still a decrease the concentration H_2S was observed. Still, after alkylation a two time higher concentration was needed to reach the same reduction in sulfurous volatiles. This study shows that the presence of disulfide containing peptides can reduce the presence of (heat-induced) sulfurous volatiles.

Introduction

Sulfurous volatiles can be formed during heating of food products, resulting in off-flavors as the cooked flavor of milk. The main components responsible for these off-flavors are hydrogen sulfide (H_2S), methanethiol (MeSH), dimethyl sulfide (DMS), dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS).[1-4] These components are formed by chemical conversion of the cysteine (resulting in H_2S) and methionine (resulting in MeSH, DMS, DMDS, DMTS) residues in proteins, peptides and free amino acids. It has been suggested[5, 6] that the formation of sulfurous volatile compounds from methionine and cysteine is formed due to the presence of a α-ketone. The formation of these components has been used as to produce flavors for meat-analogues[6]. In this study, cysteine was mixed with ninhydrin or dehydroascorbic acid and boiled to form H_2S via enaminol (Strecker degradation). Another study[7] found that MeSH was formed from methionine in the presence of ninhydrin at 80°C.

Both in the case of the milk off-flavor and flavor formation for meat-analogues the question is how the formation of sulfur-containing volatiles can be controlled. The cooked flavor intensity in heated milk, was found to be inhibited by the addition of thiosulfates and thiosulfonates.[8] An interesting observation was that the addition of L-cystine to milk considerably reduced the level of H_2S and cooked flavor after heating.[9] The authors suggested a mechanism in which the disulfide reacts with the H_2S to form an additional free thiol group available for further H_2S removal, but this work has not been followed up. However, these components (thiosulfates, thiosulfonates and L-cystine) are not allowed as food additives in most countries.

In our recent studies on the effect of peptides on the heat-induced aggregation and gelation of intact proteins[10] it was observed that the soluble peptide fraction from a protein hydrolysate made by the enzyme *Bacillus licheniformis* could also reduce the smell of sulfurous volatiles formed. In this chapter it is investigated how the addition of protein hydrolysate to β-lactoglobulin can be used to decrease the presence of these sulfurous compounds. A secondary aim of this work is to provide understanding of the formation of these compounds during the heating of proteins.

Material and Methods

β-Lactoglobulin was isolated from fresh bovine milk, purified and characterized as described previously.[11] Ovalbumin was isolated and purified as described previously.[12] Whey protein isolate (WPI) was obtained from Davisco (Le Sueur, MN, USA). Gluten was obtained from Basic Supply Group (Emmen, The Netherlands). A β-lactoglobulin hydrolysate (DH 6.8%, hydrolysed by *Bacillus licheniformis* protease (BLP)) was produced as described previously.[10] Lysozyme, dipyridyl disulphide (PDS), 5,5'-dithiobis-2-nitrobenzoic acid (DNTB), L-methionine, L-cysteine, L-cystine, dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Sigma (St. Louis, MO, USA).

Reduction and alkylation of disulfide bonds in hydrolysate

The hydrolysate of β-lactoglobulin was separated into a supernatant and a pellet as described previously.[10] The freeze dried peptides from the supernatant (200 mg) were dissolved 100 mM ammonium bicarbonate buffer, pH 7.8 to a final concentration of 5 mg/mL (40 mL). One part (20 mL) of the sample was reduced and alkylated. To this end, dithiothreitol (DTT) was added (154.25 mg) to the sample (final concentration is 50 mM DTT) and the mixture was incubated for 60 min at 54°C. Subsequently, the solution was diluted twice with 100 mM ammonium bicarbonate buffer, pH 7.8 and 700 mg iodoacetamide (IAA) was added to 40 mL to obtain a final IAA concentration of 90 mM. This mixture was incubated 120 min at room temperature. The other part of the sample (20 mL) was treated without DTT or IAA as a control. Next, the alkylated and non-alkylated samples were extensively dialyzed in Float-A-Lyzer dialysis tubes (Spectro/Por G2, cut-off 100-500 Da, SpectrumLabs, Breda, The Netherlands) and denoted as soluble alkylated peptides and soluble peptides, respectively.

Heat induced formation of sulfurous volatiles

Intact proteins (WPI, β-lactoglobulin or lysozyme) were dissolved in milliQ water to a final protein concentration of 60 mg/mL. Stock solutions of 13.5 mg/mL (2,019 μM SS, see table 3) soluble (alkylated) peptides, 0.2 mg/mL (868 μM) L-cystine,

0.2 mg/mL (910 µM) PDS, 1.0 mg/mL (8,260 µM) L-cysteine, 3.38 mg/mL (25,762 µM) L-methionine in milliQ water were prepared and adjusted to pH 7.0 with 0.1 N HCl or NaOH. Subsequently, the protein solutions were diluted twice by mixing 1 mL protein solution to 1 mL of (alkylated) peptide solution or L-cystine (diluted to 10-1,000 µM) or 1 mL stock solution of PDS, L-cysteine or L-methionine. Each solution (2 mL) was mixed with 100 µL of internal standard stock solution (2 mg deuterated dimethyl sulfide (D6-DMS) in 1 L of MilliQ water and transferred to airtight test tubes. The test tubes were heated in a waterbath at 80°C for 30 min and 2 hours. After heating, the samples were cooled down to room temperature by running tap-water and analysed by gas chromatography. Randomly selected duplicates were analysed to determine the average error in the measurements. The standard deviation of the duplicates was found to be < 5%.

Masking of pre-formed sulfurous volatiles at room temperature

A stock solution containing 50 µg/L (1.47 µM) H_2S and 25 µg/L (0.54 µM) MeSH was prepared by dissolving 317.52 mg/L $Na_2S.9H_2O$ and 38.34 mg/L MeSNa in milliQ water. From this solution, 20 mL was acidified by addition of 20 µL 50% (v/v) HCl (final pH = 2) to form H_2S and MeSH. The solution was incubated for 10 min before re-adjusting the pH to pH 7. Then, 1 mL of this solution was mixed with 1 mL peptide solution (0.1-1,000 µM), 1 mL alkylated peptide solution (2-1,000 µM) L-cystine (0.1-1,000 µM) and incubated for at least 0.5 hours at room temperature, before analysis by GC-MS.

Gas chromatography-Mass Spectrometry (GC-MS)

Volatile sulfurous compounds were analyzed in all the samples by gas chromatography (Trace, Thermo Scientific, Waltham, MA, USA) in combination with static headspace sampling and mass spectrometry (Trace DSQII, Thermo Scientific). The samples were cooled to 4 °C prior to analysis. After an incubation of 15 min at 60 ºC, 1 mL of the static headspace was injected on the GC system in which the compounds were refocused at -150 ºC. The cold trap was heated rapidly

to 200 ºC and the captured compounds were injected directly onto the column. After trapping, the compounds were separated on a VF 1MS 30 m x 0.25 mm GC-column, d_f = 1 µm (Agilent Technologies, Santa Clara, CA, USA). The GC separation started at 40 °C for 2 min, thereafter the temperature was raised with 20 °C/min till 150 °C and kept at 150 °C for 2 minutes in a constant flow mode of 1.5 mL/min. Mass spectra were recorded in full scan over a range of m/z 30-150 in selected ion monitoring mode.

Results and Discussion

Formation of sulfurous volatiles from protein

Heating a 30 mg/mL β-lactoglobulin solution (1,639 µM, containing 1 cysteine and 5 methionine residues) resulted in the formation of 58 µg/L (1.7 µM) hydrogen sulfide (H_2S) and 19 µg/L (0.41 µM) methanethiol (MeSH) (table 1). The molar amount of cysteine residues (-SH) in 30 mg/mL β-lactoglobulin is 1,639 µM, while the molar amount of H_2S (1.7 µM) formed after heating β-lactoglobulin is approximately 5,000 times less (0.02%). The molar amount of methionine residues ($-SCH_3$) in 30 mg/mL β-lactoglobulin is 8,200 µM, while the molar amount of MeSH formed (0.41 µM) is approximately 20,000 less (0.005%).

Tabel 1. Presence of H_2S and MeSH in GC headspace after 2 h heating of components at 80ºC, pH 7.0.

Components*	Concentration component (mg/mL)	Concentration component (µM)	Concentration H_2S (µM)	MeSH (µM)
β-LG	30	1,639	1.7	0.41
L-cysteine	0.50	4,130	1.4	<0.002
L-methionine	1.69	12.881	<0.003	0.22
β-LG + L-cystine	30 + 0.1	1,639 + 434	<0.003	<0.002
β-LG + PDS	30 + 0.1	1,639 + 555	<0.003	<0.002
β-LG + soluble peptides	30 + 6	1,639 + 1,000	<0.003	<0.002
β-LG + L-cysteine	30 + 2	1,639 +16,529	6.7	<0.002
WPI	30	1,639	1.5	0.53
WPI + soluble peptides	30 + 6	1,639 + 1,000	0.32	0.009
Lysozyme	30	2,083	0.22	0.07
lysozyme + soluble peptides	30 + 6	2,083 + 1,000	0.01	<0.002

* β-LG = β-lactoglobulin; WPI = whey protein isolate; PDS = dipyridyl disulfide

The concentrations of the volatiles H_2S and MeSH formed are higher than the threshold values for olfactory detection of the volatiles (0.29 µM (10 µg/L), and $0.43*10^{-3}$ µM (0.02 µg/L) in water, for H_2S and MeSH respectively.[13] Hence, heating β-lactoglobulin (30 mg/mL) at a concentration which is comparable to that of milk, leads to formation of the volatiles H_2S and MeSH, which are sensorial detectable. In addition to the formation of H_2S and MeSH, also dimethyl sulfide (DMS), dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) were identified in and quantified β-lactoglobulin. The amounts were a factor 10-50 times lower (<0.02 µM) than the amount of MeSH. Therefore, in the rest of this study, only the amounts of H_2S and MeSH are used as indicators for the formation of sulfurous volatiles.

Tests with heating different proteins showed that the amount of H_2S formed depends on the protein (table 2). To compare the results, the amount of formed H_2S was recalculated using the amount of free cysteine, or total cysteine present in the proteins. After heating β-lactoglobulin, $1.04*10^{-3}$ mole H_2S is formed per mole free SH present (0.1%). In WPI, less than half of this amount of H_2S is formed ($0.45*10^{-3}$ mole H_2S/mole SH (0.05%)). Considering the number of total S (SH+SS) from the cysteine residues (= 5) present in β-lactoglobulin and WPI, $0.21*10^{-3}$ and $0.09*10^{-3}$ mole H_2S/mole total S was formed, respectively. Heating 30 mg/mL lysozyme, which contains no free SH groups, resulted in the formation of $0.03*10^{-3}$ mole H_2S/mole total S. Although the amount of H_2S present in a heated lysozyme (no SH) solution is low, the presence cannot be explained at this moment.

Table 2. Molar amount of H_2S formed per mole free SH or mole total S (SH+SS) from all cysteine residues in heated proteins/amino acid (2 hour at 80°C, pH 7.0).

Protein/amino acid	#SH/[# total S]	mole H_2S/mole SH	mole H_2S/mole total S
β-Lactoglobulin	1[5]	$1.04*10^{-3}$	$0.21*10^{-3}$
WPI	1[5][a]	$0.45*10^{-3}$	$0.09*10^{-3}$
Lysozyme	0[4]	n.p.[b]	$0.03*10^{-3}$
L-Cysteine	1[1]	$0.42*10^{-3}$	$0.42*10^{-3}$

[a] based on β-lactoglobulin as main component of WPI
[b] not present

Form these results it could be suggested that the presence of H_2S decreases with the decreased purity of the sample (WPI < β-lactoglobulin < L-cysteine). From literature it is known that in milk systems β-lactoglobulin is the main source for the formation of these volatiles.[14, 15] It was suggested that a diketone, for instance from reducing sugars, has to be present for the formation of H_2S and MeSH via the so-called Strecker degradation. Since there is little information on the reactivity of the related amino acids (cysteine and methionine) in pure systems and the above mentioned observation of H_2S formation in β-lactoglobulin systems without the presence of diketones, model experiments were performed.

Formation of sulfurous volatiles from single amino acids

Heating of pure L-cysteine (4,130 μM) and L-methionine (12,881 μM) solutions resulted in the formation of only H_2S (47 μg/L, 1.4 μM) in the case of L-cysteine and only MeSH (10 μg/L, 0.22 μM) in the case of L-methionine (Table 1). These values correspond to the formation of $0.33*10^{-3}$ mole H_2S/mole SH and $0.02*10^{-3}$ mole MeSH/mole SCH_3, respectively. These values indicate that the conversion of free L-cysteine into H_2S is 3 times less effective than for the cysteine residues present in β-lactoglobulin. This could be due to the presence of the charged amino and carboxylate group in the vicinity of the SH group in free L-cysteine. These experiments confirmed the above mentioned hypothesis that heating of pure protein or amino acid solutions (without the presence of a diketone) in water is already sufficient to form significant amounts of these sulfurous volatiles.

Effect of disulfides components on volatile formation during heating

When β-lactoglobulin was heated in the presence of solutions of L-cystine (434 μM) or dipyridyl disulfide, PDS (555 μM) no H_2S and MeSH were detected (table 1). The above described results confirmed previous experiments where L-cystine was added to heated milk and milk products.[9] Those authors proposed a mechanism in which the removal of one mole H_2S by a disulfide yields two mole of L-cysteine as a reaction product. While this seems plausible, it does not explain

why the two formed cysteine residues do not react further to form H_2S as was shown to happen in the experiments described above.

Similarly to L-cystine, in the presence of soluble peptides (500 μM SS) no volatiles were formed. From previous work[10] it is known that the soluble peptide fraction of a β-lactoglobulin hydrolysate contains six different disulfide containing peptides (table 3). The addition of peptides also reduced the amount of volatiles formed after heating WPI solution.

Table 3. Disulfide containing peptides identified in the soluble fraction of a β-lactoglobulin hydrolysate.[10]

Observed mass (Da)	Theoretical mass[a)] (Da)	Possible fragment	Peptide sequence[b)]	Concentration in 13.5 mg/mL soluble peptide fraction (μM)[c)]
876.3	876.5	B [f63-67]=[f159-161]	E-NGECA=QCH	412
984.5	984.5	B [f106-108]=[f115-119]	E-CME=QSLAC	135
1499.8	1499.8	AB [f66-74]= [f159-162]	E-CAQKKIIAE=QCHI	388
979.3	979.4	B [f63-68]=[f158-160]	E-NGECAQ=EQC	694
1800.0	1800.0	B [f63-74]=[f159-162]	E-NGEQACKKIIAE=QCHI	355
3572.6	3572.8	A [f97-112]=[f115-128]	D-TDYKKYLLFCMENSAE= EQSLVCQCLVRTPE	35

a) Theoretical mass derived from ExPASy, SIB, Switzerland ($[M+H]^+$).
b) Peptide sequence starting with residue after which the BLP enzyme has cleaved. The "=" sign represents the disulfide bond.
c) The concentrations of the peptides were calculated according to equation 4 in chapter 3.

In other experiments, the peptides showed also more than 4 times reduction of H_2S and MeSH formed after heating of 30 mg/mL ovalbumin and 30 mg/mL gluten (data not shown). This indicates that the soluble peptides react with the sulfurous volatiles formed after heating.

To test this in more detail, β-lactoglobulin was heated in the presence of different concentrations of either L-cystine or soluble peptides (figure 1). Even though pure β-lactoglobulin alone forms 1.7 μM H_2S (table 1), 434 μM L-cystine and 500 μM (based on moles of SS) soluble peptides are needed to completely reduce the amount of detected volatiles. This shows that the suggested 1:1 molar ratio[9] is an underestimation.

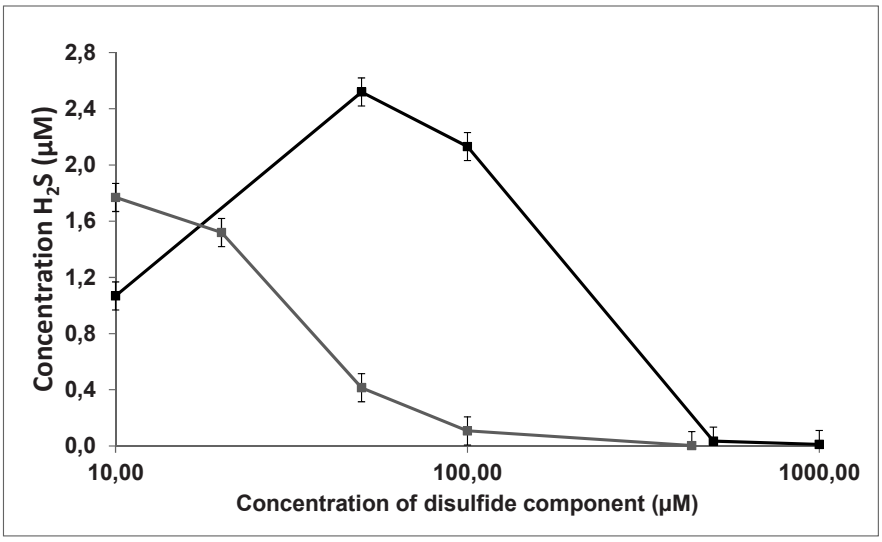

Figure 1. The concentration H_2S formed after heating (2 hours 80°C) of 1,639 µM β-lactoglobulin in the presence of different concentrations L-cystine (grey), or soluble peptides (black) were added to the protein before heating.

Reduction of sulfurous volatiles by disulfide containing peptides at room temperature

While the addition of L-cystine or soluble peptides during the heating of proteins clearly showed a decrease in the amount of volatiles detected, the mechanism is not clear. To test whether the volatiles formed upon heating react with the disulfide components, tests were performed at room temperature. To investigate the minimal amount of disulfides needed for the complete removal of the volatiles, L-cystine or soluble peptides were added in concentration series of 10-1,000 µM (based on SS content) to cooled down solutions containing H_2S formed from heating a 1,639 µM β-lactoglobulin solution (figure 2A).

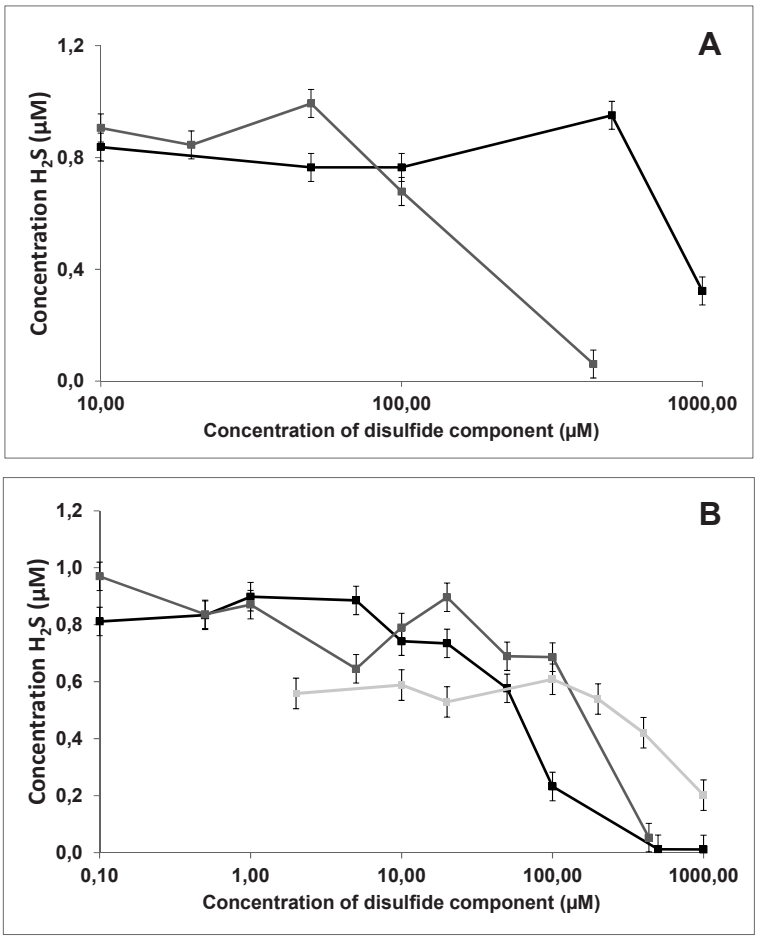

Figure 2. A: The concentration H_2S determined in the presence of L-cystine (grey) and soluble peptides (black) added to heated 1,639 µM β-lactoglobulin (2 hours 80°C). **B:** The concentration H_2S determined in the presence of L-cystine (grey), alkylated peptides (light grey) and soluble peptides (black) added to preformed H_2S.

From figure 2A it is clear that 500 µM of L-cystine was needed to remove the H_2S formed, while 1,000 µM soluble peptides was not enough to remove H_2S completely. This difference in efficiency was not so clear in the case of adding concentration series of 0.1-1,000 µM L-cystine and soluble peptides to 0.73 µM preformed H_2S (figure 2B) instead of heated β-lactoglobulin. The minimal concentrations for the complete removal of H_2S by L-cystine and the soluble

peptides were 434 µM and 500 µM, respectively. Removal of the pre-formed H_2S shows that the disulfide components can react with the volatiles once they are formed. The effect of the peptides in the β-lactoglobulin hydrolysate is thought to be predominantly due to the presence of disulfide bridges within some peptides. This was tested by reducing the disulfide bridges in the peptides and subsequent alkylation of the reduced cystine residues (figure 2B). Even in the presence of alkylated peptides the amount of H_2S is reduced. However, the efficiency of the alkylated peptides seems to be 5 and 10 times less than the soluble peptides and L-cystine, respectively. The observation that alkylated peptides also reduce the presence of H_2S indicates that H_2S is not only reacting to disulfide bridges, but also interacts other groups present in the peptides.

Conclusion

In this study it was shown that sulfurous volatiles can be formed by heating free cysteine and methionine in aqueous solutions. Interestingly, the presence of L-cystine, or other disulfide-containing peptides reduces the amount of formed volatiles. Disulfides do not prevent the formation of the volatiles, but react with them once they are formed. The hydrolysate from β-lactoglobulin was found to be as effective as L-cystine in removing H_2S in the stock solution, but less effective in reducing the volatiles formed after heating β-lactoglobulin. Thus, disulfide-containing peptides can be used as a natural ingredient in food to reduce the presence of unwanted sulfurous volatiles.

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Chapter 7

General Discussion

As described in the introduction of this thesis, the aim of this work was to study the effects of peptides and protein hydrolysates on the heat-induced aggregation and gelation of (concentrated) protein systems. The background for this aim is the observation that in high protein foods, the product hardness increases during storage due to protein-protein interactions.[1] At the start of this thesis work it was not known how the peptides in the hydrolysate affect these properties. The peptides could bind to the native protein, thereby affecting the denaturation process. Alternatively, the peptides could bind to the unfolded protein. In both cases, the binding of the peptides can be due to either specific or non-specific non-covalent interactions with the protein, or by formation of covalent bonds between the peptides and the proteins. To investigate whether individual peptides in the hydrolysate have specific contributions to the aggregation and gelation, the binding of peptides to intact proteins and the subsequent effects on aggregation and gelation were studied.

For these studies, a hydrolysate (DH 6.8%) of β-lactoglobulin made by *Bacillus licheniformis* protease (BLP) was used. At this degree of hydrolysis, most of the intact protein is hydrolyzed, while at the same time the peptides formed are not completely hydrolyzed. At pH 7, the proteinaceous material in this hydrolysate is not 100% soluble, whereas the parental protein is. In previous work[2], a whey protein isolate (WPI) hydrolysate (DH 6.8%) obtained with the same enzyme was separated by centrifugation (21,000 * g; 20 min; 10 °C) into a soluble and an insoluble fraction. The whey protein isolate used in that study contained mostly β-lactoglobulin (β-LG, 74.0% (w/w)), next to α-lactalbumin (α-La, 12.5% (w/w)), bovine serum albumin (BSA, 5.5% (w/w)) and immunoglobulins (5.5% (w/w)). In that work, the effects of the insoluble fraction on protein aggregation were studied. This choice was based on the hypothesis that peptides that aggregate with each other, and hence yield a precipitate, could also aggregate with proteins. Indeed, peptide-protein aggregates were identified upon the addition of the insoluble fraction of the hydrolysate to the parental protein. As a continuation of that

research, the current study focuses on the soluble fraction of a β-lactoglobulin hydrolysate.

Solubility of the peptides

The β-lactoglobulin hydrolysate made by *Bacillus licheniformis* protease (BLP) incubation was separated into a soluble and insoluble fraction at pH 7.0 when centrifuged (21,000 * g; 20 min;10 °C). Using a newly developed approach to quantify the peptides in RP-UPHPLC[3], the molar concentrations of the peptides in the total hydrolysate and in the supernatant and pellet fractions was determined (chapter 3). The results showed that 5 out of 28 peptides were identified solely (>98%) in the supernatant. All other peptides were present in both the pellet and the supernatant. The relative abundance of these peptides in the supernatant, being defined as mole peptide supernatant/mole total hydrolysate, in the supernatant varied from 12-98 %. This shows that, contrary to the suggestions from Creusot and co-workers[4], no clear distinction can be made between 'soluble' (non-aggregating) or 'insoluble' (aggregating) peptides in the hydrolysate. Moreover, two peptides ([f1-45] and [f135-158]) that were identified by Creusot and co-workers[4] as dominating the properties of the 'insoluble' fraction were found to be present in the supernatant of our hydrolysate for 12 and 41%, respectively. Interestingly, in chapter 4 it was found that in an isolated system the peptide [f135-158] was still soluble at 250 μg/mL, while in the hydrolysate the concentration in the supernatant was only 34 μg/mL. This indicates that, taking into account that 59% of the peptide was present in the pellet, the insolubility of this peptide in the hydrolysate is due to complexation with other peptides, rather than to the solubility characteristics of the peptide itself.

Hydrolysis of β-lactoglobulin by BLP and V8

At the start of the thesis work, purified β-lactoglobulin was hydrolyzed with the highly pure and Glu-specific protease *Staphylococcus aureus* V8 (V8). The use of both a pure protein and a pure enzyme allows the correct mass spectrometric (MS)

identification of the peptides formed enabling the introduction of SELDI-TOF-MS as analysis technique (chapter 2). In later work, the enzyme was changed to *Bacillus licheniformis* protease (BLP), used for hydrolysis of β-lactoglobulin and whey protein isolate (WPI). BLP is also a Glu-specific enzyme, but available in larger quantities enabling to produce large quantities of protein hydrolysate necessary to determine the effect on gelation (chapter 5). It has been described that BLP has a small side-activity towards Asp residues (1,000 times less than towards Glu residue).[5] In addition, the use of WPI also enables us to investigated whether the effects observed in the β-lactoglobulin system also occurs in the WPI system, which is representing industrial whey protein preparations

The activities of the proteases V8 and BLP were determined using an azocasein substrate, and found to be quite similar (0.07, and 0.09 μmol substrate*min^{-1}*μL^{-1}, respectively). While both the known enzyme specificity and the activity on azocasein are equal, the hydrolysis rate on β-lactoglobulin for BLP was 50 times faster (figure 1A). A difference between the two enzymes was also reflected in the in the visual turbidity of the solutions during and after hydrolysis. While the protein BLP-containing protein solution became turbid, the V8-containing protein solution remained transparent. As mentioned above, BLP exhibits side activity towards Asp residues, but to a 1,000 times lesser extent than towards Glu residues. Hence, we postulate that this side activity could not be reason for the faster increase in DH as function of time. Further analysis of the hydrolysates showed differences in the decrease of intact β-lactoglobulin as function of the degree of hydrolysis (DH) (figure 1B).

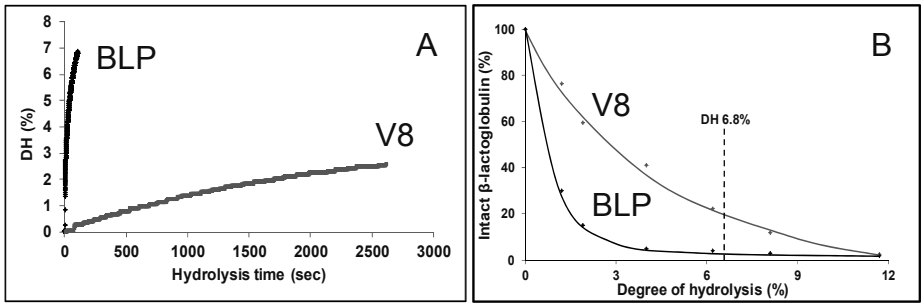

Figure 1. Degree of hydrolysis versus time for hydrolysis of β-lactoglobulin (5 mg/mL) by BLP (black) and V8 (grey) (pH 8, T=40 °C, based on same activity) **(A)**. The relative amount of intact β-lactoglobulin after hydrolysis by the protease BLP (black) and V8 (grey) as function of the degree of hydrolysis. The dotted line indicates DH 6.8% **(B).**

The amount of intact protein as function of DH decreased faster in the case of BLP than for V8 at the same system conditions (temperature, pH). Since the DH represents the number of peptides bonds split, this suggests that the affinity of BLP is higher towards the intact protein than towards the peptides formed, while for V8 this is the opposite. The peptide profiles of both hydrolysates were determined using RP-UHPLC and shown in figure 2.

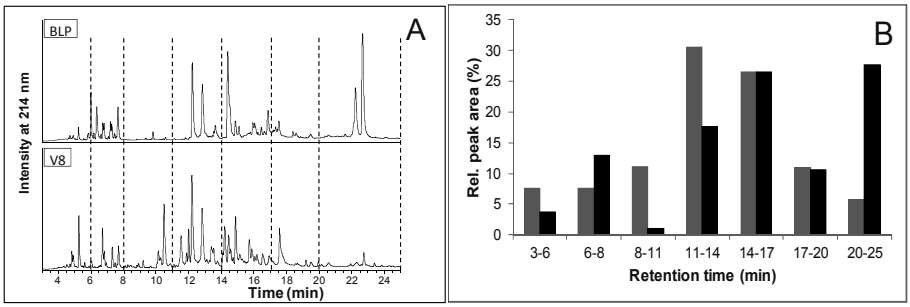

Figure 2. RP-UPLC chromatogram of β-lactoglobulin hydrolysate made by BLP and V8. The dotted lines indicate the retention time intervals**(A)**. The relative peak areas of V8 (grey) and BLP (black) at these intervals **(B)**.

In general, in RP-UPLC chromatograms peaks with shorter retention times can be related to smaller peptides[6] which possess relatively more charge than large peptides. The RP-UHPLC chromatograms showed that the elution pattern of the V8 shifted towards shorter retention times and hence, smaller peptides in

comparison to the BLP hydrolysate (figure 2A). This is once more illustrated by relative peak areas at different retention time intervals (figure 2B). The differences in elution patterns were also seen by analyzing the peptide composition of the V8 and BLP hydrolysates on MS (table 1). Although the hydrolysate were analyzed by different techniques (LC-MS for BLP and MALDI-TOF-MS for V8), it was clear that the peptide composition was (partially) different. A comparison of both hydrolysates with the same MS analysis technique should be made in order to see whether those results unambiguously confirm the RP-UPLC data (figure 2).

Table 1. Peptides identified in the hydrolysates of β-lactoglobulin obtained by the BLP and V8 enzyme.

Nr.	theoretical mass (Da)	possible fragment	peptide sequence	BLP (LC-MS)	V8 (MALDI)
1	432.2	AB [f52-55]	E-GDLE	X	
2	485.3	AB [f56-59]	E-ILLQ	X	
3	589.3	AB [f59-62]	L-QKWE	X	
4	683.4	AB [f46-51]	E-LKPTPE	X	
5	789.4	AB [f128-134]	E-VDDEALE	X	
6	876.5	B [f63-67]=[f159-161]	E-NGECA=QCH	X	
7	948.5	AB [f82-89]	V-FKIDALNE	X	
8	979.4	B [f63-68]=[f158-160]	E-NGECAQ=EQC	X	
9	979.4	AB [f131-138]	D-EALEKFDK	X	
10	984.5	B [f106-108]=[f115-119]	E-CME=QSLAC	X	
11	1026.6	AB [f66-74]+Na	E-CAQKKIIAE		X
12	1258.8	AB [f75-85]	E-KTKIPAVFKID	X	
13	1475.7	A [f115-127]	E-QSLVCQCLVRTPE		X
14	1499.8	AB [f66-74]= [f159-162]	E-CAQKKIIAE=QCHI	X	
15	1557.9	AB [f76-89]	K-TKIPAVFKIDALNE	X	
16	1800	B [f63-74]=[f159-162]	E-NGEQACKKIIAE=QCHI	X	
17	2309.2	B [f46-65]	E-LKPTPEGDLEILLQKWENGE		X
18	2436.3	AB [f138-158]	D-KALKALPMHIRLSFNPTQLEE		X
19	2553.4	AB [f90-110]	E-NKVLVLDTDYKKYLLFCMENS	X	
20	2555.1	B [f109-131]+Na	E-NSAEPEQSLACQCLVRTPEVDDE		X
21	2814.4	AB [f105-129]	L-FCMENSAEPEQSLACQCLVRTPEVD	X	
22	3606.9	AB [f1-33] +Na+2MeO	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLD		X
23	3695.9	AB [f12-45]	D-IQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEE	X	X
24	4006.2	AB [f1-38]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAP	X	
25	929.5	AB [f56-62]	E-ILLQKWE	X	X
26	966.5	AB [f45-53]	E-ELKPTPEGD (-H2O)	X	
27	1361.7	AB [f34-45]	D-AQSAPLRVYVEE		X
28	1482.7	AB [f12-24]	D-IQKVAGTWYSLAM (Met ox)	X	
29	1687	AB [f75-89]	E-KTKIPAVFKIDALNE	X	X
30	1903	AB [f29-45]	D-ISLLDAQSAPLRVYVEE		X
31	2335.2	AB [f90-108]	ENKVLVLDTDYKKYLLFCME		X
32	2696.6	AB [f135-157]	E-KFDKALKALPMHIRLSFNPTQLE	X	
33	2826.5	AB [f135-158]	EKFDKALKALPMHIRLSFNPTQLEE	X	X
34	2917.5	AB [f138-162]	D-KALKALPMHIRLSFNPTQLEEQCHI	X	
35	2993.6	B [f63-89]+Na	E-NGECAQKKIIAEKTKIPAVFKIDALNE		X
36	3307.7	AB [f135-162]	E-KFDKALKALPMHIRLSFNPTQLEEQCHI		X
37	3572.8	A [f97-112]=[f115-128]	D-TDYKKYLLFCMENSAE=EQSLVCQCLVRTPE	X	
38	4895.6	AB [f1-45]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLR VYVEE	X	X

Based on these differences, we propose a different hydrolysis mechanism for V8 than for BLP. This is schematically represented in figure 3. The mode of action of BLP is mainly based on the preference towards the intact protein, while V8 has a preference for cleaved proteins. This difference corresponds with the *one-by-one* and *zipper* reaction models according to the Linderstrøm-Lang theory as elaborated by Adler-Nissen (1986)[7]. In the *one-by-one* reaction model, the hydrolysate will consist of intact protein and small peptides. The lower initial rate of hydrolysis of β-lactoglobulin by V8 indicates that V8 has lower efficiency in hydrolyzing the intact globular protein than BLP. In addition, in the case of BLP aggregating peptides are formed. This will reduce the action of BLP towards the peptides. According to Creusot et al. (2006)[2], peptides from form aggregates upon hydrolysis and will not be hydrolyzed by the enzyme BLP.

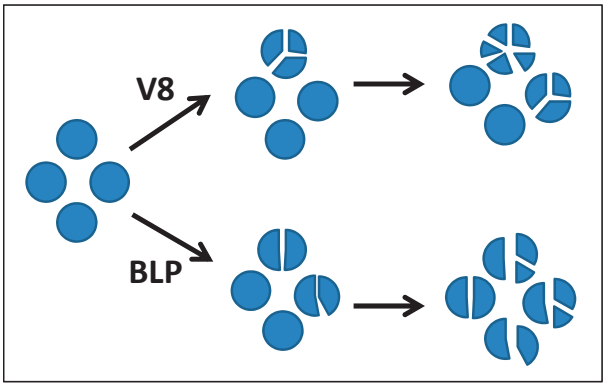

Figure 3. Schematic illustration of the different hydrolysis mechanisms between V8 and BLP.

Binding of peptides to native proteins

The first step in this study was to identify if certain peptides in the hydrolysate could bind native proteins. Such information (e.g. binding energy, stoichiometry) can be obtained from isothermal titration calorimetry (ITC).[8] This method was applied to study the binding of the peptides [f135-158] and [f135-162]-SH that were used in

chapter 4. In the measurements, a concentrated solution of peptides or of protein is added to the solution of the other component. In both cases, the titration of the concentrated solution in the measuring cell resulted in endothermic peaks, indicative of dissociation of complexes or aggregates (data not shown). Dissociation of peptide [f142-148] aggregates (endothermic peak) after titration into a buffer or a β-lactoglobulin solution was also found by Roufik et al.[9] Nonetheless, from the remaining exothermic signal after subtraction of a blank, these workers suggested a three-site binding model for this peptide. In our study this approach showed that the remaining exothermic signal was too low to interpret. Hence, association between proteins and peptides could not be clearly identified. Therefore, another technique was needed.

In chapter 2 surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) was found to be a fast and sensitive method to identify peptides that bind to proteins.[10] In this technique, the protein is bound covalently to a solid support. Next, the hydrolysate is added on the spot, allowing the peptides to bind to the protein. The non-bound peptides are subsequently washed away, after which the remaining peptides can be identified by matrix assisted laser ionization desorption mass spectrometry. In both the V8 and the BLP hydrolysate, only a small fraction of the peptides present were found to bind to the intact protein (six out of sixteen for V8, and five out of twenty-eight for BLP, table 2). In chapter 3 a principle component analysis was applied to identify whether the binding of peptides could be predicted based on the peptide properties (e.g. hydrophobicity, net charge).

Table 2. Identified β-lactoglobulin peptides binding to β-lactoglobulin at ambient temperature (20 °C) and elevated temperature (80 °C).

	Peptides binding at 20°C	Peptides binding at 80°C	Enzyme	$H\Phi_{tot}[H\Phi_{av}]$ (kcal) [(kcal/res)]	Net charge at pH 7.0
AB[f29-45]	X		V8	21.3[1.3]	-2.0
AB[f90-108]	X		V8	28.6[1.5]	-0.1
B[f63-89]	X		V8	33.1[1.2]	+0.9
AB[f45-53]		X	BLP	9.6[1.1]	-2.0
AB[f56-62]		X	BLP	12.3[1.8]	0.0
AB[f34-45]		X	BLP	15.4[1.3]	-2.0
AB[f12-24]		X	BLP	17.7[1.4]	+1.0
AB[f75-89]		X	BLP	21.7[1.4]	+1.0
A[f97-112]=[f115-128]	X		BLP	22.8[1.0]	-2.0
AB[f135-162]	X		V8	35.5[1.3]	+1.0
AB[f138-162]	X		BLP	31.4[1.3]	+1.1
AB[f135-157]	X	X	BLP	31.6[1.4]	+2.1
AB[f135-158]	X	X	V8/BLP	31.6[1.3]	+1.1
AB[f1-45]	X	X	V8/BLP	53.6[1.2]	-2.0

For those peptides that bound (at pH 7), SELDI-TOF could even be used to obtain an indication of the different affinities of binding. For this, the SELDI plate was washed with increasing concentrations of Triton-X 100 (Fig 5, chapter 2). Using this modification of the method, it was found that peptide [f1-45] bound more strongly than peptide [f135-158], indicative for the more hydrophobic character of peptide [f1-45]. These peptides were identified by others[4, 11] to be responsible for initiating aggregation capacities.

SELDI-TOF was also used to investigate the binding of peptides to ovalbumin, glycinin and β-casein (chapter 2), and the binding of pure peptides ([135-158] and [f135-162]-SH to ovalbumin and lysozyme (chapter 4). In both cases, the peptides that bound to β-lactoglobulin were also found to bind to the other proteins. This shows that the specificity for binding does not depend strongly on specific molecular details of the protein, but more on the properties of the peptides. Principal component analysis of the binding peptides (compared to the non-binding

peptides) indicated that the total net charge as well as the total hydrophobicity are important factors determining whether a peptide binds to the native protein. The effect of charge was studied by varying the pH of the peptide solution during binding to native protein on the SELDI plate. The amount of peptides that bound to the protein varied with the pH, and at pH 2.8 no binding of peptides was observed.
As described above, the association (binding) of the peptides from the hydrolysate could be dissociated by washing with Triton X-100, indicating non-covalent interaction between the peptides and the proteins. For the pure, cysteine-containing peptide [f135-162]-SH, an indication for covalent binding to the protein was observed when the peptide was applied to different proteins. The SELDI-TOF-MS signal intensity from the peptides decreased with the number of free sulfhydryl groups present in the protein. This indicates that the covalent disulfide binding between the protein and peptide was not broken during the MS analysis.
The observation that a significant number (but not all) of peptides in a hydrolysate can bind to intact proteins is quite new and indicates that binding of peptides to protein is a generic rather than a specific phenomenon.

Effects of binding peptides during protein unfolding upon heating

The binding of peptides was found not to lead to changes in the secondary structure of β-lactoglobulin, before or after heating (chapter 3). Small changes in the near-UV CD showed that the residues tyrosine and tryptophan were less solvent exposed, indicating binding of peptides in the vicinity of these residues. Since these residues are located on the backside of the calyx, this is another indication that the calyx is not involved. Hence, the peptide binding does not depend on this specific property of β-lactoglobulin. The denaturation temperature of the protein was not affected by the binding of peptides, but the enthalpy of unfolding of β-lactoglobulin decreased by 50% upon the addition of the soluble peptide fraction. (chapter 3). This observation seems to be in agreement with the reports from Barbeau et al.[12] They studied heat-induced unfolding of β-lactoglobulin in the presence of a total and a fractionated (by hydrophobic

interaction chromatography and anion exchange chromatography) tryptic hydrolysate. For the total hydrolysate, a decrease (9%) in the enthalpy was found, while for most of the hydrolysate fractions tested a small increase in the denaturation temperature was found, as well as an increase (approx. 30%) in the enthalpy of unfolding compared to β-lactoglobulin alone.

We attribute the decrease in enthalpy of unfolding to the binding of peptides to the unfolded protein. This is based on the identification of peptides present in the pellet obtained after heating (and centrifugation) of protein in the presence of hydrolysate (chapters 3 and 4). Eight peptides in the BLP hydrolysate were identified in the pellet of β-lactoglobulin-peptide mixture after heating (chapter 3). Of these peptides, only three were also found to bind to non-heated β-lactoglobulin. While for the five peptides from the BLP hydrolysate that bind at room temperature, a correlation was found with the total hydrophobicity, this correlation was not present for the peptides that bind to the unfolded protein. The importance of this is that apparently more peptides can bind upon the unfolding of the proteins, which indicates the possibilities for these peptides to influence the subsequent processes of aggregation and gelation.

Effects of binding peptides on heat-induced protein aggregation

Heating of β-lactoglobulin at 80°C, pH 7.0 in the presence of hydrolysate was found to result in formation of aggregates (chapter 3), while heating of the pure protein resulted in less aggregate formation (under these conditions). Since the hydrolysate contains many peptides, this effect could not be directly related to a specific property of binding peptides. The distribution of the hydrophobicity and charge over the binding peptides at 80 ºC is illustrated in figure 4.

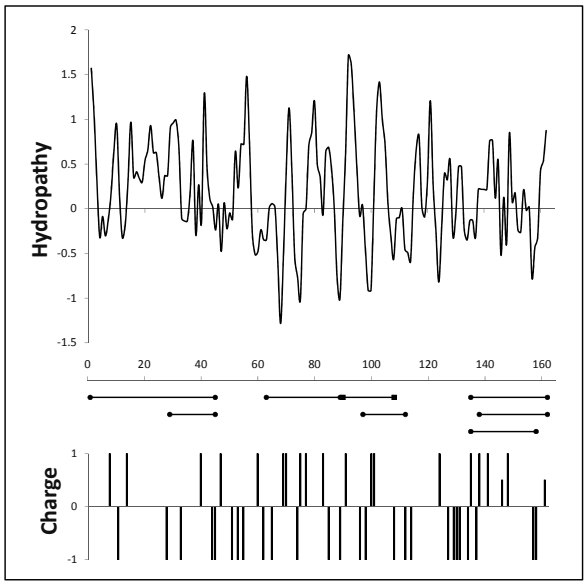

Figure 4. Distribution of hydrophobicity and charged residues in β-lactoglobulin (1-162) and the binding peptides at 80 ºC (as indicated by the horizontal lines).

From this graph it is clear that the binding peptides are originated from sequences distributed over almost the complete sequence of β-lactoglobulin. Peptide [f1-45] consists of mainly hydrophobic residues (hydropathy >0) and relatively low amounts of charged residues. This explains the poor solubility of this peptide and consequently, the abundant presence of this peptide in the insoluble peptide fraction (chapter 3). Peptides originated from the C-terminal end of the β-lactoglobulin sequence (135-162) consist of mainly positively charged residues, which is reflected in the high pH value for the isoelectric point (≈ pH 8).

In chapter 3 a principal component analysis (PCA) was performed to identify which peptide properties are correlated with their propensity to bind to intact proteins at room temperature. This analysis is now performed using all peptides identified in the V8 and in the BLP hydrolysates (figure 5). In the analysis, the binding to peptides is included in the dataset in the parameter b20, with values of 0 or 1. The binding of the peptides (b20) is most strongly correlated with the total length, total

hydrophobicity and the total positive charge. However, it must be noted that some peptides (25, 26, 27 and 28) are binding to the proteins, but have a negative charge, and apparently also a lower total hydrophobicity. Certain peptides (18, 22, 23, and 24) have not been identified as binding peptides. These peptides come from the same parts of the primary sequence and also share some of the properties. That these have not been identified in the SELDI-TOF-MS experiments can be due to low signal intensities (low ionisability), or a competition with the other binding peptides. It is clear that the combination of PCA can be used to adapt the experimental analysis techniques to further improve the understanding of the peptide properties that dominate the binding to intact proteins.

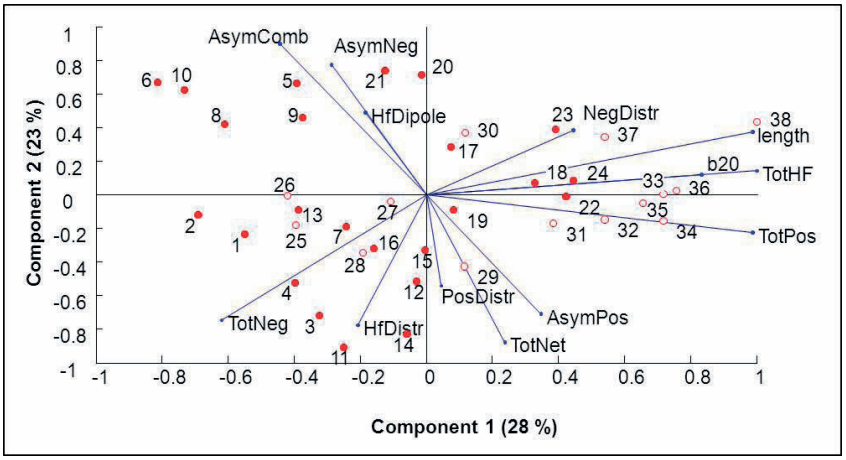

Figure 5. Principal component analysis plot of the peptides in the V8 and BLP hydrolysate. The numbers in the plot correspond to the numbers in table 1.

Based on the identification of peptides that bind to native protein by SELDI-TOF, one of the binding peptides was synthesized and used in chapter 4. This peptide (f135-158) also induced aggregate formation during heating with β-lactoglobulin, indicating that the binding of the peptide indeed altered the tendency of the unfolded proteins to aggregate. The effect of peptides on aggregation was studied

in more detail with respect to the role of charge, and the presence of free sulfhydryl groups.

Role of charge The effect of the presence of peptides on the amount of aggregated β-lactoglobulin varied with the pH applied (chapter 3, Fig. 7). At pH <3, the same amount of β-lactoglobulin aggregation was observed in the absence and presence of peptides. These results agree with the SELDI-TOF, where also no binding at this pH was observed. This suggests that the aggregation of β-lactoglobulin is (partially) driven by electrostatic interactions of the peptides with the protein. Also in literature, it has been observed that at these low pH values, little or no effect of the peptides was observed on the aggregation.[12] This is in contrast to the work of others.[13] They found that peptides were the building blocks of fibrils formed after heating β-lactoglobulin (20 h, 85 °C, pH 2). In addition, it was found that fibril formation could also be achieved by the hydrolysis of β-lactoglobulin by AspN endoproteinase, followed by lowering the pH to pH 2.[14] Probably at 85 ºC, chemical modifications of amino acid residues at pH 2 could play a role here.

Role of free sulfhydryl (SH) groups The effect of free sulfhydryl (SH) group of β-lactoglobulin on its aggregation has often been described.[15-17] From this, the general view has emerged that the free SH group play an important role in inducing the aggregation of the unfolded proteins. For example, the heating of bovine β-lactoglobulin was found to lead to aggregation. When the free cysteine residue (C121) was modified in the presence of iodide[18], the heating does not result in aggregation and the modified protein refolds upon cooling down.[19-21] Also, in mixed protein solutions the effect of the presence of free SH groups on the aggregation has been shown. Heating of α-lactalbumin (containing no free SH) does not result in aggregation. The aggregation was induced by the addition of ovalbumin (containing 4 free SH groups) in a 1:1 (w/w) ratio.[22] While the effect of the SH groups (from intact proteins) on the heat-induced aggregation of proteins has been described widely, only a few studies examined the exact role of the presence of (free) SH groups in the solution. Addition of cysteine to α-lactalbumin (molar ratio 2:1[23] and 5:1[24]) or BSA[25] was found to result in increased

aggregation upon heating. At even higher concentrations of L-cysteine (molar ratio β-lactoglobulin:cysteine = 1:50) a reduction in the amount of (pressure induced) aggregation has been reported.[26]

In this study, the effect of free SH groups in the solution was studied by comparing the aggregation of β-lactoglobulin in the presence and absence of the synthesized peptides [f135-158], [f135-162]-SH, and the NEM-treated peptide [f135-162]-SX. The presence of [f135-162]-SH resulted in a marked increase of the aggregation, already at the lowest concentration of peptides (β-lactoglobulin:[f135-162]-SH = 1:0.1). In these experiments, an increase in the peptide concentration to a ratio of 1:5.6 further increased the aggregation. Both peptides that lacked the reactive SH group showed a similar amount of aggregation, which was approximately 10 times higher than in the absence of the peptides, but 10 times lower than in the presence of [f135-162]-SH. The addition of a small amount of peptides with a free SH group can increase the aggregation, while a similar increase in SH groups by the addition of more β-lactoglobulin does not. This shows that not only the presence of the reactive SH groups is important, but also the environment of this group. Addition of the peptide [f159-162]-SH resulted in a lower amount of aggregates of β-lactoglobulin compared to peptide [f135-162]-SH, but higher amount of aggregates compared to peptide [f135-158] and [f135-162]-SX. Once more, this indicates that the free sulfhydryl group is important in heat-induced aggregation.

Effect of binding peptides on gelation

The gelation of β-lactoglobulin was studied in the absence and presence of the pure peptides [f135-158], [f135-162]-SH, and the NEM-treated peptide [f135-162]-SX, since the former two were found to bind to the native protein. While the presence of all three peptides resulted in increased aggregation upon heating, it did not affect the critical gel concentration (C_{gel}) at a constant ratio of protein: peptide (1:48 on weight basis). Moreover, at concentrations > C_{gel}, addition of the peptides lacking the free SH group ([f135-158], [f135-162]-SX) showed gel strengths comparable to that of β-lactoglobulin alone. Even though these peptides

bind, and affect the aggregation, the gel strength measured by small deformation rheology was not affected. This could mean that other techniques should be applied to better characterize the gel properties, since small deformational rheology reflects only part of the characteristics of the gel.[27] A large effect on the gel strength was observed in the presence of peptide [f135-162]-SH with the reactive SH group (chapter 4). At a molar protein:sulfhydryl group ratio of 9:1 a 2.5 times increase in the gel strength was observed. A similar increase in gel strength upon the addition of cysteine to ovalbumin (at similar prot:SH ratios) has been observed by others.[28] The effect of reactive SH groups on gelation has also been observed for genetically modified variants of β-lactoglobulin showed increased gel strength with increasing number of cysteine residues introduced in the molecule.[29] In addition to the experiments with the synthesized peptides added to pure β-lactoglobulin, the effect of WPI hydrolysates with free or blocked sulfhydryl groups on gelation of WPI was studied (chapter 5). It was found that a gel formed from 75 mg/mL of hydrolysate (-SH) and 150 mg/mL WPI had the same gel strength as a 225 mg/mL WPI gel. Blocking of the reactive SH groups decreased the gel strength to similar values as the 150 mg/mL WPI gel. This again shows the very significant influence of the reactive SH groups. It must be noted, that the molar efficiency of the pure peptide [f135-162]-SH on β-lactoglobulin (ratio prot:SH = 9:1) is lower (approx. 10 times) than that of cysteine-containing peptides on WPI solution. In the same line, blocking the free sulfhydryl group of β-lactoglobulin results in decreased gel strength.[29-31]

Based on the light scattering experiments, it is concluded that the free SH groups in peptides have more effect on the aggregation than the free SH group of the intact β-lactoglobulin. However, in the gelation experiments with WPI this effect is less clear, since the gel strength reaches similar values after the addition of (SH) hydrolysate or of WPI. On the one hand, one might state that then the SH groups seem to be as reactive, as on the other hand, the average molecular weight of the peptides in the hydrolysate is 1/3 of the molecular weight of the intact protein,

which leads to the conclusion that the presence of the SH groups does yield an extra increase in gel strength.

The role of disulfide containing peptides in the removal of sulfur volatiles

Besides the effect of peptides on aggregation and gelation of proteins, it was found that peptides can affect the formation or release of sulfurous volatiles, such as dihydrogen sulfide (H_2S) and methanethiol (MeSH) in heated β-lactoglobulin solutions (chapter 6). While the formation of sulfurous volatiles has been studied often, [32-34], the exact mechanisms behind the formation of these volatiles is unknown. Furthermore, it is not clear exactly how the peptides could reduce the formation of such volatiles. It has been suggested that cysteine and methionine in the presence of diketones[34] yield the volatiles H_2S and MeSH, respectively. This is why most of the studies (e.g. Golovnja et al., 1980)[35] on the formation of these compounds by heating protein hydrolysates use reducing sugars during the heating step. Furthermore, these studies only focused on heating in dry state at high temperatures (>100 °C), while the formation of these volatiles is also known to result in the cooked flavor of milk.[32, 36] In our work on the formation of these volatiles, it was shown that they were already formed during heating of aqueous solutions of pure (>99%) L-cysteine and L-methionine, respectively. This indicates that for the formation of these volatiles the presence of diketones is not required, as has been suggested in literature.[34]

The presence of the soluble fraction of the BLP hydrolysate of β-lactoglobulin during the heating step resulted in a decrease in the amounts of H_2S and MeSH detected in the gas phase. This prompted the question how these peptides could affect the formation and/or release of these volatiles. It has been described[32] that the cooked flavor formed during heating of milk could be reduced by the addition of the disulfide component L-cystine (molar ratio β-lactoglobulin:cystine = 1:2). Therefore, the role of the disulfides present in the hydrolysate was studied. It was shown that addition of peptides to reach a molar ratio of protein:peptide cystine of 3:1 indeed completely removed the presence of H_2S from 30 mg/mL heated β-

lactoglobulin. Based on these results, the expectation was that during the heating of cysteine and methionine residues of the protein, the H_2S and MeSH formed would react with the disulfide bridges (cystine) present in the peptides. In addition, experiments showed that addition of peptides to a solution containing preformed H_2S and MeSH also reduced the amount of H_2S and MeSH present in the gas phase. At the moment it is not certain if this is solely due to chemical reactions between these compounds and the disulfide bridges, or that the volatiles might bind in other ways to the peptide material.

Concluding remarks

The starting point of this thesis was to identify whether individual peptides in a protein hydrolysate would have specific contributions to the heat induced aggregation and gelation of proteins. In this work, it was indeed found that certain, but not all, peptides in the hydrolysate show binding to native or unfolded proteins. Since the peptides were shown to bind to not only β-lactoglobulin, but also other proteins, it can be concluded that the binding does not depend on specific molecular details of the protein. With the hydrolysates, as well as with two synthesized peptides (modelled on those found in the hydrolysate) it was confirmed that the addition of these binding peptides has significant effects on the heat-induced aggregation. Not only textural properties, but also the presence of sulfurous volatiles from heated proteins was found to be affected/reduced by the addition of peptides. In this study, it was found that for each of the processes studied, different molecular properties were important. This is summarized in table 3.

Table 3. Overview of the effect and functionality of peptides on different parameters.

	binding at ambient T	binding at elevated T	denaturation T_d	amount aggregated material	gel strength G	presence sulfurous volatiles
effect of peptides	**0/+**	**0/+**	**0**	**+/++**	**-/+**	**-**
property of peptides	HΦ, z	unknown	unknown	HΦ, z	SH	SS

+ = positive effect; 0 = no effect; - = negative effect; HΦ = hydrophobicity; z = charge; SH = sulfhydryl group; SS = disulfide.

The hydrophobicity and charge were found to be important in determining the binding and the effect on aggregation. In the gelation experiments performed in this study a dominant effect was found for peptides containing free SH groups. While it is expected that the changes in aggregation behaviour, induced by the binding of non-SH containing peptides also affects the gel properties, this was not found with the techniques used. Since only certain, although not specific peptides show the binding to intact proteins, it may be expected that control over the hydrolysis process and, thereby the concentrations of such specific peptides, can be used to produce hydrolysates with specific functionalities in this respect.

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Summary

In this thesis the effects of peptides, or protein hydrolysates on the heat-induced aggregation and gelation of (concentrated) protein systems were studied. First, it was investigated whether specific peptides could influence the heat-induced denaturation and aggregation of intact proteins, and which peptide properties dominated the different interactions. Next, the effects of the peptides on the heat-induced gelation of intact proteins as a model for a potential high protein food system were studied.

In **chapter 2**, the use of SELDI-TOF-MS was introduced to identify which peptides present in a protein hydrolysate are capable to bind non-covalently to intact food proteins. A purified and well characterized β-lactoglobulin preparation was extensively hydrolyzed by the Glu-specific enzyme V8 from *Staphylococcus aureus*. The hydrolysate was composed of sixteen peptides, which covered 98% of the primary sequence of β-lactoglobulin (as determined from MALDI-TOF-MS). The complete hydrolysate being soluble at pH 7.0 was applied to ovalbumin, glycinin, β-lactoglobulin and β-casein covalently bound on a SELDI ProteinChip. Six peptides (AB [f29-45], AB [f90-108], AB [f138-158], B [f63-89], AB [f1-45], AB [f135-162]) were bound to these four different proteins with decreasing affinity to glycinin>ovalbumin>β-lactoglobulin>β-casein. Since the same six peptides bound to these four different proteins, it is concluded that the binding does not depend on unique, structural properties of the protein, but rather on properties of the peptides.
The same was observed in with peptides from β-lactoglobulin hydrolysate prepared using the Glu-specific enzyme *Bacillus licheniformis* protease (BLP) in (**chapter 3**). This hydrolysate was fractionated at pH 7.0 by centrifugation into a soluble and an insoluble fraction. Identification and quantification of the peptides in all three fractions by LC-MS showed that most of the peptides were present both the supernatant as the pellet. Therefore, no clear distinction between ‘insoluble’ and ‘soluble’ peptides can be made. From the twenty-six peptides identified in the soluble fraction, five peptides (A[f97-112]=[f115-128], AB[f1-45], AB[f135-157], AB[f135-158] and AB[f138-162]) bound to β-lactoglobulin at room temperature.

After heating of β-lactoglobulin in the presence of peptides, eight peptides were identified in the pellet formed, three of them belonging to the previously mentioned peptides. Principle component analysis revealed that binding at room temperature to β-lactoglobulin was related to the total hydrophobicity and the total charge of the peptides. Although only a few peptides were found to participate in heat-induced aggregation, suggesting specificity, principal component analysis was unable to identify specific properties responsible for this. The presence of the peptides caused a 50% decrease in denaturation enthalpy, while no change in secondary structure or denaturation temperature was observed. At temperatures between 75 and 85 °C, the addition of peptides resulted in a 30-40% increase of precipitated β-lactoglobulin. At pH < 6 no differences in the amount of aggregated β-lactoglobulin were observed, which indicates the lack of binding of peptides to β-lactoglobulin at those pH values as was also observed by SELDI-TOF-MS (**chapter 2**).

In **chapters 2 and 3** it was shown that certain peptides in a hydrolysate can bind to intact proteins, in **chapter 4**, two of these peptides; [f135-158] and [f135-162]-SH were used to study the binding of the peptides, as well as the subsequent effects on aggregation and gelation of intact β-lactoglobulin. The interaction between the peptides [f135-158] and [f135-162]-SH and the β-lactoglobulin was observed in the solubility behaviour of β-lactoglobulin at different pH's. The pure β-lactoglobulin was soluble over the total pH range (3-9) investigated. The pure peptides showed minimal solubility between pH 7 and 9. The β-lactoglobulin-peptide mixture showed a decreased solubility between pH 5 and 9. The binding was confirmed using SELDI-TOF MS. In this experiment it was found that [f135-158] bound equally to β-lactoglobulin, ovalbumin and lysozyme, once again indicating that the binding mechanism does not depend to specific molecular properties, or even the net charge of the protein. The mass intensity observed for peptide [f135-162]-SH decreased in the order ovalbumin>β-lactoglobulin> lysozyme. The number of free sulfhydryl groups in the protein decreases in the same order, indicating an additional contribution of SH/SS interactions.

While heating of either pure protein or pure peptide solutions did not result in aggregation, the mixtures did show heat-induced aggregation. Furthermore, the aggregation in the presence of [f135-162]-SH is 10 times higher than in the presence of [f135-158], which shows the additional influence of the free sulfhydryl group. A clear difference between these two peptides was also observed in the gel strength of heat-induced gels. The replacement of additional β-lactoglobulin by [f135-158] resulted in a decrease in gel strength, while replacement by peptide [f135-162]-SH increased the gel strength.

In **chapter 5**, the role of cysteine-containing peptides present in a whey protein isolate (WPI) hydrolysate (DH 6.8% and 9.2%) in the aggregation and gelation behavior of a WPI protein system was studied. Four cysteine containing peptides (A/B[f159-162]; A/B[f90-108]; B[f115-127] and B[f63-89]) were identified in the soluble fraction of the WPI hydrolysate. The presence of the hydrolysate during heating of intact WPI (80°C, pH 7.0) resulted in 5 times more aggregation of WPI compared to the WPI alone. Blocking of the free sulfhydryl groups in this hydrolysate by NEM did not change the amount of aggregates. Heating of a mixture of 150 mg/mL WPI and 75 mg/mL hydrolysate (80°C, pH 7.0) resulted in a slight increase of the storage modulus (1.4 times) compared to 225 mg/mL WPI solution. Heating of WPI in the presence of hydrolysate in which the free SH groups were blocked, resulted in a G' that was 16 times lower than that of the 225 mg/mL WPI solution, reaching the same values as a 150 mg/mL WPI solution. These results showed that the heat-induced aggregation and gelation of a WPI protein system can be affected by the presence of free sulfhydryl group.

During the studies on the effect of peptides on heat-induced aggregation and gelation of proteins, it was observed that they also had another effect. In **chapter 6**, the effect of disulfides-containing peptides on formation and presence of the sulfurous volatiles dihydrogen sulfide (H_2S) and methanethiol (MeSH) after heating of protein solutions was investigated. Heating of β-lactoglobulin (2 hours at 80°C, pH 7.0) resulted in the formation of H_2S and MeSH. It was shown that H_2S and MeSH was formed from the amino acids L-cysteine and L-methionine, respectively,

without the presence of diketones. The addition of L-cystine, dipyridyl disulfide and soluble peptides during the heating of β-lactoglobulin resulted in the complete absence of H_2S and MeSH. Alkylation of the soluble peptides led to an increase of the concentration of peptides (approx. 2 times) needed to decrease the concentration H_2S present. Finally, the removal of H_2S formed from β-lactoglobulin, WPI and lysozyme was also observed when disulfide containing peptides and L-cystine was added prior to heating at 80°C. In the case of β-lactoglobulin, L-cystine was more effective in the removal of H_2S than the soluble peptides. This study shows that the presence of disulfide-containing peptides reduces the presence of (heat-induced) sulfurous volatiles.

The main results obtained in the several chapters are discussed in **chapter 7** and a comparison of the enzymes used is made. Since only certain peptides exhibit the binding to intact proteins, it is expected that control over the hydrolysis process and, thereby the concentrations of such specific peptides, can be used to produce hydrolysates with specific functionalities in this respect.

Samenvatting

In dit proefschrift zijn de effecten van peptiden of eiwit hydrolysaten op de hitte-geïnduceerde aggregatie en gelering van (geconcentreerde) eiwit systemen bestudeerd. Allereerst is onderzocht of specifieke peptiden de hitte-geïnduceerde denaturatie en aggregatie van intacte eiwitten kunnen beïnvloeden en welke peptide eigenschappen de verschillende interacties domineren. Vervolgens zijn de effecten van peptiden op de hitte-geïnduceerde gelering van intacte eiwitten bestudeerd als model voor potentiele voedingsmiddelen met hoge eiwit gehalten.

In **hoofdstuk 2** is het gebruik van SELDI-TOF-MS geïntroduceerd om peptiden te identificeren die via niet-covalente interactie in staat zijn te binden aan intacte eiwitten. Een gezuiverd en goed gekarakteriseerd β-lactoglobuline preparaat werd gehydrolyseerd met behulp van het Glu-specifieke enzym V8 afkomstig van *Staphylococcus aureus*. Het hydrolysaat bestond uit zestien peptiden die 98% van de primaire sequentie omvatten (bepaald m.b.v. MALDI-TOF-MS). Het complete hydrolysaat dat oplosbaar is bij pH 7.0 werd toegevoegd aan ovalbumine, glycine, β-lactoglobuline en β-caseine, welke covalent gebonden waren op een SELDI ProteinChip. Zes peptiden (AB [f29-45], AB [f90-108], AB [f138-158], B [f63-89], AB [f1-45], AB [f135-162]) bonden aan de vier eiwitten met afnemende affiniteit voor glycinine>ovalbumine>β-lactoglobuline>β-caseine. Omdat dezelfde peptiden binden aan deze vier verschillende eiwitten kan worden geconcludeerd dat de binding niet afhankelijk is van unieke, structuureigenschappen van een eiwit, maar eerder van de eigenschappen van de peptiden.
Hetzelfde werd in **hoofdstuk 3** waargenomen met peptiden van een β-lactoglobuline hydrolysaat dat gemaakt was met behulp van het Glu-specifieke enzyme *Bacillus licheniformis* protease (BLP). Dit hydrolysaat werd bij pH 7.0 gefractioneerd door middel van centrifugatie in een oplosbare en onoplosbare fractie. Identificatie en kwantificering van de peptiden door middel van LC-MS in alle fracties gaf aan dat de meeste peptiden zowel in het supernatant als in het pellet aanwezig waren. Daarom kan er geen duidelijk onderscheid gemaakt worden tussen oplosbare en onoplosbare peptiden.

Van de zesentwintig peptiden die in de oplosbare fractie geïdentificeerd zijn, bonden er vijf (A[f97-112]=[f115-128], AB[f1-45], AB[f135-157], AB[f135-158] en AB[f138-162]) aan β-lactoglobuline bij kamertemperatuur. Na verhitten van β-lactoglobuline in de aanwezigheid van peptiden, werden acht peptiden in de gevormde pellet geïdentificeerd. Drie van deze peptiden behoorde tot de eerder genoemde peptiden. Principal component analyse liet zien dat binding bij kamertemperatuur aan β-lactoglobuline gerelateerd was aan de totale hydrophobiciteit en de totale netto lading van de peptiden. Alhoewel maar een paar peptiden deelnemen in de hitte-geïnduceerde aggregatie, duidend op specificiteit, was principal component analyse niet in staat om specifieke eigenschappen te identificeren. De aanwezigheid van peptiden veroorzaakte 50% reductie in de denaturatie enthalpie, terwijl geen verandering in de secundaire structuur en denaturatietemperatuur werd waargenomen. Het toevoegen van peptiden resulteerde in 30-40% toename van geprecipiteerd β-lactoglobuline bij temperaturen tussen de 75 en 85 °C. Bij pH < 6 werden geen verschillen in de hoeveelheid geaggregeerd β-lactoglobuline waargenomen. Dit duidt op het ontbreken van binding van peptiden aan β-lactoglobuline bij deze pH waarden, zoals ook door SELDI-TOF-MS analyses is waargenomen (**hoofdstuk 2**).

In hoofdstuk 2 en 3 werd aangetoond dat bepaalde peptiden in een hydrolysaat kunnen binden aan intacte eiwitten. In **hoofdstuk 4** werden twee van deze peptiden([f135-158] en [f135-162]-SH) gebruikt om de binding van peptiden alsmede de effecten op de aggregatie en gelering van intacte β-lactoglobuline te bestuderen. De interactie tussen de peptiden [f135-158] en [f135-162]-SH en β-lactoglobuline werd waargenomen in het oplosbaarheidsgedrag van β-lactoglobuline bij verschillende pH waarden. De pure β-lactoglobuline was oplosbaar over de totale pH range (3-9) die onderzocht werd. De pure peptiden lieten een minimale oplosbaarheid zien tussen pH 5 en 9. De β-lactoglobuline-peptide mix liet een verminderde oplosbaarheid zien tussen pH 5 en 9. De binding werd bevestigd door SELDI-TOF-MS. In dit experiment werd waargenomen dat er

evenveel binding van [135-158] aan β-lactoglobuline, ovalbumine en lysozyme was, wat opnieuw aantoont dat het bindingsmechanisme niet afhankelijk is van specifieke moleculaire eigenschappen, of zelfs de netto lading, van het eiwit. De waargenomen massa intensiteit voor peptide [135-162]-SH nam af in de volgorde: ovalbumine>β-lactoglobuline>lysozyme. Het aantal vrije sulfhydryl groepen in het eiwit nam af in dezelfde volgorde, waaruit blijkt dat er een extra bijdrage van SH/SS interacties is.
Hoewel het verhitten van pure eiwit of pure peptide oplossingen niet in aggregatie resulteerde, lieten de eiwit-peptide mengsels wel hitte-geïnduceerde aggregatie zien. De aggregatie in de aanwezigheid van [135-162]-SH is 10 keer hoger dan in de aanwezigheid van [135-158], wat de bijkomende invloed van de vrije sulfhydryl groep aantoont. Een duidelijk verschil tussen deze twee peptiden werd ook waargenomen in de gelsterkte van hitte-geïnduceerde gelen. Het vervangen van extra β-lactolgobuline door [135-158] resulteerde in een afname van de gelsterkte terwijl vervanging door [135-162]-SH een toename van de gelsterkte liet zien.

In **hoofdstuk 5** werd de rol van cysteine bevattende peptiden, aanwezig in een hydrolysaat (DH 6.8% and 9.2%) van een wei-eiwit isolaat (WPI), tijdens het aggregatie- en geleringsgedrag van een intact WPI eiwit systeem bestudeerd. Vier cysteine-bevattende peptiden (A/B[f159-162]; A/B[f90-108]; B[f115-127] and B[f63-89]) werden in de oplosbare fractie van het WPI hydrolysaat geïdentificeerd. De aanwezigheid van het hydrolysaat tijdens het verhitten van intacte WPI (80°C, pH 7.0) resulteerde in 5 keer meer aggregatie van WPI in vergelijking tot WPI zonder peptiden. Het blokkeren van de vrije sulfhydryl groep in dit hydrolysaat door NEM gaf geen verandering van de hoeveelheid aggregaten. Het verhitten van mengsels van 150 mg/mL WPI en 75 mg/mL hydrolysaat (80°C, pH 7.0) resulteerde in een kleine toename van de opslagmodulus, G’ (1.4 keer) in vergelijking tot 225 mg/mL WPI oplossing. Het verhitten van WPI in de aanwezigheid van hydrolysaat (80°C, pH 7.0), waarin de vrij SH groep was geblokkeerd, resulteerde in een G’ waarde die 16 keer lager was dan die van een 225 mg/mL WPI oplossing en gelijk was aan

de G' waarde van een 150 mg/mL WPI oplossing. Deze resultaten tonen aan dat de hitte-geïnduceerde aggregatie en gelering van een WPI eiwit systeem kan worden beïnvloed door de aanwezigheid van vrij sulfhydryl groepen. Tijdens de studies van het effect van peptiden op de hitte-geïnduceerde aggregatie en gelering van eiwitten werden ook nog andere effecten waargenomen. In **hoofdstuk 6** werd het effect van disulfide-bevattende peptiden onderzocht op de vorming en aanwezigheid van de zwavelachtige vluchtige verbindingen diwaterstofsulfide (H_2S) en methaanthiol (MeSH) na verhitten van de eiwitoplossingen. Het verhitten van β-lactoglobuline (2 uur bij 80°C, pH 7.0) resulteerde in de vorming van H_2S en MeSH. De resultaten lieten zien dat H_2S en MeSH werd gevormd uit respectievelijk de aminozuren L-cysteine en L-methionine, zonder de aanwezigheid van diketonen. Het toevoegen van L-cystine, dipyridyl disulphide en de oplosbare peptiden na het verhitten van β-lactoglobuline resulteerde in de volledige afwezigheid van H_2S en MeSH. Het alkyleren van de oplosbare peptiden leidde tot een toename van de hoeveelheid peptiden (ongeveer 2 keer) die nodig was voor afname van de aanwezige hoeveelheid H_2S. Het verwijderen van het gevormde H_2S uit β-lactoglobulin, WPI en lysozyme werd ook waargenomen wanneer de disulfide-bevattende peptiden en L-cystine vòòr het verhitten bij 80 °C werden toegevoegd. In het geval van β-lactoglobuline was L-cystine meer effectief in het verwijderen van H_2S dan de oplosbare peptiden. Deze studie toont aan dat de aanwezigheid van disulfide-bevattende peptiden de aanwezigheid van (hitte-geïnduceerde) zwavelachtige vluchtige verbindingen verminderd.

De belangrijkste resulaten die verkregen zijn in de verschillende hoofdstukken worden bediscussieerd in **hoofdstuk 7** waarbij ook een vergelijking van de gebruikte enzymen is gemaakt. Aangezien alleen bepaalde peptiden binding aan intacte eiwitten vertonen, wordt verwacht dat controle van het hydrolyse proces, en daarbij de concentraties van specifieke peptiden, gebruikt kan worden om hydrolysaten te produceren met specifieke functionaliteiten.

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Hans

Curriculum Vitae

Hendrik Albertus (Hans) Kosters is geboren op 25 november 1968 in Heerewaarden. In 1985 heeft hij zijn middelbare school aan de RSG Buys Ballot te Zaltbommel afgerond en vervolgens heeft hij in 1989 zijn diploma Middelbaar Laboratorium Onderwijs richting Microbiologie te ’s-Hertogenbosch gehaald. Hiervoor liep hij stage bij Unilever NV te Oss en Biochem BV te ’s-Hertogenbosch. Hij vervolgde zijn opleiding aan het Hoger Laboratorium Onderwijs aan de Fontys Hogescholen in Eindhoven met als studierichting Biochemie en Microbiologie waarvoor hij in 1993 zijn diploma heeft gehaald. Hiervoor deed hij zijn afstudeeropdracht bij DSM Food Specialties (voorheen Gist-Brocades NV) in Delft. In datzelfde jaar startte hij de opleiding International Master of Science (MSc) in Biotechnology aan de De Montfort University in Leicester (UK)/Hogeschool Avans. Begin 1995 sloot hij deze opleiding af met een afstudeeropdracht bij NIZO food research BV te Ede. Na een jaar (1995) in militaire dienst te zijn geweest is hij in februari 1996 bij NIZO food research BV begonnen als Scientific Assistant in uiteenlopende projecten. Per november 2007 kreeg hij van dit bedrijf de mogelijkheid om binnen het TI Food & Nutrition (TIFN) project “High Protein Foods” promotieonderzoek te doen bij de leerstoelgroep Levensmiddelenchemie aan de Wageningen Universiteit wat geleid heeft tot het schrijven van dit proefschrift. Momenteel is hij nog steeds werkzaam bij NIZO food research BV te Ede, nu als projectmanager Proteins/Hydrolysates.

Completed training activities

Discipline specific activities

Courses

Food Enzymology, Wageningen, 2008

Dutch Peptide Symposium, Eindhoven, 2008

Food Colloids, 2008, Le Mans1.1

Mass Spectrometry, Proteomics, Protein Technology, 2008, Amsterdam

Food hydrocolloids, 2009, Wageningen

NIZO Dairy Conference, 2009, Papendal

Delivery of functionality in complex food systems, 2009, Wageningen

Synapt Spectrometry Waters, 2010, Wageningen

NIZO Dairy Conference, 2011, Papendal

Meetings

Protein meetings Food Chemistry, 2007-2011, Wageningen

TIFN meetings, 2007-2011, Wageningen

Student presentation Food Chemistry, 2007-2011, Wageningen

Colloquia Food Chemistry, NIZO, 2007-2011, Ede-Wageningen

General courses

Information Literacy, 2008, Wageningen Graduate School

Afstudeer begeleiding, 2008, WUR docenten ondersteuning

Ph.D. Introduction week, 2008, VLAG

Time Management, 2009, TIFN, Wageningen

Mobilizing your scientific network, 2010, Wageningen Graduate School

How to write a world class paper, 2011, Wageningen University

Optionals

Preparation Ph.D. research proposal

Food Chemistry Ph.D. trip, 2008, China

Food Chemistry study trip, 2009, Gent University

List of Publications

Van Kraaij, C., Breukink, E., Rollema, H. S., Bongers, R. S., **Kosters, H. A.**, De Kruijff, B., & Kuipers, O. P. (2000). Engineering a disulfide bond and free thiols in the lantibiotic nisin Z. *European Journal of Biochemistry, 267*(3), 901-909.

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