The Formation and Deformation of Protein Structures with Viscoelastic Properties

Thesis committee

Thesis supervisors
Prof. dr. R. J. Hamer
Professor of Technology of Cereal Proteins
Wageningen University

Prof. dr. ir. R. M. Boom
Professor of Food Process Engineering
Wageningen University

Co-supervisor
Dr. A. J. van der Goot
Associate professor at the Food Process Engineering Group
Wageningen University

Other members
Prof. dr. ir. M. A. J. S. van Boekel
Wageningen University

Prof. dr. ir. K. Dewettinck
Ghent University, Belgium

Prof. dr. E. van der Linden
Wageningen University

Dr. Ir. P. L. Weegels
Sonneveld Group B.V., Papendrecht

This research has been conducted under the auspices of the Graduate School VLAG

Lieke E. van Riemsdijk

The Formation and Deformation of Protein Structures with Viscoelastic Properties

Thesis
Submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 16 March 2011
at 4 p.m. in the Aula.

Lieke E. van Riemsdijk
The Formation and Deformation of Protein Structures with Viscoelastic Properties

Thesis, Wageningen University, Wageningen, NL (2011)
With references, with summaries in Dutch and English

ISBN 978-90-8585-863-8

Al wist ik alles wat er te weten is
als ik geen liefde had, was ik niets.
(1 Korintiërs 13 : 2)

Contents

Chapter 1

Introduction

This chapter describes the motivation and the approach of the project on "the formation and deformation of protein structures with viscoelastic properties". The main objective of this study is to develop a dough with similar properties as dough with gluten but then with an alternative protein as structuring agent. This is of particular relevance to patients suffering from gluten intolerance (celiac disease). While the final gluten replacing ingredient is important for helping patients of celiac disease, the insight that is created in parallel, may well open routes into structuring of other types of products as well.

1.1 The Need for a Viscoelastic Protein Network

The prevalence of gluten intolerance (celiac disease) has increased dramatically in the last 20 years [1, 2]. Gluten intolerance has become a wide-spread disorder affecting about 1 percent of most populations [1, 3, 4], of which a large part is still undiagnosed [5]. For patients that suffer from gluten intolerance, the consumption of products containing wheat, rye or barley such as bread, spaghetti or beer has a negative health effect. Currently a life long gluten-free diet is the only effective remedy [6]. Unfortunately, a gluten-free diet is difficult to comply with for several reasons. First, wheat derived products and gluten are widely used. There is a long list of products containing (traces of) gluten. Patients have to be alert for hidden gluten in products [7]. Second, most gluten-free alternative food products are less attractive as their original variants [8]. Besides, the gluten-free products are more expensive. For example daily consumed products, such as bread and pasta, are twice as expensive as their wheat-based counterparts [9]. To make it easier to comply to a gluten-free diet a new generation gluten-free products is needed.

Early studies on the development of gluten-free products were reported by Rotsch in 1954, Jongh in 1968 and Kulp in 1974. These pioneers described the use of substances such as xanthan gum or glyceryl monostearate to replace gluten [10, 11]. Since then many studies followed aiming at better gluten-free products. The main focus was on bread, and some work has been done on gluten-free cookies [12], and pasta [13]. In general, two routes are followed to develop gluten-free products. The first route focuses on the use of gluten-free cereals such as oat, corn and buckwheat [14-18].

The second route explores the use of non-cereal ingredients (mostly hydrocolloids and emulsifiers) and gluten-free starches [19-27]. Despite all scientific studies, it remains difficult to find (a mixture of) ingredients that provides the proper properties to mimic the gluten functionality as present in wheat flour.

1.2 What Makes Wheat Flour Unique

The protein fraction in wheat flour is responsible for a large part of the properties of cereal products. The main proteins in flour are the gluten proteins, which represent between 80 and 85 % of the total wheat protein fraction [28]. Gluten is the water-insoluble fraction of flour. Depending on the flour type, approximately 10 - 14 % of the dry matter is gluten. The origin of the gluten properties in dough is related to the unique elastic [29], strain hardening [30] and self healing properties of gluten, which allows the formation of a strong, reversible network [31]. All these properties are essential to develop a dough that has the ability to retain gas during proving and baking. Gluten is the structure builder in flour and therefore gluten can not be removed without having a detrimental effect on the properties of dough and resulting product [8, 32].

Gluten contains a broad range of individual proteins [33]. The term gluten is often used for two types of proteins present in gluten; the glutenins and gliadins. Gluten proteins comprise about equal parts of these two fractions [28]. The glutenins are polymeric proteins, linked by intra-chain and inter-chain disulphide bonds [34-36]. Two types of glutenins are distinguished: low molecular weight (LMW) glutenin and high molecular weight (HMW) glutenin. The gliadins are monomeric proteins, which have only some intra-chain disulphide bonds [37, 38]. It is generally accepted that the glutenins and gliadins fulfil different roles in the gluten properties.

Glutenins affect the elastic properties of dough [29, 39]. Glutenins can form a network that can be stretched and can recover after deformation [40-43]. The gliadins do not have this network forming ability, but act as a plasticizer [34, 43] thereby affecting the viscous properties of the dough [29, 39]. Dough strength is a balance between the elastic and viscous properties of a dough. The elastic and viscous properties of gluten are illustrated in figure 1.

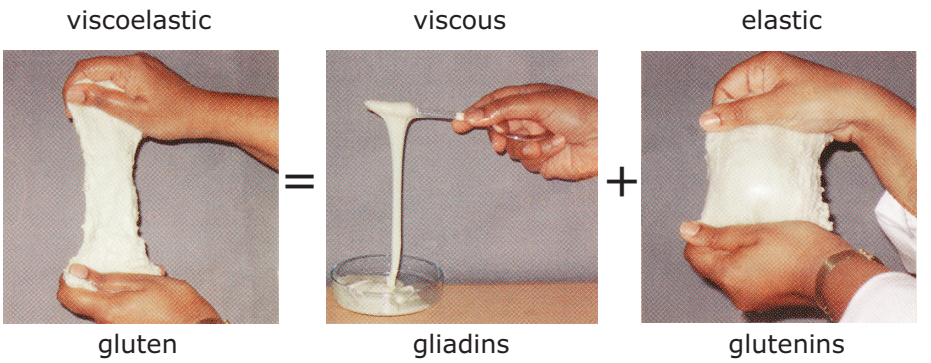

Figure 1: The viscous and elastic properties of gluten (reprinted from Uthayakumaran and Wrigley) [46]

Dough quality is not only determined by the relative amount of glutenins and gliadins, but also by the size and structure of the glutenin protein network. Especially the large insoluble glutenin polymers (glutenin macro polymer - GMP) fraction influences the dough properties. GMP consists of very large polymeric structures of both LMW and HMW glutenin subunits. This GMP-fraction is regarded a key factor, determining gluten network properties [44, 45].

1.3 The Gluten Network

The gluten network in developed dough has a complex structure that so far has not been unravelled completely. Nevertheless, there are different models describing the gluten structure. These models vary in their concept of the major causes for the formation of a gluten network. The earliest model correlates the ability to form a network to molecular properties. According to these first studies, dough properties originate from proteins which are linked via disulphide bonds thereby forming an elastic system [47, 48]. This molecular perspective was further developed by different groups. They focussed on the disulphide interactions and amount of branching crosslinks. There was a discussion between groups that stated that the glutenin consists of highly branched chains and groups stated that the glutenins have a linear character and the chains are bound by only one disulphide bond [49-54]. Others stated that glutenin have no intermolecular disulphide bonds but only intramolecular bonds. Those intramolecular disulphide bonds constrain the glutenin subunits, which favours the formation of non-covalent crosslinks (e.g. hydrogen bonds) between adjacent subunits [55, 56]. Two examples of those molecular gluten models are depicted in figure 2.

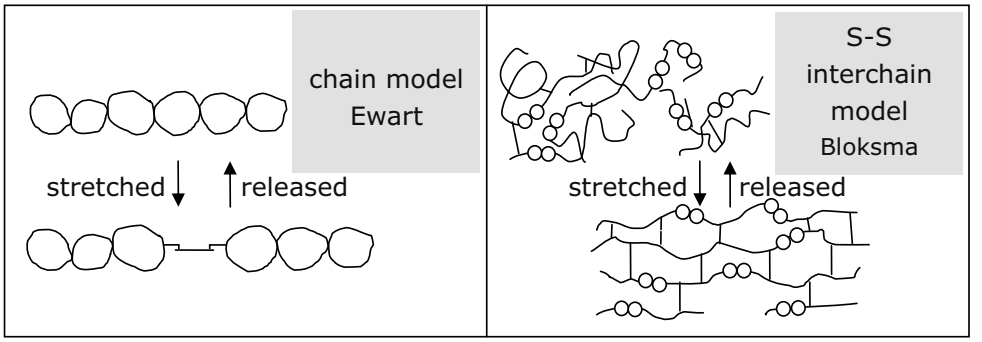

Figure 2: Some examples of molecular oriented gluten models

The earlier models were abandoned after it was generally accepted that the disulphide bonds could not account for all dough properties. More interactions have to be considered to better understand the structure formation properties of gluten. This new insight let to the development of new types of gluten models. Those models include physical interactions at a molecular scale [57, 58]. Two almost simultaneously developed models are the entanglement model [59, 60] and the loop-and-train model [61]. These gluten models are depicted in figure 3. The entanglement model is based on polymer physics. According to this model, there are small regions where the polymers interact (entanglements). Between those entanglements there are non-interacting regions. The non-interacting region can easily be deformed upon stretching. The entanglements cause regions where the polymer chains are stuck and are not able to move freely. The loop and train model is based on rubber elasticity. According to this model, there are regions with polymers interchain interactions (trains) and there are regions with polymer-solvent interactions (loops). On stretching, the network will first deform by deformation of the loops. When the extension is increased the trains are pulled apart so that the chains slip over each other [61, 62].

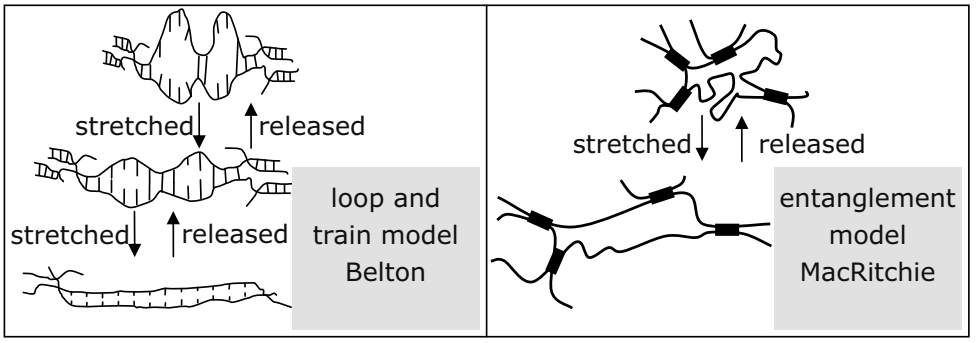

Figure 3: Some examples of physical oriented gluten models

Most of the gluten models that were previously mentioned, focus on the molecular structure of the glutenin proteins. There is one hypothesis, the hyper-aggregation model, which presents the mesoscopic structure as the essential element in building up the gluten network. According to this hypothesis, (part of) the gluten proteins are structured into mesoscopic particles [42, 63]. According to this model, the disulphide bonds between glutenin subunits are dominant at small length scales (< 1 μm), while the physical interactions (and some additional disulphide bonds) are dominant at larger length scales (1 - 100 μm). The interactions with non-protein constituents become relevant at even larger length scale (> 100 μm). This model assumes that mesoscopic (ca 10 – 100 μm) glutenin particles are the essential building blocks of the glutenin network. These soft protein particles can be deformed and disrupted to form a particle network which is held together by physical interactions [42]. Upon dilution in SDS, the protein particles form the so called GMP particles (figure 4), These GMP particles were demonstrated to be present in wheat flour [64] and wheat dough [42].

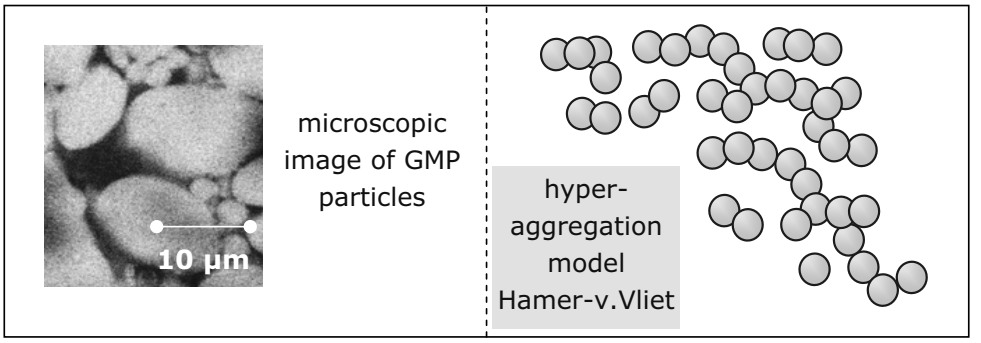

Figure 4: Microscopic image of GMP particles (left), these GMP particles are according to the hyper-aggregation model (right) the building-blocks of the gluten structure

The debate of the validity of the physical oriented models and the hyper-aggregation model in explaining the gluten behaviour is still ongoing [65-69]. The entanglement model is criticized because its explanation of gluten elasticity is not convincing [62] and it lacks a clear idea about the effect of solvent quality on gluten properties [68]. However, others state that the entanglement model has the least ad hoc assumption compared to other models (e.g. the loop and train model) [65, 66]. For both the entanglement model and the loop and train model, it is questioned if they can explain the macroscopic behaviour of gluten [66]. The Hyper-aggregation model is discussed because it does not explain how a colloidal particle gel can simultaneously produce shear thinning and strain hardening at large extensional strains. The microscopic images of GMP particles are debated, and suggested that these can be due to the preparation process [68-70].

1.4 Towards Gluten-Free Products

1.4.1 A New Approach: Creation of a Gluten Substitute

The main objective of this study is to develop a dough with similar properties as dough with gluten but then with an alternative protein as structuring agent. The route followed in this thesis is a step-by-step approach. Rather than concentrating on the analysis of gluten network properties, and only finally attempting to develop a gluten substitute, we chose for compiling the existing insight into the design of protein ingredients, and comparing their properties with those of gluten. By following this route, the design process itself leads to refinement of insight on those aspects that need improvement.

While the final gluten replacing ingredient is important for helping patients of celiac disease, the insight that is created in parallel, may well open routes into structuring of other types of products as well.

This research therefore focuses on mimicking the gluten functionality by creating a particle network on the mesoscopic level. Several authors showed that controlling the mesoscopic structure has much potential in developing a new category of food products, such as meat alternatives [71] and fat substitutes [72]. The particles to be designed should have similar properties as the gluten particles described by the hyper-aggregation model. This means that the particles have to be soft and swollen in water, and have to be susceptible to mechanical disruption upon (dough) kneading. In addition, the particle fragments obtained after mixing should be able to re-aggregate into larger structures that percolate in three dimensions, giving the required dough viscoelastic properties [42].

The first step in this research is therefore to produce particles that have the correct mesoscopic structure. Protein particles can be created by mixing solutions of protein and another biopolymer with low compatibility [73, 74], which induces phase separation, leading to swollen protein containing domains (particles) in a matrix that contains the other biopolymer. The resulting suspension can be added to a gluten-free flour, which is further processed. The method to produce protein particles and a protein particle bread are schematically depicted in figure 5.

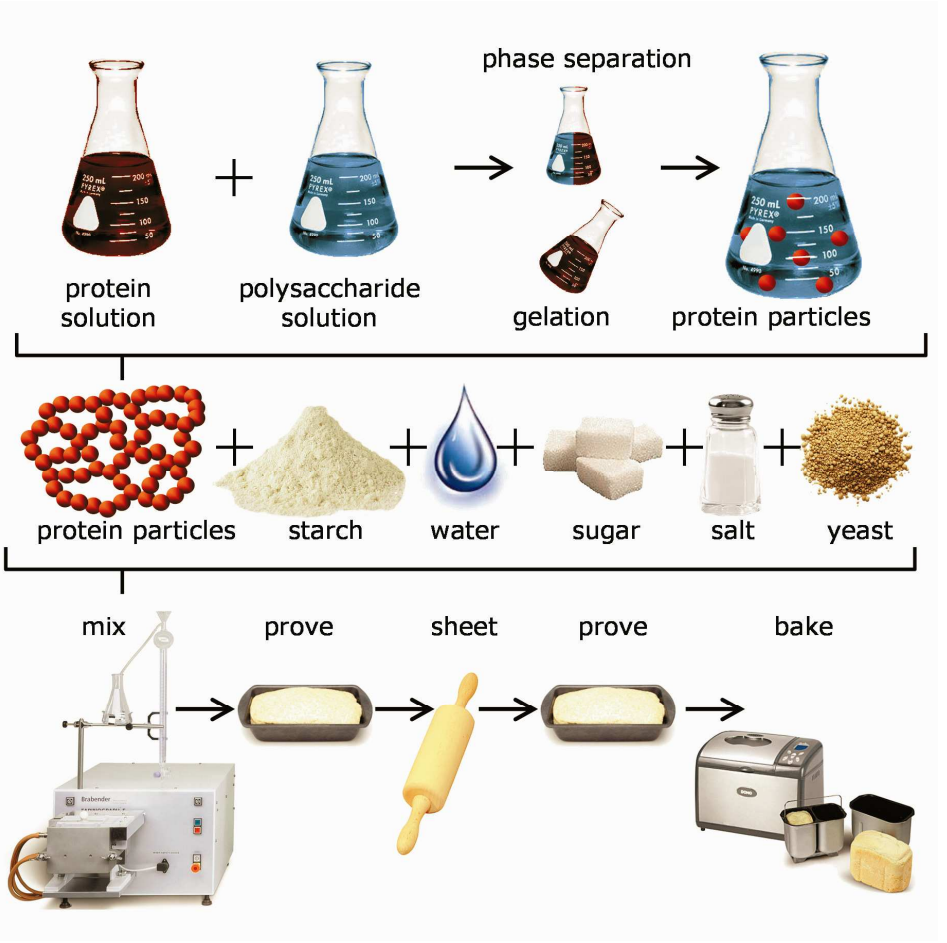

Figure 5: Schematic representation of the methodology of the research

1.4.2 Description of the Chapters

The goal of this study is to investigate the properties that are necessary to obtain a gluten substitute. The hypothesis guiding this work was that, at least part, of the functionality of gluten is due to its (mesoscopic) network. To obtain this goal this study can be divided in two parts. Part I (chapters 2 - 4) focuses on the formation of protein particles, and the characterisation of the protein particle suspensions. Part II (chapters 5 - 7) focuses on the application of the protein particle system in a gluten-free formulation. The outline of this study is summarized in figure 6.

Chapter 2 describes the formation and properties of protein particle suspensions. Two proteins with different intrinsic properties, gelatin and whey protein, were selected as model materials.

Chapter 3 describes the effects of simple shear flow on the formation and properties of gelatin particle suspensions. The application of well-defined simple shear flow during phase separation was used to control the protein particle size in a gelatin–dextran system.

Chapter 4 describes the formation and properties of whey protein particle suspensions having different particle sizes and different abilities to form disulphide bonds. Application of shear during their formation was used.

Chapter 5 describes a novel concept for making elastic dough through combining a whey protein particle suspension with native wheat starch. Three differently structured whey protein suspensions were evaluated.

Chapter 6 discusses the use of the whey protein particle suspensions prepared and used in chapter 5 for baking bread.

Chapter 7 describes the role of molecular properties on the final dough and bread that were discussed in chapters 5 and 6.

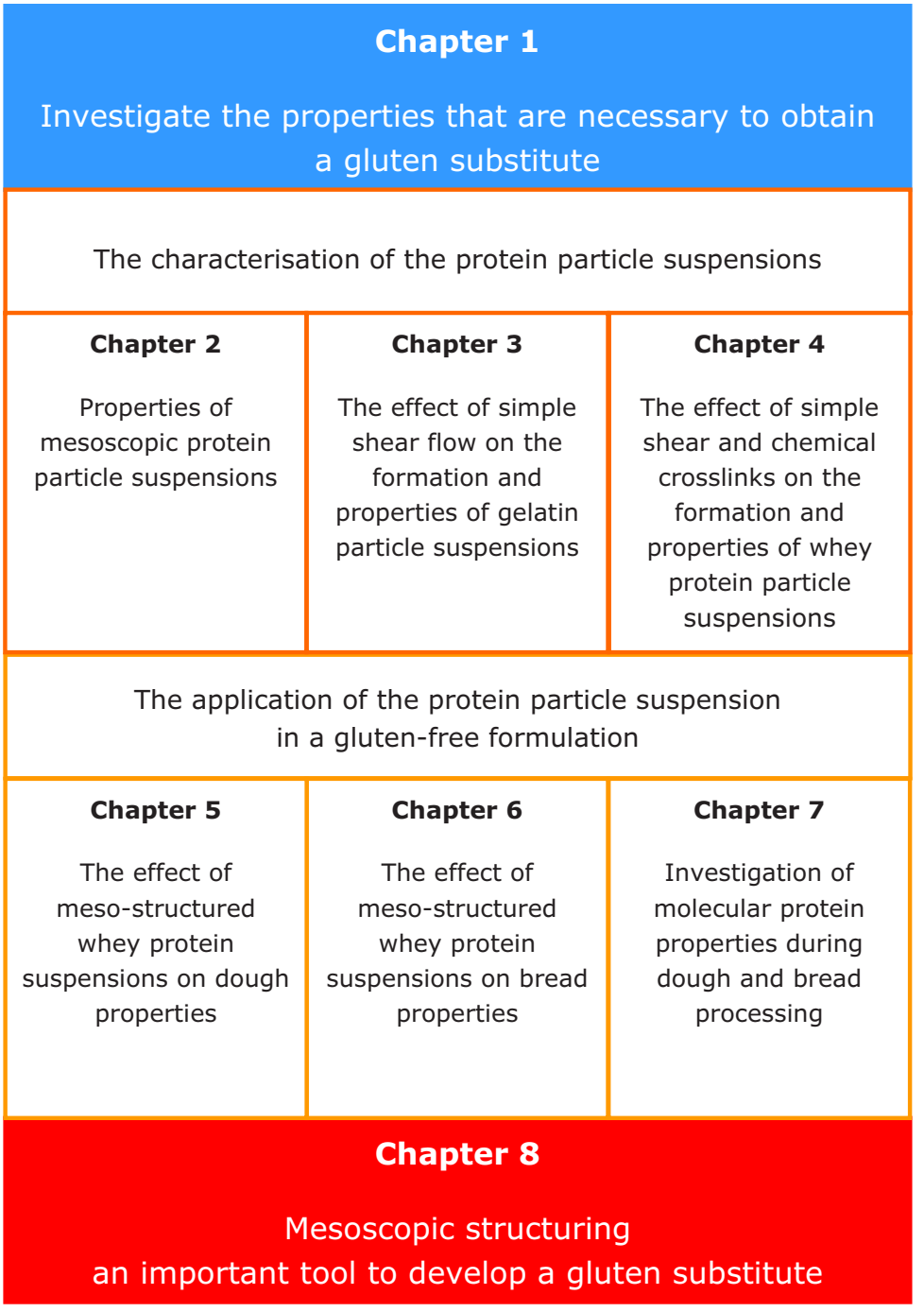

Figure 6: Schematic representation of the outline of the research

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Part I
Characterisation

Chapter 2

Elastic Networks of Protein Particles

This chapter describes the formation and properties of protein particle suspensions. The protein particles were prepared by a versatile method based on gelation of a phase separating protein–polysaccharide mixture. Two proteins were selected, gelatin and whey protein. Gelatin forms aggregates by means of reversible physical bonds, and whey protein forms aggregates that can be stabilized by chemical bonds. Rheology and microscopy show that protein particles aggregate into an elastic particle gel for both proteins. Properties similar to model systems of synthetic colloidal particles were obtained using protein particle suspensions. This suggests that the behaviour of the particle suspensions is mainly governed by the mesoscopic properties of the particle networks and to a lesser extent on the molecular properties of the particles.

This chapter was published as:
van Riemsdijk, L. E., Sprakel, J., van der Goot, A. J. & Hamer, R. J. Elastic Networks of Protein Particles. Food Biophysics 5, 41-48 (2010)

2.1 Introduction

Many materials consisting of polymer melts and colloidal suspensions show elastic behaviour. In polymer melts, elastic behaviour is caused by molecular entanglements [1]. In colloidal suspensions, elastic behaviour is, for example, caused by flocculation and subsequent network formation [2]. Elastic materials are used in many industrial applications such as thickeners, flow improvers and stabilizers of pigments [3]. Wheat gluten is an example of a biopolymer system with elastic properties, which allow wheat flour to retain gas during proving and baking [4, 5]. Gluten has self healing properties [6-8], which are uncommon in synthetic polymers [9]. The elastic properties of gluten are hypothesized to be a result of a glutenin particle network structure [6]. It is therefore of interest to understand more about the rheological behaviour of biopolymer particle systems. Limited information is available about the properties of suspensions containing protein particles. Therefore, in this study, the behaviour of suspensions containing protein particles is compared with the properties of synthetic colloidal particles found in other studies [10-17].

Protein particles can be involved in several types of interactions, for example, hydrophobic, Van der Waals and hydrogen-bonds type interactions. These interactions are mainly reversible and weak. However, if these interactions exist on a larger, cooperative scale, the overall interaction can be strong. Interactions can allow the formation of disulphide bonds, leading to covalent stabilization of the resulting particle aggregate. In addition, entanglement and depletion type interactions may exist. Depending on the type of protein used to form the particles, different combinations of these interactions may exist.

In this study gelatin and whey protein particles are used as model protein materials. These proteins have different intrinsic properties. Gelatin is a protein that forms aggregates via reversible, hydrogen-type bonds. The formation of these bonds is very fast, because hydrogen bonds form within milliseconds [18]. Whey proteins (mainly β-lactoglobulin and α-lactalbumin) have a high content of the amino acids glutamine, leucine and asparagine. Cystine residues are characteristic in whey proteins [19]. Whey protein forms aggregates on heating or acidification, which can be stabilized through disulphide bonds. The formation of these disulphide bonds takes seconds to minutes depending on the pH [18] and other properties, which is much slower than the formation of hydrogen bonds. Protein particles can be created by mixing a protein and a biopolymer with low compatibility [20, 21]. The rate and onset of phase separation and gelation are important characteristics for the morphology of the protein structure produced [22, 23], and are critically dependent on the concentration, temperature and molar mass of the continuous phase [24-28]. Creation of protein particles is possible for a limited number of biopolymers using specific process conditions [22, 29, 30]. Gelatin particles are formed by inducing phase separation by temperature quenching. Whey proteins form small aggregates by mild heating of a whey protein solution. Bringing a pre-aggregated solution to its isoelectric point (ca 4.5) leads to gel formation. This process is often defined as cold gelation [31].

The aim of this study is to demonstrate that protein particle suspensions can show elastic behaviour through aggregation of protein particles. We used gelatin and whey protein to prepare particles and characterized the behaviour of the resulting particle suspensions. The results are compared with results from studies on non-biopolymer, colloidal particle systems.

2.2 Experimental Section

2.2.1 Materials

The proteins used were gelatin type A, bloom number 175 and a gel point (for a 5 % solution) at 14 °C (Bio-Rad Laboratories, The Netherlands) and whey protein (Davisco Foods International Inc., USA). All proteins were used without further purification. Both protein materials contained about 90 % (w/w) protein, according to Dumas measurements (using N=5.55 for gelatin and N=6.38 for whey protein). The polysaccharides used were dextran (Mw 2000 kDa, Sigma Chemicals, The Netherlands) and locust bean gum (Danisco Holland BV, The Netherlands). Glucono-delta-lacton (GDL; Sigma Chemicals, The Netherlands) was used for pH regulation. Rhodamine B (Sigma Chemicals, The Netherlands) was used for confocal laser scanning microscope (CLSM) analysis. All chemicals were of analytical grade.

2.2.2 Preparation of Protein Particles

Protein particles were prepared using cold gelation in a phase separating biopolymer system [22, 23, 29-34]. A 10 % (w/w) gelatin stock solution was prepared by stirring a gelatin solution for 2 h at 50 °C. A 10 % (w/w) stock solution of dextran was prepared by stirring for 1 h at 80 °C. The dextran and gelatin stock solutions were kept at 50 °C before mixing (approximately 2 h). A mixture of gelatin (5 % (w/w)) and dextran (5 % (w/w)) was gelled by cooling from 50 to 30 °C in approximately 1 h. After 16 h, the mixture was cooled further to 25 °C in approximately 30 min.

A 9 % (w/w) whey protein stock solution was prepared by stirring for 2 h at 25 °C followed by heating the solution at 68 °C for 2.5 h. Heating the whey protein samples resulted in the formation of small protein

aggregates of 40 – 100 nm [35], without forming a gel. A 1 % (w/w) locust bean gum stock solution was prepared by stirring at 80 °C for 1 h. The whey protein and locust bean gum stock solutions were cooled to 25 °C before mixing. A mixture of whey protein (3 % (w/w)) and locust bean gum (0.45 % (w/w)) was gelled by adding GDL (0.20 % (w/w)), as a result of a gradual decrease in pH.

After incubation of the protein–polysaccharide mixtures for 16 h, the samples were diluted with distilled water and the continuous phase was removed by centrifugation (15 min at 2000×g). The pellet was re-dispersed in distilled water up to the original volume and then centrifuged at 2000×g for 15 min. The pellet was re-dispersed to obtain a sample with the required concentration (6.5 ± 0.6 % (w/w)). The samples were used within 1 day after processing. Three samples per protein were prepared for analysis unless stated otherwise.

2.2.3 Analysis of Protein Particles

Shape and Size of Suspensions

Confocal Laser Scanning Microscopy (CLSM) was used to analyze the shape and spatial distribution of the protein particles. After processing, the sample was transferred into a chambered cover glass (Nunc, Naperville, IL, USA). Rhodamine B was added to a concentration of 2×10^{-3} % (w/w) for non-covalent labelling of the proteins. The samples were visualized with an LSM 510 microscope (Zeiss, Oberkochen, Germany). The 543-nm laser line was used for excitation to induce a fluorescent emission of Rhodamine B, detected between 600 and 650 nm. Image analysis of two images per sample obtained at 10-fold magnification was used to calculate the average volume fraction occupied by the particles using Image-J software.

The average particle diameter was measured by calculating the mean diameter of eight particles, four particles per sample.

The particle size distribution of a highly sheared diluted protein particle suspension was analyzed by light scattering using a Mastersizer (Malvern Instruments Ltd. 2000, Worcestershire, UK) particle size analyzer. The refractive index used was 1.347 for gelatin particles and 1.334 for whey protein particles. The average particle diameter was calculated from the measurements of two samples.

Rheological Characterization of Suspensions

Shear rate sweeps were carried out at 25 °C in a cone/plate geometry (angle 4°/diameter 50 mm). The polysaccharide was almost all removed by washing and the sample was diluted with water until a protein concentration of 6.5 ± 0.6 % (w/w). After equilibrating the sample for 15 min, the shear rate was increased logarithmically over the range 1 – 300 s^{-1}. One measurement consisted of 21 steps with ten measuring points of 10 s for each step, a total duration of 35 min per measurement. From the measurements, the shear stress and apparent viscosity were calculated as a function of shear rate.

Steady shear measurements were performed in the concentration range 1 – 5 % (w/w) protein at 25 °C in a cone/plate geometry (angle 1°/ diameter 75 mm). One sample per concentration was measured. After equilibrating the sample for 15 min, the apparent viscosity was measured at a shear rate of 0.001 s^{-1} for 8000 s. Each sample was measured twice with an equilibration time of 20 s between the two measurements. From the measurements, the apparent viscosity was calculated as a function of time.

A rheomicroscope, which is a combination of a light microscope and a rheometer equipped with a quartz parallel plate geometry, was used to observe the particle structure during steady shear. The apparent viscosity of one sample was measured at a shear rate of 0.001 s^{-1} for 8000 s and at a shear rate of 0 and 100 s^{-1} for 10 s.

Strain sweeps were performed at 25 °C in a plate/plate geometry (diameter 50 mm). The polysaccharide was almost all removed by washing, and the sample was diluted with water until a protein concentration of 6.5 ± 0.6 % (w/w). After equilibrating the sample for 15 min, the strain was increased logarithmically from 0.01 % to 300 % at a frequency of 1 Hz. The limit of linearity of the suspension was determined from the amplitude sweep. From the measurements, the storage (G') and loss (G") moduli and the loss tangent (tan δ) were calculated as a function of the strain.

Frequency sweeps were performed at 25 °C in a plate/plate geometry (diameter 50 mm). The polysaccharide was almost all removed by washing, and the sample was diluted with water until a protein concentration of 6.5 ± 0.6 % (w/w). After equilibrating the sample for 15 min, the frequency was increased logarithmically from 0.01 to 100 Hz at a strain of 0.1 %. This range was within the linear viscoelastic region, as determined by preliminary strain sweep experiments. From the measurements, the storage (G') and loss (G") moduli and the loss tangent (tan δ) were calculated as a function of the angular frequency.

Wall slip is often observed in microgel suspensions. We checked the steady shear measurement using a rheomicroscope to verify these experiments, but when interpreting the rheological results it is important to be aware of possible wall slip.

The shear rate sweep measurements were carried out using a Paar Physica MCR 501 (Anton Paar, Austria) stress-controlled rheometer; for rheomicroscopy, a Paar Physica MCR 300 (Anton Paar, Austria) stress-controlled rheometer was used; for all other rheological characterizations, a Paar Physica MCR 301 (Anton Paar, Austria) stress-controlled rheometer was used.

2.3 Results

The gelatin and whey protein particles produced were characterized using CLSM and light scattering. A characteristic size distribution profile of the protein particles is presented in figure 1, showing that the gelatin particles (137 ± 19 μm) were larger than the whey protein particles (18 ± 1 μm). The gelatin and whey protein particles retained their spherical shape for at least 1 day after processing.

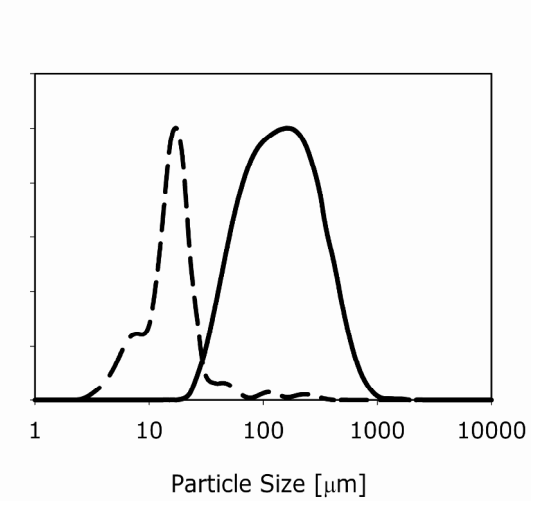


Figure 1: Characteristic particle size distribution curves for gelatin (solid line) and whey protein (dashed line) particles analyzed by light scattering.

An overview of the CLSM pictures for the protein particles is presented in figure 2. These pictures were also used to determine the typical particle size for each protein used. For gelatin, an average particle size of about 120 ± 17 μm was found, which was slightly smaller than the particle size obtained by light scattering. For whey protein, the size measured by CLSM analysis was somewhat larger (20 ± 4 μm). To calculate the average volume fraction occupied by the particles using Image-J software, we

assumed that the plane section of the two CLSM images were representative of the sample. The volume fractions occupied by the particles were 46 ± 1 % and 53 ± 1 % for gelatin and whey protein, respectively. These volume fractions were well below random close packing (which occurs at a volume fraction of 63 %), when jamming phenomena occur [36].

CLSM was also used to check the physical stability of the individual protein particles. It was found that both gelatin and whey protein particles could withstand all deformation forces exerted during preparation and rheological analysis.

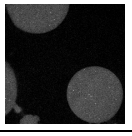
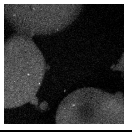
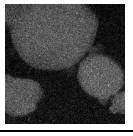
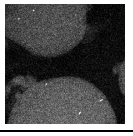
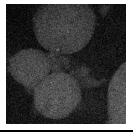
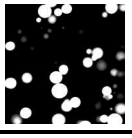
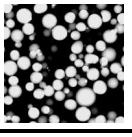
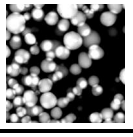
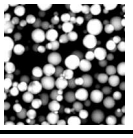
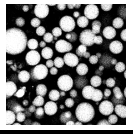
Protein Source	Size (μm)	Centrifugation		After Shear Test		
		Before	After	Rotational	Oscillatory	
					Strain	Frequency
Gelatin 20 μm	120 ± 17					
Whey Protein 20 μm	20 ± 4					

Figure 2: Overview of the size and structure of gelatin and whey protein particles before and after centrifugation and after the rheological tests. The average particle diameter was calculated from particle size analysis.

Rheology can be used to obtain information about particle interactions present in a particle suspension. Figures 3 and 4 show the shear stress as a function of shear rate for gelatin and whey protein particle suspensions in the range 1 – 300 s^{-1}. The insets show the viscosity for gelatin and whey protein particles. The graph in the inset also shows the viscosity of

2.5 % (w/w) dextran and 0.225 % (w/w) locust bean gum (50 % of the weight percentage needed for the preparation of protein particles). This graph shows that polysaccharide behaves at this concentration as a Newtonian liquid. As a result of washing, it is expected that the polysaccharide concentration in the suspension will be much lower, also leading to Newtonian behaviour. The viscosity of the protein particle suspensions is high compared with the continuous phase viscosity.

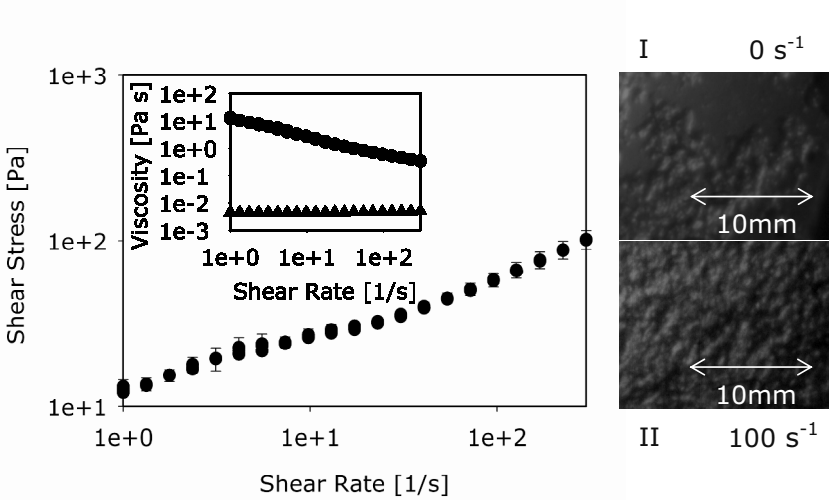


Figure 3: Shear stress as a function of the shear rate (up-sweep) of gelatin particle suspensions (6 – 7 %). The inset shows the viscosity as a function of shear rate (up-sweep) of gelatin particle suspensions 6 – 7 % (circles) and a dextran solution 2.5 % (triangles). I A rheomicroscopy image of gelatin particles at rest; II a rheomicroscopy image of gelatin particles at a shear rate of 100 s^{-1}.

In addition, the viscosity profile of the whey protein particle suspensions show a yield stress followed by shear thinning behaviour. We did not observe a yield stress for gelatin, but extrapolation of the shear rate sweep for gelatin particle suspensions to zero indicated a yield point of 12 Pa. The yield point for whey protein particle suspensions is

approximately 16 Pa. When the shear rate was increased beyond 5 s^{-1}, gelatin particle suspensions showed a shear rate dependency comparable with whey particle suspensions.

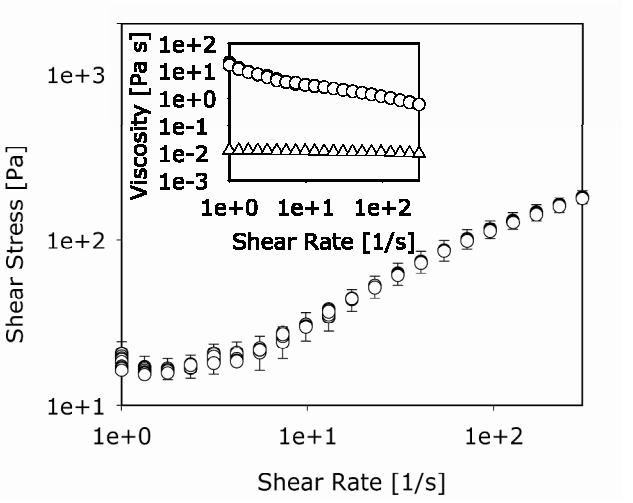


Figure 4: Shear stress as a function of the shear rate (up-sweep) of whey protein particle suspensions (6 – 7 %). The inset shows the viscosity as a function of the shear rate (up-sweep) of whey protein particle suspensions 6 – 7 % (circles) and a locust bean gum solution 0.225 % (triangles).

Particle aggregation can lead to the formation of a macroscopic sample-spanning network. Rheomicroscopy indeed shows that gelatin particles at rest form a particle network (figure 3 (I)). The application of shear breaks up the network, leading to unclustered particles (figure 3 (II)).

To investigate the effect of particle interactions at near-static conditions, we measured the shear stress as a function of shear time at a constant low shear rate (0.001 s^{-1}). The gelatin and whey protein particle suspensions were measured at different protein concentrations (gelatin 2 %, 3 %, 4 % and 5 % (w/w) and whey protein 1 %, 2 %, 3 %, 4 % and 5 % (w/w)).

Figure 5 shows that the viscosity of the gelatin particle suspension increased over time with low shear rate indicating rheopectic behaviour. An increase in the protein concentration gave an increase in the final shear stress for gelatin. The time needed to reach the final shear stress increased with gelatin concentration.

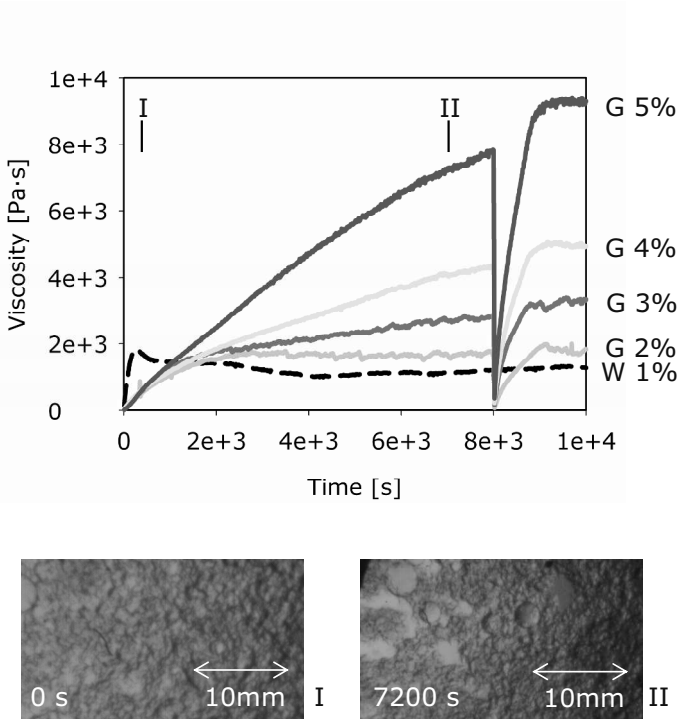


Figure 5: Viscosity as a function of the shear time of gelatin (solid lines) and whey protein (dashed line) particle suspensions. Four different concentrations for gelatin particle suspensions (2 %, 3 %, 4 %, 5 % (w/w)) and one concentration for whey protein particle suspensions (1 % (w/w)) are shown. The shear stress is measured at a constant deformation of 0.001 s^{-1} for 8000 s. After 8000 s, the shear is stopped for 20 s and then continued with a constant deformation of 0.001 s^{-1}. I A rheomicroscopy image of gelatin particles at a constant deformation of 0.001 s^{-1} after 0 s; II a rheomicroscopy image of gelatin particles at a constant deformation of 0.001 s^{-1} after 7200 s.

The viscosity profile of the whey protein particle suspension did not show a clear correlation with the protein concentration (results not shown). For clarity, only one whey protein concentration is shown in figure 5. Whey protein particle suspensions showed an initial increase in the shear stress with time, followed by a decrease in the shear stress. Resuming the shear after 20 s resulted in a constant shear stress.

Microscopic pictures of a gelatin suspension were used to investigate the structure formation of the gelatin particles during constant deformation of 0.001 s^{-1}. At 0 s, the gelatin particles were distributed homogenously (figure 5 (I)). After 7200 s, clusters of gelatin particles were present (figure 5 (II)). This particle aggregation suggests a significant interaction between the gelatin particles.

The strength and ability of the protein network to recover after deformation was measured with oscillatory frequency and strain experiments. Figure 6 shows G′ and G″ as a function of the strain for gelatin and whey protein particle suspensions in the strain range of 0.01 – 300 %. Both gelatin and whey protein particle suspensions showed network fracture at higher strain values. For gelatin particles, there is fracture at a strain of 16 (maximum deviation was 1 %). The whey protein particles fracture at a lower strain of 0.54 (maximum deviation was 1 %). The loss factor showed a steeper increase in the non-linear regime for gelatin particle suspensions, indicating that the network was affected more abruptly at high strain values.

Figure 7 shows G′ and G″ as a function of the frequency of gelatin and whey protein particle suspensions in the range 0.1 – 100 s^{-1}. The storage and loss moduli of both suspensions were slightly dependent on the frequency. Gelatin particle suspensions were more frequency dependent

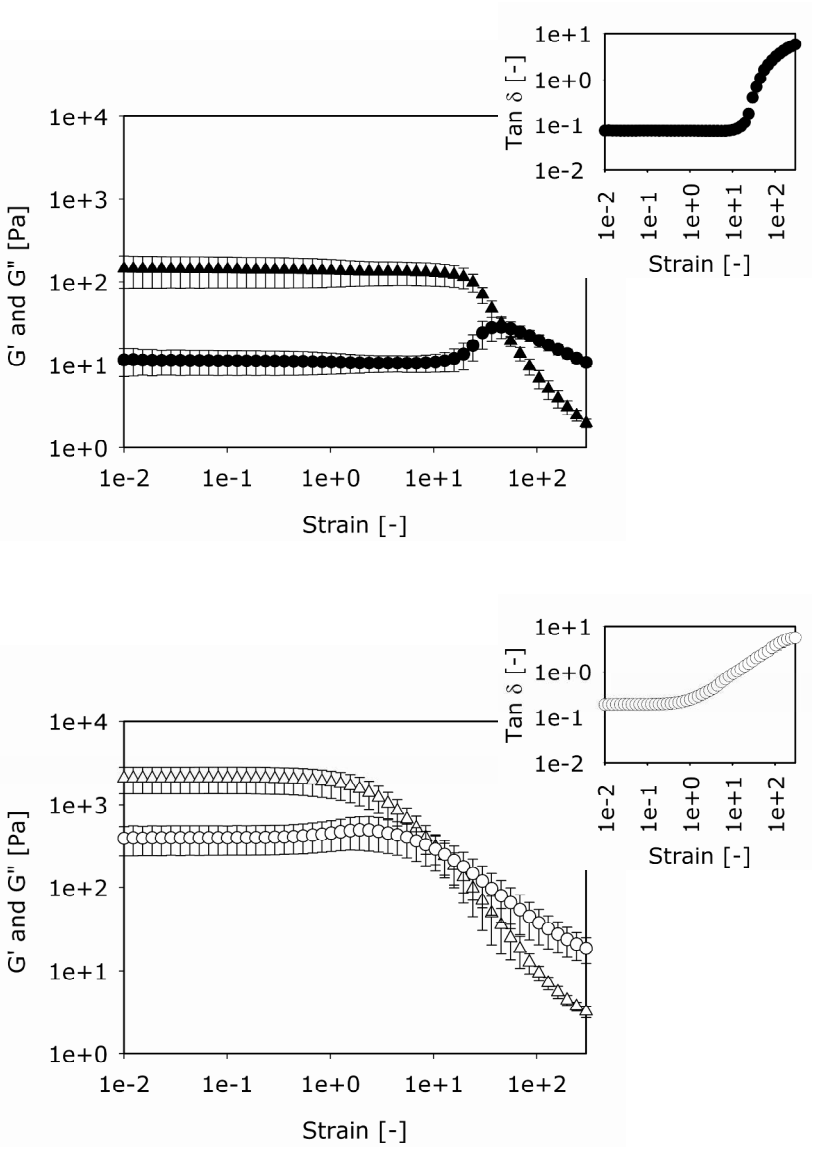


Figure 6: Storage modulus G′ (triangles) and loss modulus G″ (circles as a function of the strain of gelatin (closed symbols) and whey protein (open symbols) particle suspensions (6 – 7 %). The inset shows tanδ as a function of the strain of gelatin (closed symbols) and whey protein (open symbols) particle suspensions (6 – 7 %).

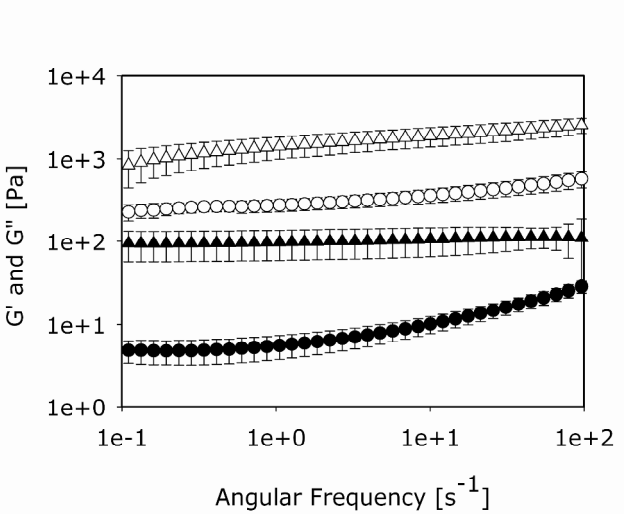


Figure 7: Storage modulus G' (triangles) and loss modulus G" (circles) as a function of the angular frequency (ω) of gelatin (closed symbols) and whey protein (open symbols) particles (6 – 7 %).

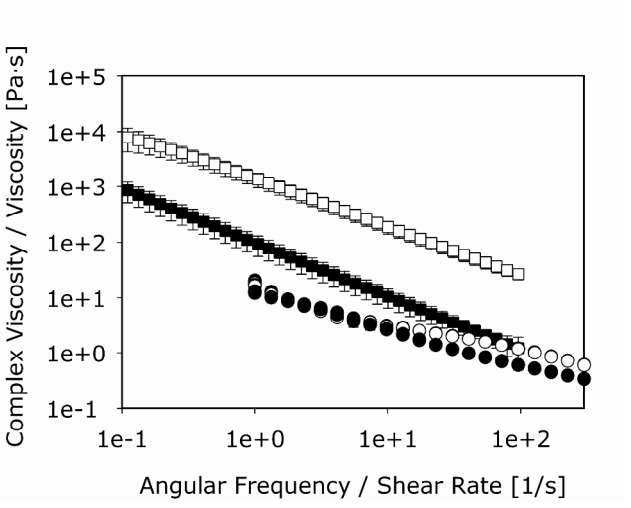


Figure 8: Complex viscosity (squares) and viscosity (circles) as a function of the angular frequency and shear rate of gelatin (closed symbols) and whey protein (open symbols) particles (6 – 7 %).

compared with the whey protein particle suspensions. Both gelatin and whey protein particle suspensions had a larger value for G′ than for G″ over the whole frequency range. Figure 8 shows the complex viscosity as a function of the frequency in the range 0.1 – 100 s^{-1} and the viscosity as a function of the shear rate in the range 1 – 300 s^{-1} for gelatin and whey protein particles. The complex viscosity decreased with increasing frequency over the frequency region measured.

2.4 Discussion

The purpose of this study was to demonstrate the unique properties of protein particles suspensions. Even though the microstructure of gelatin and whey proteins is different and the molecular properties of the proteins differ widely, the behaviour of gelatin and whey protein particle suspensions show similarities. Both protein systems show elastic behaviour and similar particle gel characteristics. The macroscopic behaviour of the particle gel systems seems to depend on the mesoscopic structure of the suspension rather than the specific chemical nature of the constituent material.

Gelation during phase separation was used to produce protein particles, because this technique is known as a suitable method to produce spherical protein particles [22, 23, 29-34]. However, this technique has some limitations because it requires the use of a polysaccharide to induce phase separation. We washed the protein particle suspension to remove most of the polysaccharide present in the system. However, even at this low residual concentration of polysaccharide, depletion flocculation can occur [37]. It was not possible to use the same polysaccharide for both systems. The combination of whey protein and dextran did not result in protein particles. Studies show that it is possible to produce gelatin particles with locust bean gum [32]. However, in that study, the concentration of gelatin was very low. We did not succeed in producing gelatin particles with locust bean gum using higher gelatin concentrations. In addition, and probably as a consequence, it was not possible to prepare particles of whey protein with the same size as gelatin particles.

The whey protein particles were always smaller (ca eight times) than the gelatin particles (see figures 1 and 2). The large gelatin particles show a broad size distribution compared with the small whey protein particles.

The importance of particle interaction can be shown by estimating the effect on viscosity assuming that no particle interaction is present. When the protein particles behave as inert hard spheres, the viscosity can be estimated using the volume fraction indicated by CLSM images and the viscosity of the continuous phase of 50 % of the polysaccharide (i.e. 2.5 % (w/w) dextran and 0.225 % (w/w) locust bean gum, respectively). According to the Krieger–Dougherty equation, with an intrinsic viscosity of 2.5 and maximum packing fraction of 0.63, the viscosity of the suspension would be 0.037 and 0.205 Pa·s for gelatin and whey protein particles, respectively. The measured viscosity of the suspensions is almost a factor 100 larger. As the volume fraction of the particles is well below the jamming transition, this high viscosity must be linked to particle interactions.

The yield stress observed in both protein particle suspensions is characteristic for colloidal suspensions that form a network [16, 38]. The yield stress is related to the force that the network can withstand before gel rupture [39]. A further increase of the shear forces leads to detachment of particles and shear thinning behaviour [40]. Shear-induced collisions can rebuild the particle network [40]. This was observed for the gelatin particle suspension. Gelatin particles showed rheopectic behaviour, which indicates the formation of interactions in the system [41-43], leading to the (re)formation of an interparticle structure [41].

Both protein particle suspensions show strain-dependent behaviour. At low strain, the particle gels are strain independent, but at higher strains they show strain softening. A particle gel of non-biopolymer particles shows comparable strain dependency; the strain independent region is an indication of the particle interaction in the gel [40, 44]. Gels with a high degree of interaction are brittle and brake at low strain values [45].

As the interaction decreases, the strain-independent region increases and the gel becomes deformable [40, 44]. Gelatin particle suspensions show high deformability, which is comparable with weakly interacted particle gels. The whey protein particle suspensions are more brittle and behave as a strong interacted particle gel [45]. Both protein particle suspensions show frequency-independent deformation behaviour. A particle gel of non-biopolymer particles shows comparable independence on frequency [40, 44, 46, 47]. The storage modulus is larger than the loss modulus over the whole frequency range, indicating that the particles form an elastic gel with an infinite relaxation time [40, 47].

The presence of structural ordering is supported by the rheomicroscopy images in figures 3 and 5 and by the invalidity of the Cox–Merz rule ($\eta(\gamma)=\eta^*(\omega)$) [40, 48], shown in figure 8. The $\eta^*(\omega)$ curves were greater than the $\eta(\gamma)$ curves throughout the measured shear region, indicating a structured system [48, 49]. But the $\eta^*(\omega)$ and $\eta(\gamma)$ curves did not show parallel behaviour, which makes it impossible to use a shift factor [50]. The unparallel behaviour was observed previously in particle gel systems, but no explanation has yet been found for this behaviour [51].

The rheological behaviour of the suspensions described above is comparable with the rheological behaviour of particulate networks of non-biopolymer model-particles. Shear thinning [52] and yielding [16, 38]

were observed, and the oscillatory rheology was comparable with other particle networks. Like other particle networks, our protein particle networks are strongly elastic at small strain values as seen from the slightly frequency-dependent behaviour [10, 46]. The strain dependence of the gelatin particle network was compared with weakly interacted particle gels [44], and the strain dependency of the whey protein particle network was comparable with strong interacted particle gels [45].

Generally, the particles investigated in other studies were much smaller. For example, the radius of carbon black particles was 14 nm [10], and the radius of carboxylated latex particles was 90 nm [46]. The particles in this study were 100 (whey) to almost 1000 (gelatin) times larger. More comparable particle sizes are observed in studies on depletion-flocculated emulsions; the particles (droplets) were only ten (whey) to 100 (gelatin) times smaller [37, 52, 53]. Generally, those model studies indicate that the minimum volume fraction necessary for elastic gel behaviour decreases with decreasing particle size [12, 13]. This implies that the protein particles show a remarkably high degree of interaction. The nature of the interaction present in the protein systems is not yet fully understood. Remaining polysaccharide might cause depletion interactions, but other interactions such as hydrogen bonds and Van der Waals forces cannot be excluded. Most likely, a combination of these interactions accounts for the high degree of interactions present in the system.

Although the behaviour of the two systems is qualitatively the same, some differences in properties can be observed. Those differences are probably caused by specific features of the protein, such as charge density and distribution along the protein molecules, the importance of hydrophobic interactions and the ability to stabilize superstructures formed by creating

additional disulphide bonds. The main differences between gelatin and whey protein particle suspensions are related to the strength of the interactions and the ability to form new interactions. Gelatin particles form a loose network that can easily be reformed provided it has sufficient time to relax. This reformation is supported by its rheopectic behaviour. The whey protein particle network shows a higher degree of structure that can withstand a small deformation. The higher degree of structure is supported by the higher yield stress and the higher value of the complex viscosity [13, 14].

2.5 Conclusions

Protein particles, created from gelatin and whey protein, can form an elastic particle network in suspension as a result of the high degree of interactions present between the protein particles. The presence of a network structure is evident from the yield stress and shear thinning behaviour. Strain dependency measurements also indicate the presence of a network. The properties of both suspensions suggest that the behaviour of the protein particles in the suspension depends to a large extent on the mesoscopic properties of the protein. The differences in the behaviour of gelatin and whey protein suspensions, such as response to oscillation and low shear rate, are probably caused by difference in their microstructure and molecular properties.

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Chapter 3

Particle Size Effects in Colloidal Gelatin Particle Suspensions

This chapter describes the effects of simple shear flow on the formation and properties of colloidal gelatin particle suspensions. Microscopy and light scattering show that simple shear flow of a phase separating gelatin–dextran mixture gave smaller particles with a narrower size distribution. Upon gelation due to a temperature decrease, the viscosity of the gelatin increased, which altered the coalescence and break-up behaviour of the particles formed. The small particles obtained by a high shear during processing aggregated into larger particle clusters, once particle solidified upon gelation. The particle size can be predicted using correlation with droplet break-up and coalescence considering the properties before gelation. The sizes of the clusters can be predicted with the coalescence behaviour using the properties after gelation. Clusters originating from small particles resist more deformation, resulting in pronounced rheological effects (e.g. increase viscosity, increased strain softening point).

This chapter was published as:
van Riemsdijk, L. E., Snoeren, J. P. M., van der Goot, A. J., Boom, R. M. & Hamer, R. J. Particle size effects in colloidal gelatin particle suspensions. Journal of Food Engineering 101, 394-401 (2010)

3.1 Introduction

Proteins can form ordered structures, governed by the tendency of proteins to aggregate into larger structures [1, 2]. The formation of larger structures by protein aggregation via thermal or enzymatic gelation is a key parameter in (food) product development, even though protein aggregation is difficult to control [3-5]. Generally, the product properties are changed by modifications at a molecular scale rather than at a mesoscopic or macroscopic length scale. However, several authors showed that there is much potential in controlling the product properties using the structure formation process at a mesoscopic length scale [6-8]. In the previous chapter, we showed that protein particle suspensions obtained via phase separation show a remarkably high degree of particle–particle interactions compared with non-biopolymer model systems [9]. A high degree of particle interaction can be useful for the development of elastic, self healing networks which can be used for different applications, e.g. biodegradable rubbers or gluten replacement. The high degree of particle interaction of those protein particles can probably be further increased by reducing the particle size [10, 11]. The protein particle size obtained in our earlier study depended on the rate and onset of phase separation and the rate of gelation, both induced by temperature quenching [9]. To investigate the effect of protein particle size on the particle network strength, we need a method to alter the mesoscopic particle size without altering the composition of the sample. Structuring using well-defined simple shear flow has been described as a promising method to control the structure of protein suspensions at the mesoscopic length scale without altering the composition of the sample [5].

Particle formation due to simple shear flow can be described by a model using deformation and break-up on the one hand and coalescence on the other [12-14]. Particle break-up occurs when the shear rate results in higher viscous forces than the interfacial and other cohesive forces of the particles can withstand. Coalescence of particles occurs when the particles collide and stay together sufficiently long to allow fusion [15]. A number of studies focussed on the effect of simple shear flow on protein particle formation [13, 16-19]. If protein particles were formed and subsequently exposed to shear, they follow the trends that were described for synthetic polymer blends [13]. Shear flow enhances coalescence, in case the shear rate was too low to cause particle break-up. At shear flow rates where particle break-up and particle coalescence occurred, particles with a narrow distribution in particle size were formed. An even higher shear rate causes particle elongation [16, 17]. When shear and gelation occurs simultaneously the behaviour seams different and to a great extent depending on the composition and procedure used. Gelation during shear can stimulate the formation of elongated particles even at low shear forces [19], while in another study, non-elongated particles were observed, that formed stable particle clusters eventually [18]. Besides, it has been reported that gelation could cause phase inversion [17]. Till now, most of the studies done used relatively low shear rates (a maximum of 100 s^{-1}) [13, 16, 18], were done without solidification [13, 16, 20], or started with particles already being formed before application of shear flow [13, 18, 20]. We, however, are interested to investigate how the combination of well-defined flow and a solidification mechanism (here gelation) can be used to create novel structures in a gelatin–dextran system. Previous research [21] showed that this combination is very promising and industrially relevant for protein structuring purposes.

The application of well-defined shear flow with the aim of creating structures requires different equipment than generally used. For examples, several studies were done using parallel plates [16, 18, 20], which does not give similar deformation throughout the whole sample. Other studies used a cone and plate geometry [19], which is better because it gives an (almost) constant deformation throughout the sample, but the gap in the tip might be too small to allow complete coalescence. Therefore we developed a range of shearing devices dedicated for structuring purposes [21-25]. Here we introduce an in-house developed device based on a Taylor–Couette geometry. The shearing device was developed in such a way that the whole sample experiences a constant deformation during shear and that the gap was significantly larger than the protein particles formed.

The aim of the current study is to investigate how simple shear flow is related to the protein particle size and to the final rheological properties in a gelatin–dextran system. The shearing of the samples started before phase separation is induced through a temperature quench. Besides, the effect of gelatinization is included. Subsequently, it is quantified to which extend mesoscopic changes of gelatin particles influences the particle–particle interaction through determining the effect of these changes on the rheological behaviour of the protein particle suspensions.

3.2 Experimental Section

3.2.1 Materials

The gelatin used was a type A from pig skin with a bloom number of 175 (Bio-Rad Laboratories, The Netherlands). The protein content was determined to be ca 90 % (w/w), according to the Dumas method (nitrogen factor 5.55). The dextran used had a molecular weight (Mw) of 2000 kDa, (Sigma Chemicals, The Netherlands).

3.2.2 Preparation of Protein Particles

Gelatin particles were prepared using the previously reported method of gelation in a thermally induced phase separating biopolymer system [16, 26]. A 10 % (w/w) gelatin stock solution was prepared by stirring at 50 °C for 2 h. A 10 % (w/w) dextran stock solution was prepared by stirring at 80 °C for 1 h. To prepare the particles, one part of the 10 % (w/w) gelatin stock solution was mixed with one part of the 10 % (w/w) dextran solution. The weight percentage is used because the particles were prepared by phase separation and consequently the volume fraction of the particles is not a primary parameter. Both stock solutions were kept at 50 °C prior to mixing. The mixture of gelatin (5 % (w/w)) and dextran (5 % (w/w)) was then cooled from 50 to 30 °C in 5 min and kept at this temperature for 8 h. In a second cooling step, the mixture was cooled to 25 °C in 5 min. This temperature was maintained for an additional 8 h. During cooling, the gelatin–dextran mixture was subjected to a simple linear shear profile at different shear rates (0, 54, 108, 216, 539 and 1079 s^{-1}). The suspensions were prepared in duplicates. The obtained suspensions were analyzed within 1 day after processing.

The shearing treatment was carried out using an in-house shearing device based on Couette geometry (figure 1). The inner cylinder is made of polyetherimide and the outer cylinder is made of polycarbonate. The diameter of the rotating inner cylinder is 40 mm. The diameter of the stationary outer cylinder is 42 mm. The bottom of the shearing device is designed in a cone/plate geometry with an angle of 2.8°. This angle guarantees that the shear rate between the cone and the plate is comparable with the shear rate between the two cylinders [24]. The shearing device is temperature controlled using a water bath. Temperature and torque are registered during processing.

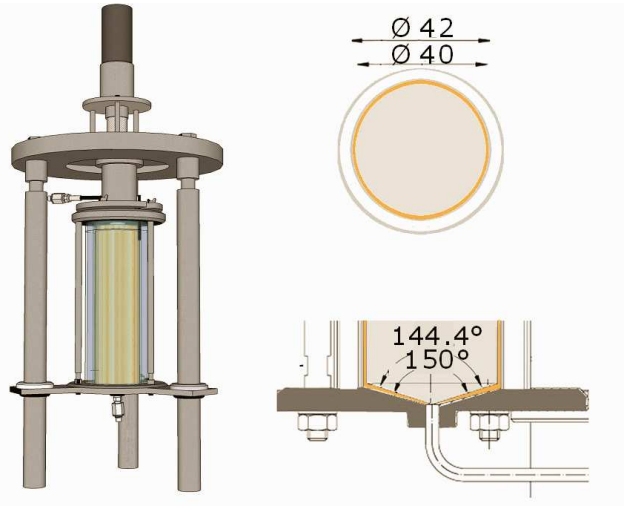

Figure 1: Schematic drawing of the shearing device. The diameter of the rotating inner cylinder is 40 mm. The diameter of the stationary outer cylinder is 42 mm. The bottom of the shearing device is designed as a cone/plate geometry with an angle of 2.8°.

3.2.3 Analysis of Gelatin Particles

Protein Content

The protein concentration was determined by the Dumas method using a nitrogen factor of 5.55. The measured average protein content in the sheared samples was 4.0 ± 0.3 % (w/w) for the samples processed with a shear rate of 54, 108, 216, 539 and 1079 s^{-1}. Only the unsheared (0 s^{-1}) sample showed a significantly lower average protein content of 2.6 ± 0.7 % (w/w), as a result of protein gel formation on the cylinder surfaces.

Shape and Size of Suspensions

A stationary confocal scanning light microscope (CLSM; LSM 510. Zeiss, Oberkochen, Germany) was used to analyze the shape and spatial distribution of the protein particles. In addition, a dedicated CLSM with cone/plate geometry (Nizo Food Research, Ede, The Netherlands) was used to analyze the behaviour of the protein particles on shear flow. Rhodamine B (Sigma Chemicals, The Netherlands) was added to a concentration of 2 $\times 10^{-3}$ % (w/w) for non-covalent labelling of the proteins. The 543 nm laser line was used for excitation to induce a fluorescent emission of Rhodamine B, detected between 600 and 650 nm. The average particle fraction and the average particle diameter D_{32} were calculated with Image-J software using two images per sample. For the average particle fraction a 10-fold magnification (921 × 921 µm) was applied for all samples. For the average particle diameter a 10-fold magnification (921 × 921 µm) was applied for the samples processed at 0, 54, 108, and 216 s^{-1}, and a 40× magnification (230 × 230 µm) was used for the samples processed at 539, and 1079 s^{-1}.

A Mastersizer (Malvern Instruments Ltd. 2000, Worcestershire, UK) particle size analyzer was used to measure the particle size distribution of the diluted protein particle suspensions. The refractive index used was 1.347. The average particle diameter D_{32} was calculated from the measurements of two samples.

Rheological Characterization of Suspensions

Shear rate sweeps were performed in a Paar Physica MCR 301 (Anton Paar, Austria) stress-controlled rheometer, at 25 °C using cone/plate geometry (angle 1°/diameter 75 mm). After equilibrating the sample for 15 min, the shear rate was increased in 21 steps from 1 to 300 s^{-1} logarithmically. Each step consisted of 10 measuring points of 10 s. Transient effects were not observed during the measurements.

The shear stress and apparent viscosity were calculated from the measurements as a function of shear rate. The shear stress and apparent viscosity were calculated using the single measurements of two separate samples.

Strain sweeps were performed in a Paar Physica MCR 301 (Anton Paar, Austria) stress-controlled rheometer, at 25 °C using plate/plate geometry (diameter 50 mm – gap 1 mm). After equilibrating the sample for 15 min, the strain was increased from 0.01 % to 100 % logarithmically at a frequency of 1 Hz. The storage (G') and loss (G") moduli, and the loss tangent (tan δ) were calculated from the measurements as a function of the strain. The limit of the strain independent region (yield point) was determined using a maximum deviation of 5 %. The moduli, loss tangent and yield point were calculated using single measurements of two separate samples.

3.3 Results and Discussion

3.3.1 Effect of Shear Rate on Particle Size

In this study, we investigate the effect of simple shear flow on a gelatin–dextran solution. The temperature of the solution at the start of the experiment was 50 °C, which made the solution homogeneous. Then, the temperature was reduced to provoke phase separation. In a next step, the solution temperature was further reduced leading the gelatinization of gelatin. The shear stress as function of processing time is depicted in figure 2. This figure also includes the temperature profile used and a schematic overview of possible droplet changes as described in earlier studies. The shear stress increased upon temperature decrease, from 50 °C to 30 °C and from 30 °C to 25 °C, which is related to the increase in viscosity upon cooling.

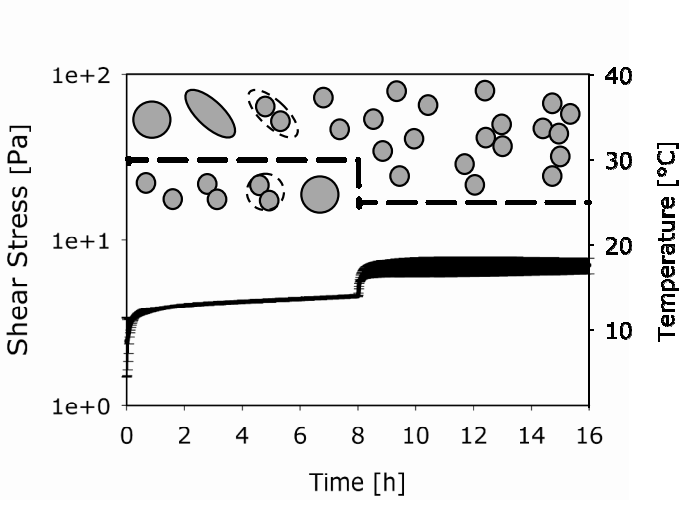

Figure 2: The shear stress profile during processing at 108 s^{-1} (solid) and the temperature profile during processing (dashed) as a function of the processing time. The inserted pictures represent the possibilities for non-gelled particles (first 8 h) and the possibilities for gelled particles (last 8 h).

In the first 8 h, the gelatin is still a liquid, implying that the gelatin particles formed can elongate, break or coalescence. After a further temperature decrease to 25 °C (i.e. the last 8 h) the gelatin gelled, which prevented elongation, break-up and coalescence, leading to particle rotation, clustering or alignment [13, 16-19].

From the experiments performed, it became clear that simple shear flow affects the size and shape of the gelatin particles obtained. The CLSM pictures in figure 3 show a variation in particle shape for different shear rates. Uniform and spherical particles were formed at shear rates of 54 and 108 s^{-1}; non-spherical particles were formed at a shear rate of 216 s^{-1}. The small particles formed at shear rates of 539 and 1079 s^{-1} aggregated into clusters.

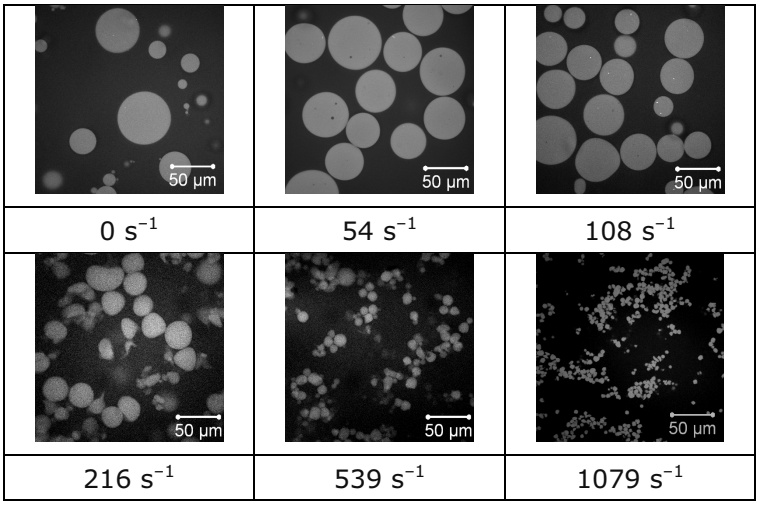

Figure 3: Overview of the size and structures of gelatin protein particles. The particles are prepared by gelation of a phase separating gelatin–dextran mixture processed under different shear rates (0, 54, 108, 216, 539 and 1079 s^{-1}).

Figures 3 and 4 presents the effect of the shear rate applied during production on the particle size and the particle size distribution, respectively. Figure 5 depicts the average particle sizes calculated from the CLSM images and light scattering data. Figure 4 shows that the particle size distribution as obtained by light scattering became narrower through the application of simple shear. Under simple shear flow, there is an equilibrium between particle break-up, and particle coalescence [13]. A large region of possible particle sizes exists at low shear rate, where the break-up of particles occurs only for large particles. In case of zero shear conditions, additional effects such as drainage of small particles cause a broad particle size distribution. The application of shear is therefore expected to narrow the particles size distribution. The CLSM images (figure 3) confirm this decrease in particle size with increasing shear rate. However, the comparison of the results of the light scattering with the CLSM measurements (figure 5) showed a number of evident differences. The light scattering measurements showed a minimum particle size at 539 s^{-1}, while the CLSM images indicated a further particle size reduction above a shear rate of 539 s^{-1}. However, the CLSM images made clear that the small particles aggregated into clusters, which are probably able to withstand the dilution and the mixing forces during the light scattering particle size measurement. Light scattering therefore measured the sizes of the clusters instead of the particles.

Up to a shear rate of 108 s^{-1} the particles were spherical, and rather similar in size. The average particle size decreased upon increased shear rate. This was also observed in the study of Van Puyvelde et al. (2003) [13]. Gelation of the particles did not change the break-up and coalescence behaviour in this shear rate range. At higher shear rates, the particles become less spherical, and aggregated into particle clusters.

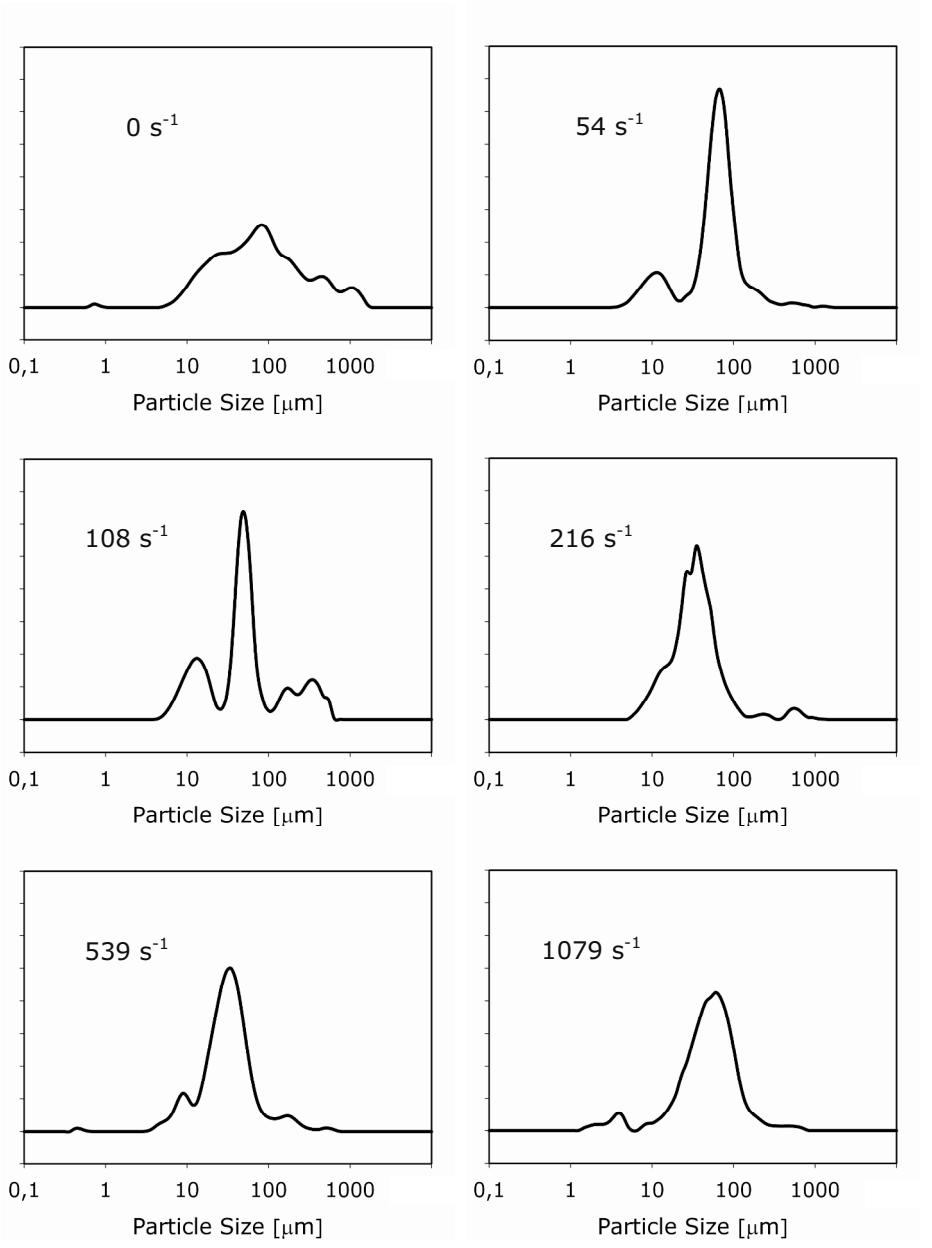

Figure 4: Overview of the particle size distribution of gelatin particles. The particles are prepared by gelation of a phase separating gelatin–dextran mixture processed under different shear rates (0, 54, 108, 216, 539 and 1079 s^{-1}).

This was not observed in non-gelling particle systems. The changes in gelatin particle size and shape can probably be related to the gelation process. Gelation (by cooling) alters the viscosity of both the continuous phase and the viscosity of the dispersed phase. The viscosity change of the continuous phase (dextran) is faster and less than that of the dispersed phase (gelatin). The changes in viscosity alter the break-up and coalescence behaviour to which the particles will adjust their size.

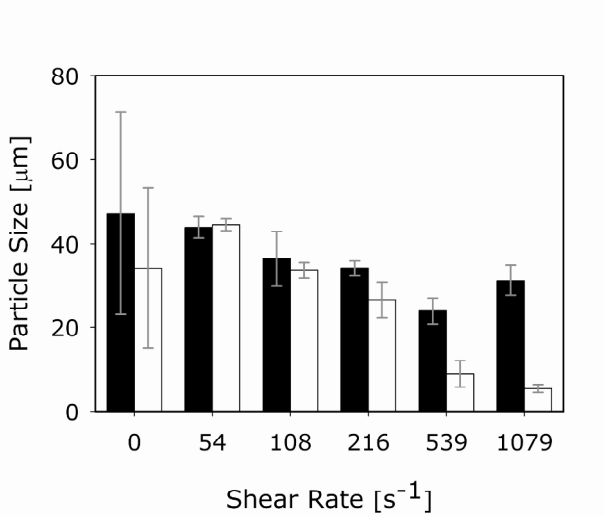

Figure 5: Overview of the average size (D_{32}) of gelatin protein particles analyzed by light scattering (black) and CLSM (white). The particles are prepared by gelation of a phase separating gelatin–dextran mixture processed under different shear rates (0, 54, 108, 216, 539 and 1079 s^{-1}).

However, it is likely that the particles do not have sufficient time to adjust completely, due to the gelation of the gelatin phase leading to entrapment of the particle size and shape. The particles formed at a shear rate of 216 s^{-1} are exactly formed at a shear rate where the effect of break-up limits the effects of coalescence. At a lower shear rate, coalescence controls the particle formation; at higher shear rates the effects of break-

up become dominant. This will be shown quantitatively in the next paragraph. This means that the particles created at 216 s^{-1} will elongate upon gelation due to the viscosity increase, which causes non spherical particles. This explains why in this transition region, a suspension was obtained with irregularly shaped protein particles.

The measured values for the average particles sizes (via light scattering and CLSM) were plotted according to the Elmendorp plot, figure 6. In accordance with the Elmendorp plot, we draw two straight lines through the measured values. The line through the measured values obtained via light scattering follows a curve with the slope of the partially mobile interface (PMI) approach [13, 14]. That line, the solid line in figure 6, represents the maximum droplet size that can be formed by coalescence [27].

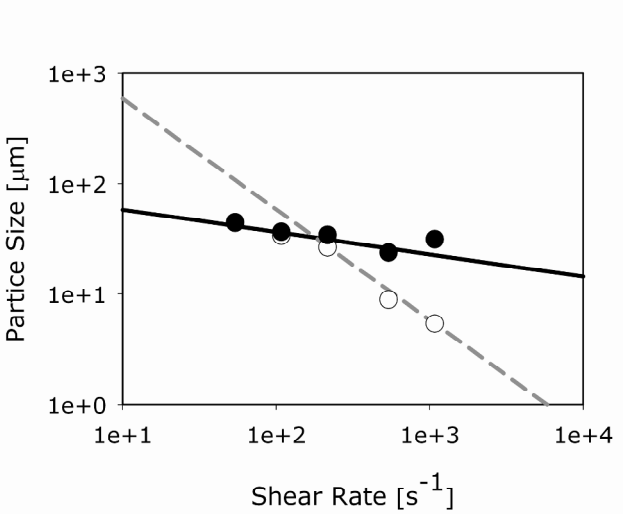

Figure 6: The droplet size analyzed by light scattering (black) and CLSM (white) as a function of shear rate and the limiting curves for break-up (dashed line) and coalescence (solid line).

The measured values via CLSM follow the same curve as the measured values for light scattering at low shear rates, but as the shear rate is increased beyond 216 s^{-1}, the line connecting measured values follow a curve with the slope similar to the slope obtained by the Weber approach [28]. That line being the dashed line in figure 6 represents the maximum droplet size that can be obtained by droplet break-up [27].

To investigate whether the particle size measured follows the PMI approach and Weber approach indeed, we estimated the values needed for these models. To calculate the break-up curve, we need to know the critical Weber number (We), the continuous phase viscosity (η_c), and the interfacial tension (σ). The critical Weber number depends on the viscosity ratio of the continuous phase (η_c) and the dispersed phase (η_d). The continuous phase viscosity and the viscosity of the dispersed phase were estimated using the assumption that the matrix only consists of dextran and the particles only consist of gelatin. Furthermore, we assumed that the plane section of the two CLSM images were representative of the sample. The particles covered 33 % of the CLSM image, analyzed using Image-J. Based on these assumptions, we estimate that the concentration of dextran in the continuous phase is 7.5 % and the protein concentration in the dispersed phase is 12 % (w/w). Both biopolymers showed nearly Newtonian behaviour at the concentrations mentioned at 30 °C. The viscosity of a 7.5 % (w/w) dextran solution is 2 $\times 10^{-2}$ Pa·s and the viscosity of a 12 % (w/w) gelatin solution is 4 $\times 10^{-2}$ Pa·s. Based on the viscosity ratio of both biopolymer phases, the critical Weber number was estimated to be approximately 4 [29].

The particle size (R), when determined by break-up in viscous laminar flow, should be inversely proportional to the shear rate ($\dot{\gamma}$) as follows:

$$R = We\frac{\sigma}{\eta_c \dot{\gamma}} \tag{1}$$

When particles become very small due to break-up, they may coalesce again into larger particles. In this case, the final particle size is somewhat less dependent on the shear rate, according to:

$$R = 1.40\left(\frac{\dot{\gamma} h_{cr}}{\lambda}\right)^{2/5}\left(\frac{\sigma}{\eta_c \dot{\gamma}}\right)^{3/5} \tag{2}$$

where h_{cr} is the so-called critical thickness of the fluid layer, λ is the viscosity ratio of the continuous phase and the dispersed phase, and σ is the interfacial tension. The interfacial tension was estimated from the literature to be 30 μN/m [30, 31]. The critical thickness of the fluid layer can be estimated from the literature to be 100 – 1200 nm [32]. Since it is not possible to measure an accurate value for the critical thickness of the fluid layer, this value is often used as an adjustable parameter [12, 13, 33]. A critical thickness of 1200 nm resulted in prediction for the coalescence curve as plotted in the figure. This curve passes through the values as obtained with particle size measurements, suggesting that the particle sizes are governed by coalescence at these shear rates.

At low shear (54 s^{-1} and 108 s^{-1}), the final particle size follows the coalescence curve, suggesting that this size is determined by coalescence. As the shear rate is further increased to 539 s^{-1} and 1079 s^{-1}, the CLSM measurements indicate that the particle sizes follow the break-up curve,

which is in agreement with earlier studies of non-gelling gelatin–dextran systems [13]. But the light scattering measurements indicated larger sizes, which suggest further coalescence. The combination of both experiments indicates that primary particle sizes are determined by a break-up process. The size of the clusters was determined by the coalescence curve. Because the size of the protein particles decreases more than the size of the protein clusters with increasing shear rate, it can be concluded that the number of particles present in a cluster increased. The amount of particles in the cluster and consequently the interaction within the cluster is therefore increased in case of a smaller particle size. We therefore conclude that the particles are formed when the gelatin is still liquid. Clusters will be formed once the gelatin particles were solidified upon gelation.

3.3.2 Effect of Particle Size on Rheological Properties

The rheological properties of suspensions depend on the interactions between the particles [34-37]. Attraction between the particles results in particle clusters, which increases the effective volume taken up, and hence increases the viscosity. Conversely, break-up of particle clusters under the influence of shear will result in a decrease in viscosity.

Figure 7 depicts the viscosity as a function of shear rate for gelatin particle suspensions processed under different shear rates and a dextran solution of 7.5 % (w/w) in the range of 1 – 300 s^{-1}. The gelatin suspensions showed shear thinning behaviour and the dextran solution was nearly Newtonian. Figure 8 shows CLSM images of the gelatin suspensions obtained after processing at 0 s^{-1} and 539 s^{-1} under shear. These images were made using a CLSM microscope system on which

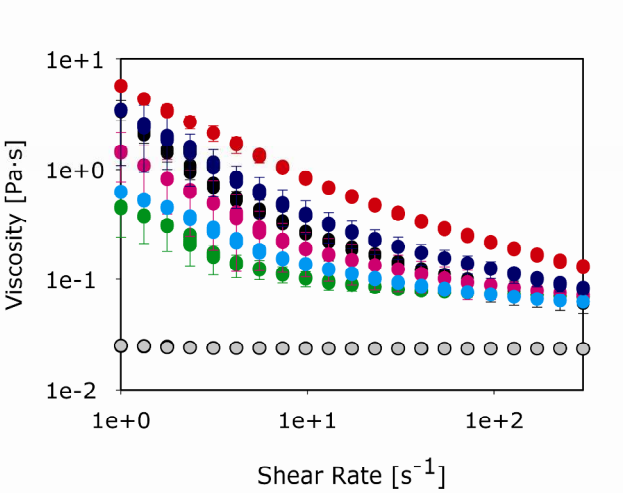

Figure 7: Viscosity as a function of the shear rate of gelatin particle suspensions processed at 0 (black), 54 (green), 108 (pink/purple), 216 (turquoise), 539 (blue) and 1079 (red) s^{-1} and a dextran solution of 7.5 w/w % (grey).

cone/plate geometry was mounted. The images (at least 10 images s^{-1}) show that gelatin particles in the suspension produced at 0 s^{-1} moved independently in the dextran solution, and the gelatin particles produced at 539 s^{-1} moved in clusters that tumbled in the dextran solution. The existence and the observed strong coherence of those clusters confirm the light scattering measurements (figure 4).

There is a clear difference in viscosity for gelatin suspensions obtained after processing under different shear rates (figure 7). The viscosity of the samples increased when the processing shear rate increased. The increase in viscosity can be correlated to the decrease in particle size with increasing processing shear rate. A suspension with small particles had a higher viscosity compared with one with larger particles [11, 38, 39], because part of the continuous phase will be trapped inside the particle clusters. This means that the clusters, increase the effective volume fraction of the dispersed phase [40, 41].

There are two exceptions to the increase in viscosity with increasing processing shear rate. The sample produced at 0 s^{-1} shows a relatively high viscosity and the sample produced at 216 s^{-1} shows a relatively low viscosity. The CLSM pictures show that these two samples have a different particle structure. The sample processed without shear has a heterogeneous particle size distribution, which leads to different particle packing. The particle size distribution has a strong effect on the viscosity [42, 43]. In our case, the viscosity increased, probably because of the presence of small particles that cluster into larger aggregates. The particles processed at 216 s^{-1} behaved unexpectedly, which might be related to the non-spherical shape of the particles [44].

When the particle suspensions are subjected to high shear rates, the effect of the particle size on the viscosity value diminishes. The behaviour of the samples is almost Newtonian at high shear rates. The particle clusters are most likely (partly) broken up. Only the suspension containing particles produced at 1079 s^{-1} had still a higher viscosity at high shear rates, which indicates a high stability of the clusters. This was confirmed using the CLSM under shear. We therefore conclude that the clusters become more stable when they are made from smaller particles.

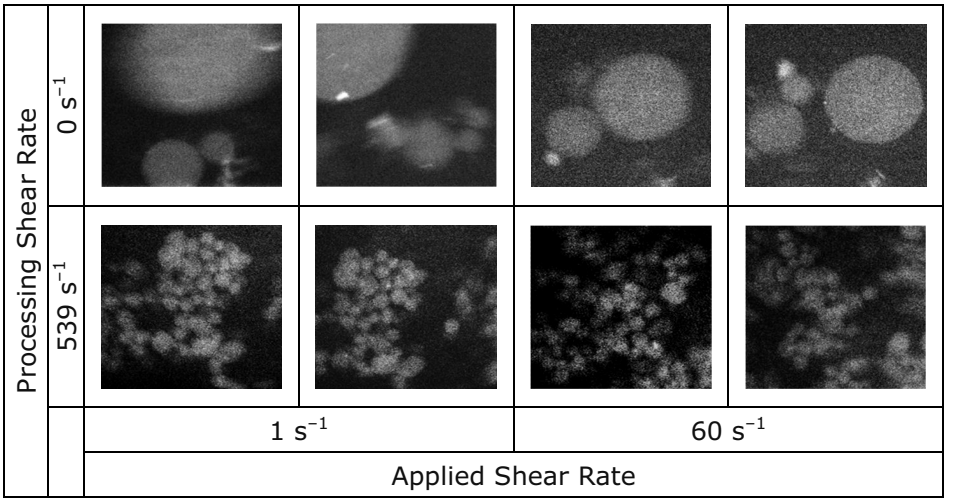

Figure 8: CLSM pictures made during shear at 1 and 60 s^{-1}: 2 CLSM pictures of a gelatin suspension produced at 0 s^{-1} and 2 CLSM pictures of a gelatin suspension produced at 250 s^{-1}.

The strength of the protein network was measured with oscillatory strain experiments in the range of 1 – 300 %. The loss factor is an indication of the deformation energy that is dissipated in the sample. Attraction between particles results in a lower loss factor, because more energy is stored in the sample.

The suspension produced without shear (0 s^{-1}) had a larger value for G″ than for G′ in the strain-independent region. Thus, the loss factor (tan δ) is slightly above 1. All other samples had a larger value for G′ than for G″, i.e. the loss factor was smaller than 1. The loss factor decreased with decreasing particle size (results not shown), which indicates that the particles show more solid-like behaviour with decreasing particle size. This is probably related to the cluster formation [11].

The length of the strain-independent region determines the maximum deformation that the clusters can handle without a noticeable difference in behaviour [45]. All particle suspensions showed strain softening at strain values between 5 % and 25 %, and this value increased with decreasing particle size. Only the sample with a broad size distribution (0 s^{-1}) and the sample with non-spherical particles (216 s^{-1}) behaved differently. Figure 9 demonstrates a correlation between the strain softening point and the inverse of the particle size measured by light scattering. A linear relation of the inverse of the particle size with the strain softening point was demonstrated in oil-in-water emulsion [46, 47]. The increase in the strain softening point with 1/R indicates that the particles showed more elastic behaviour as the particle size decreased. The particle size measured with light scattering gave a better relation with the strain softening point than the particle size measured with CLSM. Once more, we conclude that the clusters are responsible for the changes in rheological behaviour, rather than the isolated particles.

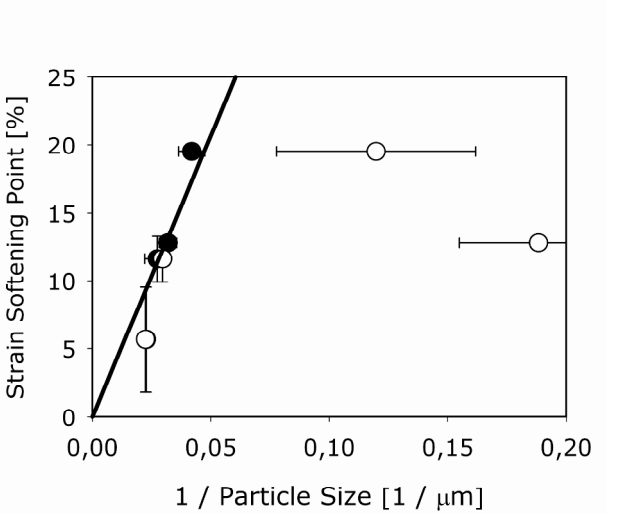

Figure 9: Strain softening point of the gelatin particle suspension as a function of the particle size analyzed by light scattering (black) and CLSM (white). Gelatin particles are processed at 54, 108, 539 and 1079 s^{-1}.

3.4 Conclusions

The application of well-defined shear flow during phase separation can be used to control protein particle size in a gelatin–dextran system. The particle size decreases when a higher shear rate is applied during processing. Upon gelation due to a temperature decrease, the viscosity of the system increases, this alters the coalescence and break-up behaviour of the particles formed. The small particles formed by particle break-up start to coalesce, but before coalescence is finished once the particles are gelled. Then, clustering of stable particle was observed at high shear rates. The sizes of the clusters depend on the primary protein particle size and consequently on the shear rate applied during processing. These protein particle clusters led to different particle suspension properties compared to the protein particles normally used.

The rheological properties of the colloidal protein particle suspension obtained were determined by the protein particle size and their ability to form stable clusters. The rheological behaviour depends on the clusters rather than on the primary particles in case of small particles. Clusters originating from small particles resist more deformation, resulting in pronounced rheological effects (e.g. increased viscosity, increased strain softening point). This size effect is probably a result of large particle interactions present within the clusters due to smaller particle size, and hence greater stability of the clusters.

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Chapter 4

New Insights on the Formation of Colloidal Whey Protein Particles

This chapter describes the formation and properties of whey protein particle suspensions having different particle sizes and different abilities to form disulphide bonds. Simple shear flow was used to control the protein particle size. The ability to form disulphide bonds was steered by blocking the reactive thiol groups of the whey proteins with N-ethylmaleimide. Microscopy and light scattering showed that simple shear flow applied during the formation of whey protein particles give irregularly shaped particles. Especially small particles aggregated into particle clusters. Microscopy and rheological measurements (strain and shear rate sweeps) showed that the protein particle clustering was favoured by the ability of the protein to form disulphide bonds and to a lesser extend by a smaller particle size. From the study it can be concluded that the formation of disulphide bonds has no effect on the formation process of protein particles, but disulphide bonds are important for the ability of the whey protein particles to form particle clusters.

This chapter was published as:
van Riemsdijk, L. E., Snoeren, J. P. M., van der Goot, A. J., Boom, R. M. & Hamer, R. J. New insights on the formation of colloidal whey protein particles. Food Hydrocolloids 25, 333-339 (2011)

4.1 Introduction

There is increasing interest in controlling product properties by structuring biopolymers at a mesoscopic (i.e. colloidal) length scale, industrially as well as scientifically. The relevance of structures having this length scale with respect to the final product properties is described for different traditional food products such as bread [1] and meat [2]. Protein structuring at the mesoscopic length scale was positively valued when it was used in mimicking food products, such as meat alternatives [3] and fat substitutes [4]. In addition, colloidal protein particles are suggested to be an interesting building block to be applied in high protein food systems [5].

Colloidal protein particles, created from gelatin and whey protein, can form an elastic particle network in suspension. This ability to form networks using both proteins suggests that the behaviour of the protein particles in the suspension depends to a large extend on the mesoscopic properties [6]. However, the strength of the networks differs. The whey protein network is stronger and hence less deformable compared to the gelatin network. The differences in network strength were attributed to specific features of the protein, such as the ability to stabilize a particle network by additional disulphide bonds. But other factors, such as size effects and solvent quality could have a certain effect on the network strength as well. In this study however, the latter parameters were kept constant to allow a good comparison of the results.

Blocking the thiol groups of the whey protein (aggregates), is a method often applied to investigate the effect of disulphide bond formation [7]. The blocking agent can influence other properties of the protein. These side effects depend in part on the size, the chemical properties of the

blocking agent and the concentration used. The side effects can be minimized by adding the lowest concentration needed for blocking the thiol groups. That is why we added only 2.25 mM N-ethylmaleimide (NEM), which is known from previous studies to have limited influence on the hydrodynamic diameter of whey protein aggregates. Besides, NEM gave comparable results as other blocking agents (iodoacetamide and p-chloromercuribenzoic acid) [7, 8].

The mechanism behind the formation of protein particles is mainly studied for a system without disulphide bond, namely gelatin particles [9-13]. In a gelatin-polysaccharide system, the formation of particles is driven by phase separation. Phase separation was mainly induced through a temperature quench [10]. After particle formation, the gelatin particles were fixated through gelation [11, 13].

The use of well-defined simple shear flow is an interesting method for influencing structures of biopolymer systems at colloidal scale [10, 14-19]. Without gelation, shear can stimulate particle coalescence at low shear rates, while it stimulates particle break-up at high shear rates. But, when particle formation and gelation happen concurrently, the overall effect of shear on particle size and shape is more difficult to predict. Particle elongation, particle clustering and phase inversion can all take place [14, 15, 17]. This makes it interesting to investigate the effect of shear on the formation of whey protein particles. In this process, particle formation and gelation happen at the same time, because particle formation and fixation by gelation are both induced by a decrease in pH, leading to coagulation of protein aggregates.

This chapter describes our new insights on the formation of colloidal protein particles obtained from a whey protein-locust beam gum system using simple shear flow conditions. The role of reactive thiol groups is studied to investigate the influence of disulphide bonds on the particle formation process as well as the strength of the resulting whey protein network.

4.2 Experimental Section

4.2.1 Materials

The whey protein used (Davisco Foods International Inc., USA) contained about 90 % (w/w) protein, according to DUMAS measurements (using N = 6.38). The polysaccharide used was locust bean gum (Danisco Holland BV, The Netherlands). Glucono-delta-lacton (GDL) from Sigma Chemicals, The Netherlands, was used for pH regulation. Rhodamine B (Sigma Chemicals, The Netherlands) was used for staining proteins to be used for confocal laser scanning microscope (CLSM) analysis. N-ethylmaleimide (Fluka, The Netherlands) (NEM) was used to block the reactive thiol groups.

4.2.2 Preparation of whey protein particles

A 9 % (w/w) whey protein stock solution was prepared by stirring at 25 °C for 2 h followed by a heating step at 68 °C for 2.5 h. The heating of whey protein samples resulted in the formation of small protein aggregates with a size of 40 - 100 nm [20]. To block the reactive thiol groups of the protein aggregates, the aggregates were treated with N-ethylmaleimide (NEM) using a concentration of 2.25 mM. After addition of the thiol-blocking agent, the reaction was allowed to proceed at room temperature for at least 30 min. The effectiveness of the NEM treatment was examined through determination of the accessible thiol groups using Ellman's reagent before and after the NEM treatment. The NEM treatment blocked 94 ± 2 % of the accessible thiol groups of the whey protein aggregates.

A 1 % (w/w) locust bean gum stock solution was stirred at 80 °C for 1 h. The whey protein and locust bean gum stock solutions were cooled to 25 °C prior to mixing. A mixture of whey protein (3 % (w/w)) and locust bean gum (0.45 % (w/w)) was gelled by adding GDL (0.20 % (w/w)),

which caused a gradual decrease of the pH from 6.8 to 5.2. This decrease in pH lowers the reactivity of the thiol groups to a large extent. In other words, the rate and extent of disulphide bond formation decreases during the process, but it does not prevent the formation of disulphide bonds completely [7].

During acidification, the whey protein-locust bean gum mixture was subjected to a simple shear flow field of different shear rates (0, 5, 11, 22, 54 and 108 s^{-1}). The shearing treatment was carried out in an in-house developed shearing device based on a Couette geometry. The diameter of the rotating inner cylinder is 40 mm. The diameter of the stationary outer cylinder is 42 mm. The bottom of the shearing device is designed in a cone/plate geometry with an angle of 2.75°. The shearing device was temperature controlled using a water bath. Temperature and torque were registered during processing. The suspensions obtained were analyzed within one day after processing.

4.2.3 Analysis of whey protein particles

Protein content

The protein concentration was determined with DUMAS, using a nitrogen factor of 6.38. The average protein content in the samples was 2.7 ± 0.05 % (w/w), and is independent of the shear rate applied during processing.

Shape and size of suspensions

A dedicated confocal laser scanning microscope (CLSM) with a cone-plate geometry (Nizo Food Research, Ede, The Netherlands) was used to analyze the whey protein particle formation during simple shear flow. A stationary CLSM (LSM 510, Zeiss, Oberkochen, Germany) was used to

analyze the shape and the spatial distribution of the whey protein particles after processing. The analysis and calculations of the average particle diameter D_{32} have been described in more detail in chapter 2 [6].

Size distribution

The particle size distribution was analyzed by light scattering using a Mastersizer (Malvern Instruments Ltd. 2000, Worcestershire, UK) particle size analyzer. The samples were diluted and stirred before measuring. The refractive index used was 1.334. The average particle diameter D_{32} was calculated from the measurements of two samples.

Rheological Characterization of Suspensions

The production of whey protein particles was mimicked in a rheometer to measure the shear stress as function of the processing time. Directly after mixing, the solution containing whey protein aggregates (3 % (w/w)), locust bean gum (0.45 % (w/w)) and GDL (0.20 % (w/w)) was transferred into a Couette geometry (diameter cup 28.9 mm/diameter bob 26.7/length bob 40 mm). The shear conditions in the rheometer (time and temperature) were similar to the conditions applied in the shear cylinder.

Shear rate sweeps were performed at 25 °C in a cone/plate geometry (angle 1°/diameter 75 mm) using the suspension containing the newly formed whey protein particles. After equilibrating the sample for 15 min, the shear rate was increased in 21 steps logarithmically distributed in the range 1 - 300 s^{-1}. Each step consisted of 10 measuring points of 10 s each. From the measurements, the apparent viscosity was calculated as a function of the shear rate.

Also strain sweeps were performed at 25 °C in a plate/plate geometry (diameter 50 mm - gap 1 mm). After equilibrating the sample for 15 min, the strain was increased logarithmically from 0.01 % to 300 % at a frequency of 1 Hz. From the measurements, the loss tangent (tan δ) was calculated as a function of the strain.

For all rheological characterizations, a Paar Physica MCR 301 (Anton Paar, Austria) stress-controlled rheometer was used.

4.3 Results and discussion

The particle formation process was followed using CLSM and rheology. Figure 1 shows an overlay of both measurements. A shear rate of 5 s^{-1} was applied in these measurements. The shear stress shows an abrupt increase, up to a maximum beyond which the viscosity decreased and smoothly leveled off. The inserted images in figure 1 represent an overview of the CLSM movie made during such a particle formation process.

At the start of the experiment, the whey protein-locust bean gum mixture was homogeneous at the length scale measurable by the CLSM image. Then, a kind of bicontinuous mixture formed from the homogeneous

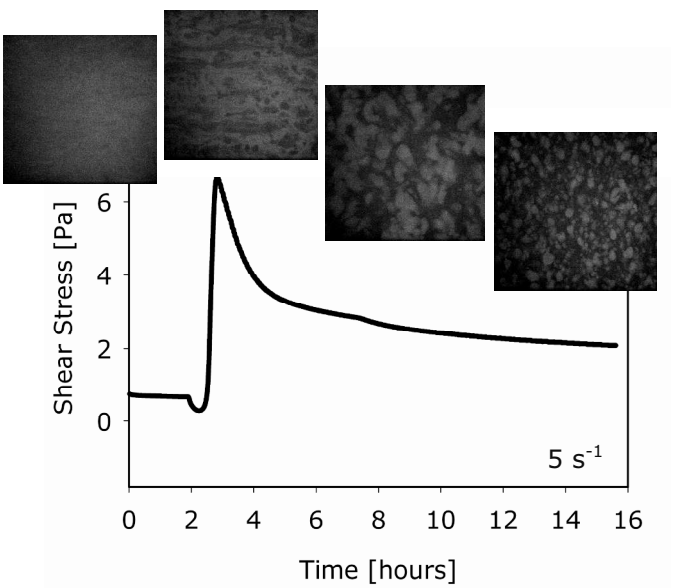


Figure 1: Shear stress as function of processing time of whey protein particles prepared by a phase separating whey protein-locust bean gum mixture processed at 108 s^{-1}. Inserted are the corresponding CLSM images of the process (protein is white - locust bean gum is black).

system. This phase separation coincided with a sudden increase of viscosity. Subsequently, the protein phase was broken up into whey protein flocks, and further into small whey protein particles. The moment at which the increase of the viscosity shown in figure 1 occurred, coincided with the moment at which the pH-value of a solution containing GDL reached the value of 5.7. At this pH, gelation of the whey protein phase occurs [21]. Eventually, the viscosity of the mixture decreased during the particle formation process.

The particle formation process and the changes in viscosity can be understood by considering the phase diagram, schematically depicted in figure 2. The initial mixture of protein and polysaccharide falls into the one-phase region of the diagram. The formation of protein aggregates from native proteins leads to a shift of the binodal in the phase diagram [22], thereby narrowing the one-phase region; the gradually acidification during the process also stimulates phase separation, because acidification decreases the electrostatic repulsion of the whey protein aggregates, and consequently provoking additional clustering of the protein aggregates [21]. Due to the shifts in the binodal, the mixture ends up in a two-phase region of the phase diagram, as a result of which phase separation occurs.

The viscosity profile during processing is typical for various mechanisms related to droplet formation. Catastrophic phase inversion [23, 24], spinodal decomposition [25], and nucleation and growth, can all result in a sharp viscosity increase followed by a viscosity decrease. The formation of protein particles is often related with spinodal decomposition or nucleation and growth [10], but for a whey protein-locust bean gum system, characteristics of catastrophic phase inversion can also be

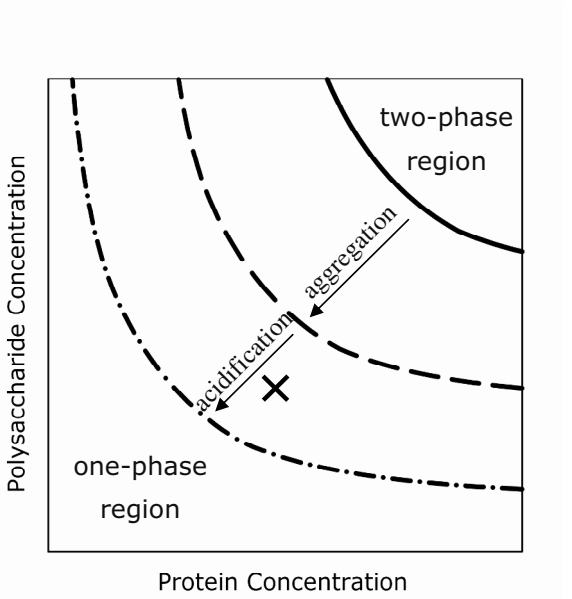


Figure 2: Schematic representation of the effects of aggregation and acidification on the phase diagram of a whey protein-locust bean gum mixture. The cross represents the whey protein-locust bean gum concentration.

observed. This is understandable, because the whey protein phase has the lowest viscosity directly after mixing. Due to GDL hydrolyses, and resulting pH-decrease, the whey protein gels and shifts from the phase with the lowest viscosity to the phase with the highest viscosity. This viscosity increase can cause disruption of the continuous protein phase (i.e. the protein phase becomes disperse), which results in the formation of large whey protein domains. Due to shear, the whey protein domains will be broken up into even smaller particles.

The particle shape confirms that phase inversion might play a role. The effect of shear on the particle shape is depicted in the CLSM images shown in figure 3. The sample produced under static conditions (0 s^{-1}) gave a uniform and spherical particle shape. Application of shear resulted

in irregularly shaped whey protein particles. The latter is characteristic for other phase separated protein-polysaccharide mixtures in which phase inversion occurred [17]. The irregular shape of the whey protein particles can have different causes. It is possible that the whey protein particles fall apart into smaller particles, due to the shear forces applied. Irregularly shaped particles will be formed, in case the formation of smaller particles due to breakage occurred more or less at the same time with the gelation of the whey protein phase. The breakage of particles will stop when complete protein gelling has occurred [21].

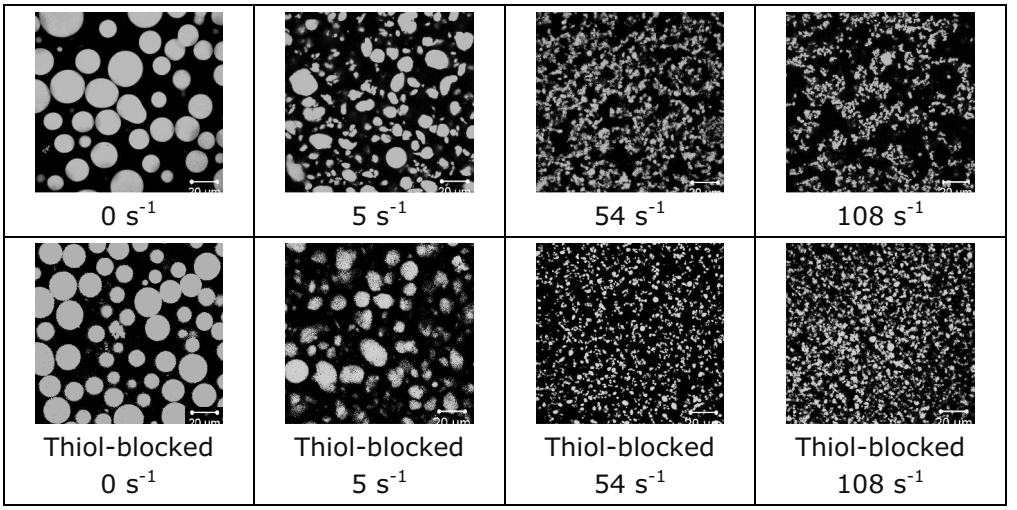


Figure 3: Overview of the size and structures of whey protein particles without and with thiol-blocking. The particles were prepared using different shear rates (0, 5, 54 and 108 s^{-1}) during processing.

Particle breakage might also explain the difference between the thiol-blocked and the unmodified particles, because the thiol-blocked whey protein particles have no additional stabilization due to disulphide bonds. Those particles will break more easily, resulting in smaller particles upon

shear (figure 4). Another cause could be that coalescence of small particles could not be completed due to fast fixation of the particles. As a result, irregularly shaped protein patches will be formed as well.

We were also interested in the role of disulphide bonds in the particle formation process and on the strength of the resulting whey protein network. The role of disulphide bonds was investigated through blocking the thiol groups of the whey protein aggregates using NEM. Figure 3 depicts the CLSM images of the unmodified and the thiol-blocked whey protein particles. From these images, it can be concluded that the shape of the whey protein particles is not affected by thiol-blocking. The size of the whey protein particles, measured with the CLSM images, is also not influenced by thiol-blocking. The particle size decreased with increasing shear rate both for the unmodified whey protein particles and for the thiol-blocked whey protein particles. However, the CLSM images show that the small unmodified whey protein particles formed particle clusters. The thiol-blocked whey protein particles were hardly clustered and homogeneously distributed over the CLSM image.

Light scattering measurements were used to obtain further inside on the effect of shear on the particle size. The particle size distribution graphs are depicted in figure 4. Those measurements confirmed that the particle size was not influenced through the thiol-blocking ingredient in case the whey protein particles are formed under static conditions. For particles produced using shear, the particle size distribution graphs of the thiol-blocked whey protein particles showed differences. The particle size decrease was confirmed for samples containing the thiol-blocking agent. The other samples gave a much larger average particle size when measured with light scattering. This difference can be explained by

considering the formation of the clusters as observed through CLSM. Most likely, these clusters were able to withstand the stirring forces and the dilution applied before the light scattering measurement. Light scattering therefore measured the sizes of the clusters rather than the size of the individual particles.

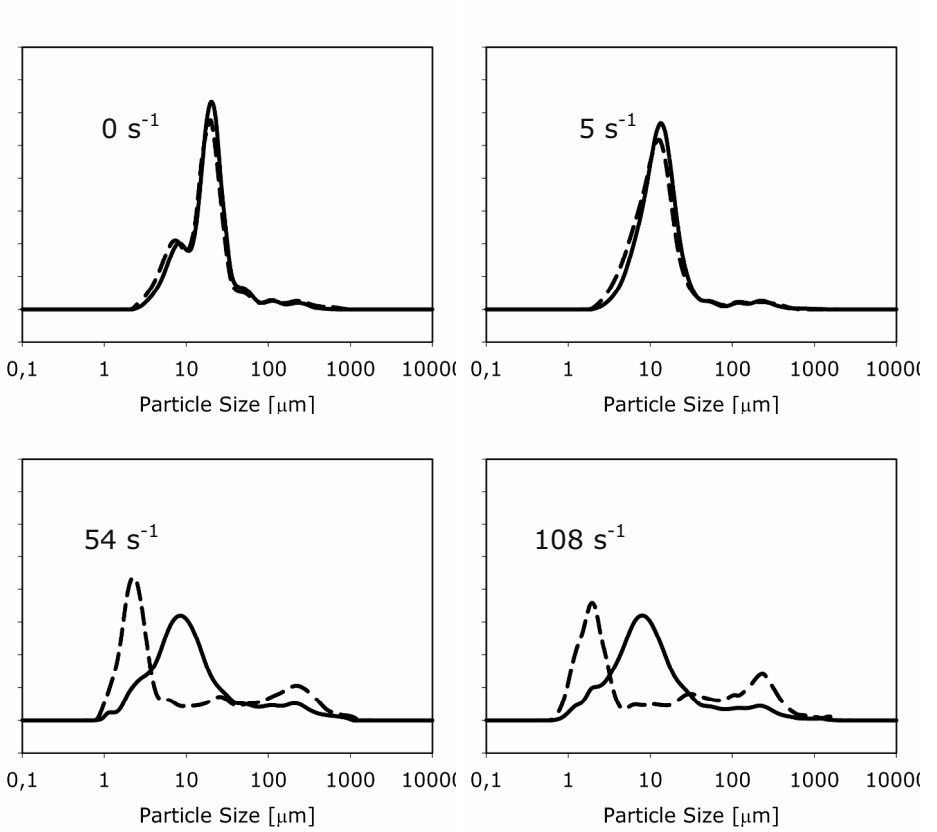


Figure 4: Overview of the particle size distribution of whey protein particles without (———) and with (— — —) thiol-blocking. The particles were prepared using different shear rates (0, 5, 54 and 108 s^{-1}) during processing.

Figure 5 compares the average particle sizes calculated from the CLSM images and light scattering data. The particle size decreased with increasing shear rate for both the unmodified and the thiol-blocked whey

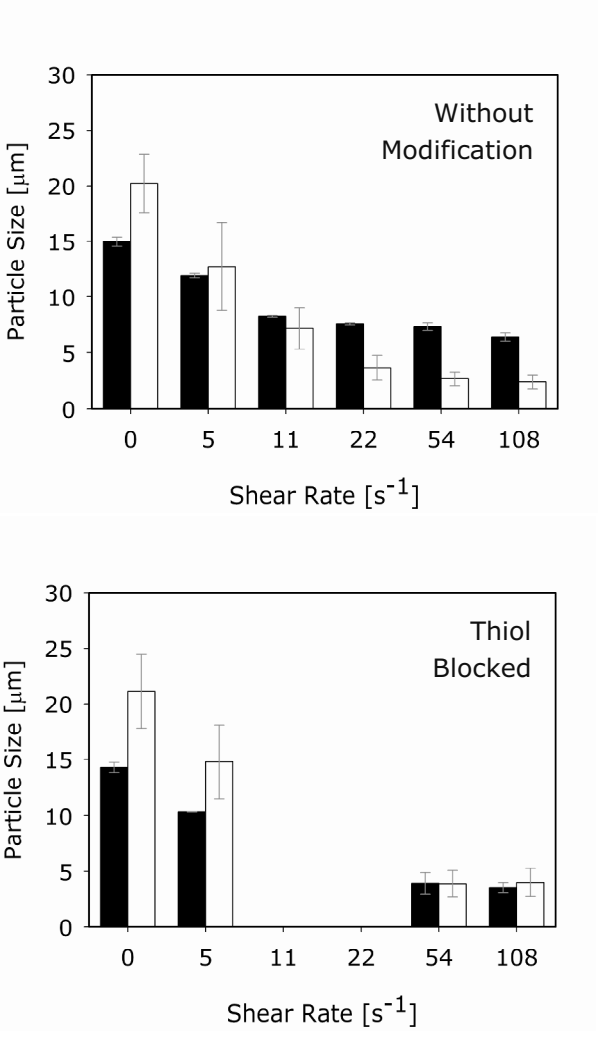


Figure 5: Overview of the size of whey protein particles without thiol-blocking (top-figure) with thiol-blocking (bottom-figure) analyzed by light scattering (black) and CLSM (white). The particles were prepared using different shear rates (0, 5, 11, 22, 54 and 108 s^{-1}) during processing.

protein particles. As stated in the previous paragraph, the results of the light scattering data show that the small unmodified whey protein particles formed clusters. The particle size measured with CLSM still decreased, while the cluster size measured with light scattering was constant from 11 s^{-1} onwards. As a consequence, the amount of particles forming a cluster increased with decreasing particle size. Thiol-blocked particles were not clustered, which explained why CLSM and light scattering gave comparable values for the average particle size.

The results above show that the thiol-blocked whey particles have a comparable non-spherical shape and they are similar in size. This strongly suggests that under the conditions applied (gradual decrease of the pH and consequently a decrease of the rate and extent of disulphide bond formation) particle formation occurs before the particle is strengthened by disulphide bonds. This seems in line with the fact that the rate of disulphide bond formation is low at low pH. Figure 1 indicated that whey protein particles were formed after approximately 3 h (though the actual particle formation process occurred even faster), while the formation of disulphide bonds takes much more time under acidic conditions [7, 26, 27].

Figure 6 compares the shear stresses during the formation processes of the thiol-blocked whey protein particles and the unmodified whey protein particles at a shear rate of 108 s^{-1}. It can be seen that the shear stress profile over time is rather similar initially. After the peak in shear stress (i.e. after particle formation), some differences were visible.

The suspensions with thiol-blocked particles had a lower viscosity compared to the unmodified particles. Besides, the thiol-blocked particles showed only one peak during phase separation, while the unmodified

particles gave an additional peak after approximately 6 h. The lower viscosity of the thiol-blocked particles could result from softer particles, or the absence of clusters.

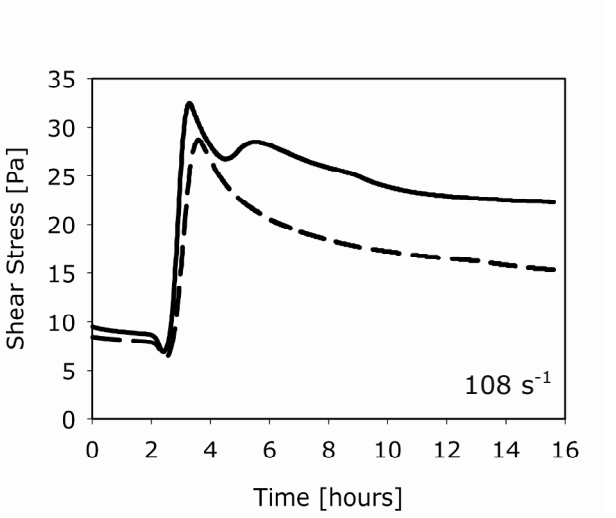
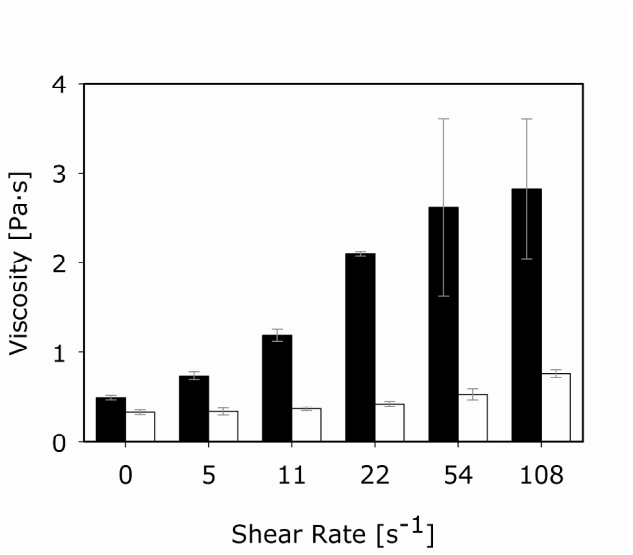


Figure 6: Viscosity as function of processing time of whey protein particles without (———) and with (— — —) thiol-blocking at 108 s^{-1}.

To conclude, the addition of a thiol-blocking ingredients did not influence the actual formation of the whey protein particles, but played an important role in the protein particle properties, which became evident from the differences in clustering behaviour.

To quantify the effect of disulphide bonds on whey protein particle properties, we investigated the rheological behaviour of both suspensions. Figure 7 shows the apparent viscosity at 1 s^{-1} and the shear thinning behaviour as a function of the applied shear rate during the formation of the thiol-blocked whey protein particles and the unmodified whey protein particles. There are similarities as well as differences for the thiol-blocked whey protein suspension and the unmodified whey protein suspension.



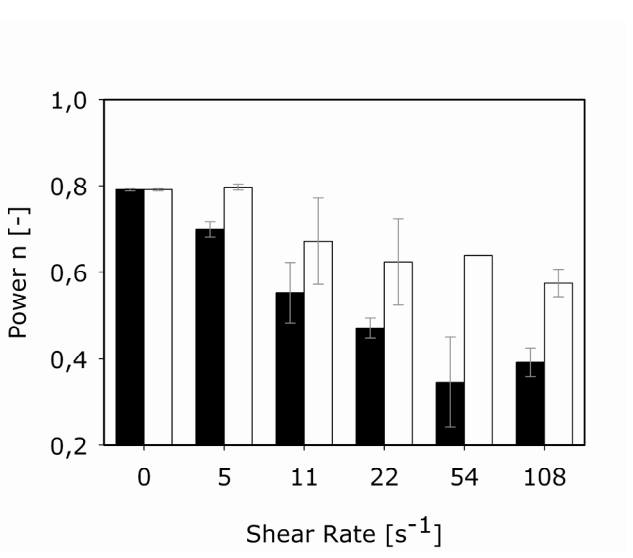


Figure 7: The apparent viscosity (Pa·s) at 1 s^{-1} and the flow behaviour index as a function of the applied shear rate (0, 5, 11, 22, 54 and 108 s^{-1}) during the formation of the thiol-blocked whey protein particles (white) and the unmodified whey protein particles (black). The flow behaviour index is calculated using the Herschel-Bulkley model.

Both particle suspensions show shear thinning behaviour. Another similarity is that the viscosity and the shear thinning behaviour increased for particles produced at a higher shear rate. This increase in viscosity could be correlated to the decrease in particle size, which led to increased particle interaction and a larger number of particles present in clusters. The latter effect accounts for higher inclusion levels of the continuous phase leading to a higher viscosity [28-30]. The shear thinning behaviour can be explained by considering the breakage of clusters and alignment of particles or clusters with the shear flow.

Differences became visible when the results were analyzed more quantitatively. Then, the unmodified whey protein particle suspensions showed significantly more shear thinning behaviour. The differences were most pronounced for the smallest particles. The rheological measurements confirmed that unmodified whey protein particles were able to form stable clusters, while thiol-blocked whey protein particles did this significantly less. This observation leads to the conclusion that the thiol groups were essential for the particle interaction and clustering in case of whey protein particles.

The elasticity of the protein network was measured with oscillatory strain experiments in the range of 0.01 - 300 %. Figure 8 shows the storage and loss moduli obtained from the linear viscoelastic region as a function of the shear rate applied. There was a clear difference in the storage modulus of the thiol-blocked whey protein suspension and the unmodified whey protein suspension. The storage moduli of the thiol-blocked whey protein suspensions were lower than the moduli of the corresponding unmodified whey protein samples, though large variations in the storage moduli were observed for suspensions produced at high shear rate.

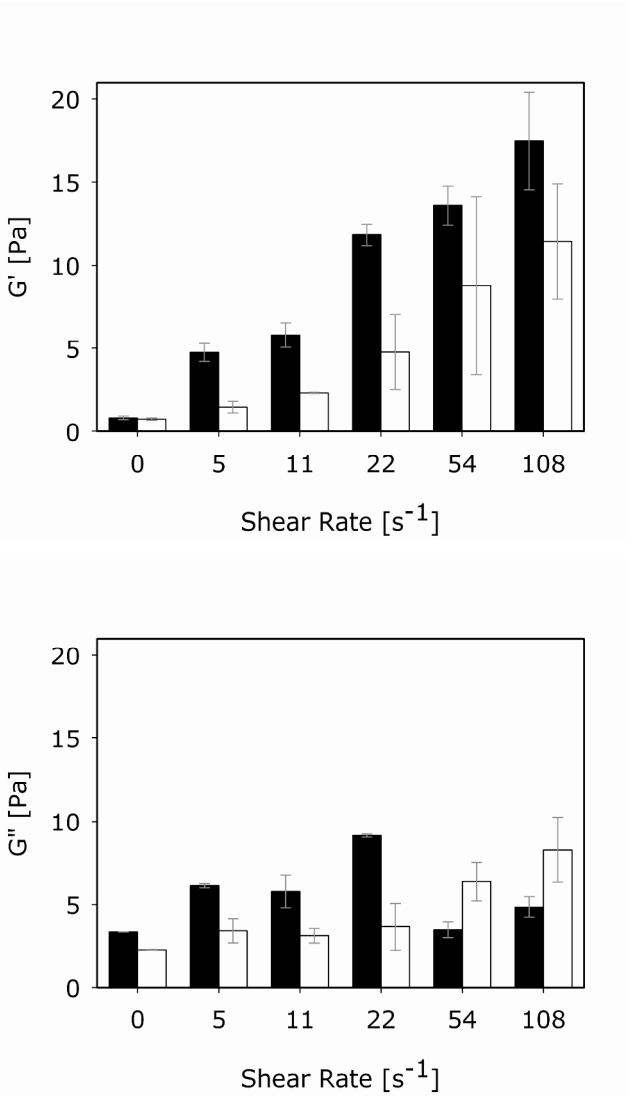


Figure 8: Storage modulus (Pa) and loss modulus (Pa) as a function of the applied shear rate (0, 5, 11, 22, 54 and 108 s^{-1}) during the formation of the thiol-blocked whey protein particles (white) and the unmodified whey protein particles (black).

The loss moduli were lower for thiol-blocked whey protein suspensions as well, except for the suspensions produced at a processing shear rate of 54 or 108 s^{-1}. The loss factor was higher for all samples containing thiol-blocked whey protein (results not shown). The higher moduli for the unmodified whey protein suspension compared to the thiol-blocked whey protein suspensions indicate that more energy was needed for the deformation of the network, most likely due to the fact that disulphide bonds were formed. The storage modulus showed a higher increase upon production shear rate than the loss modulus, indicating that the particle network became more elastic when higher production shear rates were used. Nevertheless, the results suggest that small whey protein particles can form a network due to physical interactions, even without the formation of disulphide bonds.

4.4 Conclusions

This study confirms that the formation of whey protein particle of colloidal size is provoked through gelation. The actual particle formation is fast once protein aggregates starts to form a gel. Due to gelation, the mobility of the protein phase rapidly decreased as a result of which non-spherical particles are formed when they were produced under simple shear flow conditions. Due to the fact that particle formation process is fast, the ability of proteins to form disulphide bonds does not play an important role in the particle formation process.

Whey protein particles were able to form particle clusters. Protein particle clustering was favoured by a smaller particle size due to increased particle interaction. The interaction of the whey protein particles into a network

was observed for both whey protein suspensions that could form disulphide bonds and whey protein suspensions than could only form physical interactions, but the network was stronger when disulphide bonds could be formed. We therefore conclude that the ability of protein particles to form disulphide bonds is an essential factor in understanding the behaviour of colloidal whey protein particle suspensions.

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Abbreviations

Formula

$\dot{\gamma}$	shear rate
η	viscosity
η^*	complex viscosity
η_c	continuous phase viscosity
η_d	dispersed phase viscosity
λ	viscosity ratio of the continuous and the dispersed phase
σ	interfacial tension
ω	angular frequency
G’	storage modulus
G”	loss modulus
h_{cr}	critical thickness of the fluid layer
R	particle size
tan δ	loss tangent / loss factor
We	Weber number

Text

CLSM	confocal laser scanning microscope
GDL	glucono-delta-lacton
Mw	molecular weight
NEM	N-ethylmaleimide
PMI	partially mobile interface

Part II
Application

Chapter 5

A Novel Method to Prepare Gluten-Free Dough Using a Meso-Structured Whey Protein Particle System

This chapter presents a novel concept for making an elastic dough using a structured protein suspension. The idea behind is based on the hypothesis that a number of gluten properties originates from a particle structure present in the gluten network. Three different mesoscopically structured whey protein suspensions were produced: whey protein aggregates, a whey protein cold set gel, and whey protein particles. Dough or batter mixtures were prepared by mixing the structured protein particle suspension with starch. Farinograph curves, small and large deformation experiments showed that the presence of a mesoscopic protein structure had a large impact on the properties of gluten-free starch mixtures. The whey protein that was structured into a mesoscopic particle suspension changed the starch mixture from a liquid into a cohesive material, having strain hardening properties.

This chapter was accepted for publication as:
van Riemsdijk, L. E., Pelgrom, J. M., van der Goot, A. J., Boom, R. M. & Hamer, R. J. A novel method to prepare gluten-free dough using a meso-structured whey protein particle system. Journal of Cereal Science (2011)

5.1 Introduction

People suffering from coeliac disease - gluten intolerance - cannot enjoy the structural and functional properties that gluten provides in many food products. Due to increasing number of people having an intolerance for gluten, a need is raised for the production of breads without the gluten. A huge number of recipes are available to produce gluten-free breads. Industries, scientist, and also patients themselves are designing their own gluten-free recipes. Generally, those recipes contain many different ingredients such as hydrocolloids e.g. [1, 2], dairy powders e.g. [3, 4], gelatin [5] and gluten-free cereals. The consistency of current gluten-free mixtures is not comparable with the consistency of wheat dough, though recent studies showed great progress here e.g. [2]. Nevertheless, gluten-free mixtures are often batters, which do not have the elasticity that is characteristic for wheat dough. In addition, mixtures with hydrocolloids or milk powders have low values for the storage modulus and comparable values for the loss modulus giving a higher loss factor, compared to wheat dough [4, 6, 7]. This means that those batters are not very elastic. Mechanical properties, such as strain hardening are not reported, probably due to the fact that those batters do not form a coherent mass, which makes a large deformation test not possible. However, strain hardening of dough is reported to be a good indicator for bread-making properties [8].

In this chapter, we present an alternative concept for the design of a gluten-free mixture. The gluten will be replaced by a protein (whey) structured at the mesoscopic scale. The use of a protein (in combination with a polysaccharide) is not new [5], but altering the protein structure on the mesoscopic level is a new concept. We mainly focus on the mesoscopic structure, because this scale is very promising in producing product analogues, such as fat substitute and meat alternatives [9, 10].

In those examples, the differences at molecular scale could be largely compensated by a correct structure at meso-scale. Here, we selected a protein (whey) that can be structured into particles of mesoscopic scale. As a result of the high degree of interactions present in the whey protein particle suspension, these whey protein particles can form a particle network. In chapter 2, it was shown that this whey protein particle network is strongly elastic at small strain values [11]. The use of a whey protein suspension as gluten alternative is based on the hypothesis that gluten contains a particle structure [12]. Although the debate on the role of mesoscopic gluten structure is still ongoing, a few things are commonly accepted. The main structure builder of gluten is the glutenin, which forms an elastic network in dough. This network is very resistant for stretching, and is thought to be responsible for the self-healing properties of gluten [13-16]. Both fast formation of physical linkage between the glutenin molecules, and strong disulphide bonds are considered to be important for the network properties [17].

The aim of this study is, therefore, to investigate the potential of a protein-starch mixture as a gluten-free dough formulation. The protein will be structured at the mesoscopic scale using three different methods to quantify the effects of different structures on the final properties. The protein-starch mixtures will be evaluated on rheological and mechanical properties and Farinograph characteristics.

5.2 Experimental Section

5.2.1 Preparation of Protein Structures

Three different protein structures were prepared from a whey protein (WP) solution (Davisco Foods International Inc., USA), being WP aggregates, a WP cold set gel and WP particles. The WP aggregates were prepared by heating a WP solution at 68 °C for 2.5 h. The WP particles were prepared by mixing the WP aggregate suspension with a locust bean gum (Danisco Holland BV, The Netherlands) solution and gradually decreasing the pH of the mixture through addition of glucono-delta-lacton (GDL, Sigma Chemicals, The Netherlands). The WP cold set gel was prepared similarly to the WP particles, except for the locust bean gum addition. Upon mixing with starch in the Farinograph (see next section), it is to be expected that the WP cold set gel will be ruptured into smaller gel patches. The production procedures are schematically represented in figure 1. The procedures are described in more detail in chapter 2 [11].

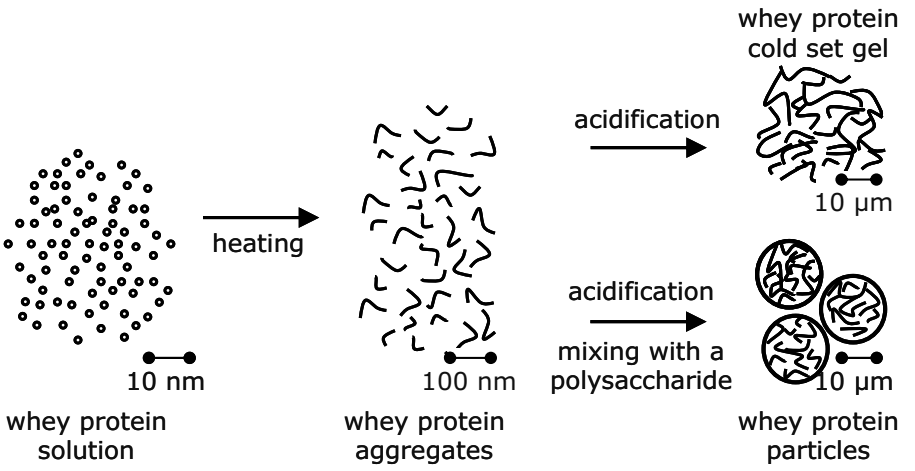

Figure 1: Conversion of native whey protein into whey protein aggregates followed by conversion into a whey protein cold set gel or whey protein particles.

5.2.2 Preparation of Starch Mixtures

Gluten-free mixtures were prepared through mixing starch (Sigma Chemicals, The Netherlands), salt (Merck, Germany) and the WP-locust bean gum suspensions in a Farinograph dough kneader. For reasons of comparison, the mixtures were diluted with locust bean gum and water to equalize the amount of WP and locust bean gum in all gluten-free mixtures. The final concentration of protein is 2.5 % (w/w db), the final concentration of locust bean gum is 0.4 % (w/w db) and the water percentage is 47 % (w/w). The production method used for the protein particles restricted the protein concentration of the starch-protein mixtures to lower amounts than the protein content of normal wheat flour. The amount of water used was such that a coherent mass was obtained. Three gluten-containing reference mixtures were made. The first reference mixture consisted of Soissons wheat flour (Meneba, Rotterdam, The Netherlands), salt and water. The specific properties of this flour are described by van der Zalm et al. (2010) [18]. The final concentration of protein was 11 % (w/w db) and the water percentage was 41 %. The second and third gluten-containing reference mixtures were obtained by mixing starch, vital wheat gluten (Roquette, France), salt and water. The final concentration of gluten in the mixture was equal to that of normal wheat dough (second reference) or equal to that of the WP starch-mixtures (third reference). The water percentage of the latter mixture was equal to that of gluten-free mixtures. Finally, a mixture was prepared without any protein to investigate the effect of locust bean gum. This means that the mixture only consisted of starch, salt and a locust bean gum solution. The water percentage of this mixture was equal to the other gluten-free mixtures.

All mixtures were prepared by combining the ingredients in a 300 g Farinograph bowl (Brabender OHG, Duisburg, Germany) for 3 min using a mixing rate of 63 rpm and a temperature of 30 °C. Soissons flour, starch, gluten and salt were added before mixing; water and protein suspensions were added during mixing within 30 s. Unless stated otherwise, each each type of mixture was prepared in duplicate and all analyses were done once for each mixture. This means that all analyses were measured in duplicate for each mixture type.

5.2.3 Analysis of Starch Mixtures

Protein Content

The protein contents of protein suspensions and starch mixtures were determined with the Dumas method (using N=5.70 for gluten and N=6.38 for whey protein).

Rheological Characterization of Suspensions

Small Deformation Measurements

Immediately after starch mixture preparation, the mixture was transferred to a Paar Physica MCR 301 (Anton Paar, Austria) stress-controlled rheometer, equipped with a serrated plate/plate geometry (diameter 25 mm – gap 1 mm) and a solvent trap. After sample loading, samples were rested for 15 min to allow relaxation of the stresses induced. This relaxation time is used more often for dough rheology [18]. Strain sweeps were performed by using a logarithmic increase of the strain from 0.001 % to 400 % at a constant frequency of 1 Hz and a temperature of 25 °C.

Large Deformation Measurements

The protein-starch mixtures were moulded into trapezium strips using a Kieffer mould coated with silicon oil immediately after the mixture preparation. The samples were allowed to rest inside the mould at 25 °C and 90 % RH for 45 min. After resting, the sample strips were elongated using a constant deformation rate of 3.3 mm/s with a texture analyzer (Instron-5564Series-Table-Model-Systems-Twin-column-design, Canton USA) equipped with a Kieffer dough-and-gluten extensibility rig and a 50 N load cell. The sample length was 18 mm and the isosceles trapezoid cross section was 16 mm^2 (3/5 × 4). At least three samples for each mixture type were tested. The force–displacement curves were transformed into stress–strain data as described by Dunnewind et al. (2004) [19], taking into account that most of the samples had a negligible banding distance, and assuming a constant volume. The stress (σ) at fracture, the Henky-strain (ε) at fracture stress, and the apparent strain hardening coefficient (n) were determined. The strain hardening coefficient was determined by applying an exponential fit $\sigma = k \cdot e^{\varepsilon \cdot n}$ on the σ – ε curve in the measured ε-range of 20 – 95 % of fracture strain.

5.3 Results

The quality of a flour is often related to the mixing parameters, such as peak consistency, mixing tolerance and dough development time determined by a Farinograph or mixograph [8]. Figure 2 depicts the Farinograph curves of the three references mixtures, the three gluten-free mixtures with WP and the mixture prepared without any protein. The Farinograph curves show for all mixtures an increase in the torque value during the first 30 s of mixing when water was added. For the Soissons mixture, the peak consistency was set at 4.9 Nm through adjusting the water content. This mixture has a large mixing tolerance, shown by the fact that the torque value decreased only slightly upon additional mixing for 3 min. The other mixtures showed different behaviour. The reference mixture with 10 % vital gluten, the gluten-free mixture with WP particles, and the gluten-free mixture with a WP gel showed a similar torque profile as the Soissons mixture. The main difference was that these mixtures showed a lower peak consistency, and a lower torque mixing tolerance than the Soissons mixture. For these three mixtures the torque-value after 3 min. was about 2 - 3 Nm. The reference mixture with 2.5 % vital gluten, the mixture prepared with WP aggregates, and the mixture prepared without any addition of proteins showed a different behaviour. These mixtures had a very low consistency after 3 min mixing, (0 - 1 Nm). The low consistency was caused either by a low peak consistency or a low mixing tolerance. The low peak consistency and the abrupt decrease in torque value were absent in normal wheat dough mixing. The typical wheat flour behaviour is associated with the self-healing properties of wheat dough. Most likely, the last three mixtures did not have a possibility to recover during mixing.

Figure 2 also shows that mixtures with a similar composition can have different Farinograph curves. Protein-starch mixtures prepared with WP aggregates showed a similar consistency as the mixture without any protein. The mixture prepared with a WP gel, however, had the highest peak viscosity which was even comparable with the reference mixture with 10 % gluten. The mixture prepared with WP particles had a slightly lower peak viscosity, but the final consistency was higher than that of the mixture prepared with a WP gel indicating a higher mixing tolerance.

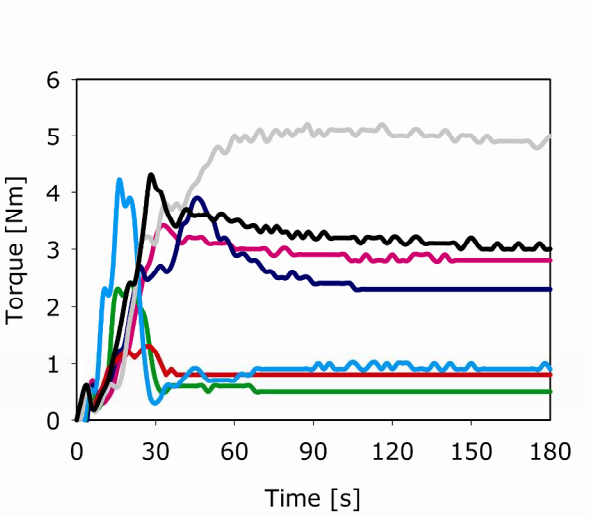

Figure 2: Torque values during preparation in a Farinograph of mixtures prepared of Soissons flour (grey) or prepared of starch and a protein source: gluten 10 % (black), gluten 2.5 % (turquoise) whey protein aggregates (green), whey protein gel (blue) and, whey protein particles (pink) or prepared of starch without any protein added (red)

The small and large deformation properties of the mixtures are depicted in figures 3 and table 1 respectively. The storage modulus values (figure 3a) are comparable for the Soissons mixture and the reference mixture with 10 % vital gluten. The results of these mixtures are in agreement with the

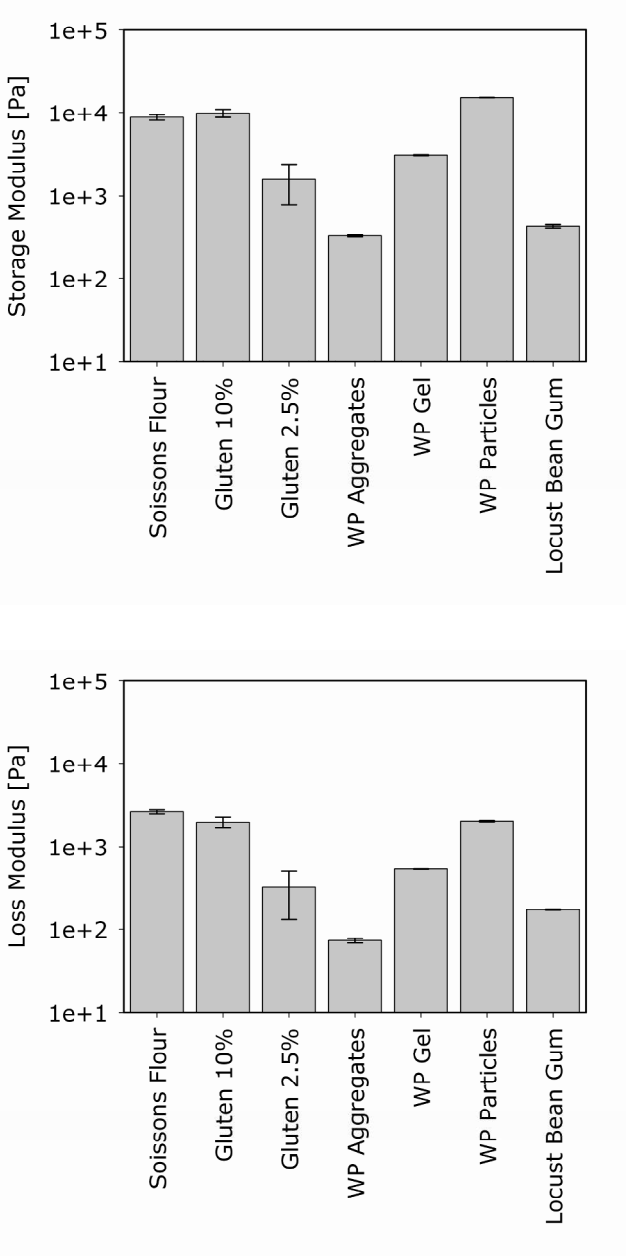

Figure 3: Storage modulus and loss modulus of small deformation measurements in a rheometer of mixtures prepared of Soissons flour, prepared of starch and a protein source: gluten, whey protein aggregates, whey protein gel, whey protein particles, and without any protein added.

results presented earlier [20]. The gluten-free mixture containing WP particles had a larger value for the storage modulus than the gluten-containing mixtures. All other gluten-free mixtures had a lower value for the storage modulus. The values were more comparable with the reference mixture with 2.5 % vital gluten and similar to the values reported in other studies on gluten-free mixtures [4, 6, 7]. The highest value for the loss modulus (figure 3b) was obtained for the Soissons mixture, the reference mixture with 10 % vital gluten and the mixture with WP particles. All other mixtures had a lower value for the loss modulus, which were similar to the values obtained in other studies on gluten-free formulations [4, 6, 7].

The loss factor (loss modulus divided by storage modulus) of the WP mixtures was similar to the references mixtures with 10 % gluten. The values of the loss factor for the WP mixtures were low compared to the values obtained in most other studies on gluten-free mixtures [4, 6, 7]. Another study on gluten-free recipes found a lower value for the loss factor, but the values for the storage and loss modulus were also high compared to wheat dough [21].

The mixtures without any protein had a high loss factor compared to the other mixtures, which indicates the importance of a protein structure for mixture stability. However, all mixtures also contained locust bean gum, which might influence the rheological properties. To investigate this effect, small deformation measurements were done after hydrolyzing the locust bean gum enzymatically using Caylase C3 (Cayla, Toulouse, France). The effects of locust bean gum conversion on the storage modulus and the loss modulus are depicted in figure 4. Except for the WP particle mixture,

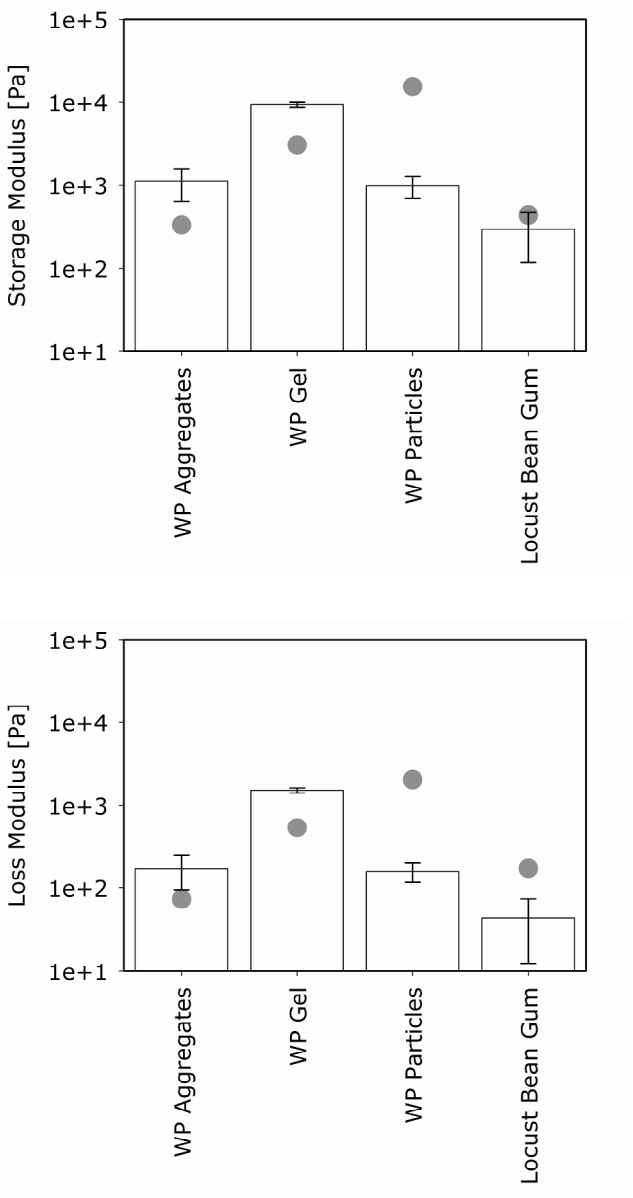

Figure 4: Storage modulus and loss modulus of small deformation measurements in a rheometer of mixtures prepared of starch and a protein source: whey protein aggregates, whey protein gel, whey protein particles, and without any protein added. Locust bean gum present in the samples is converted into oligomers with an enzyme. The circles represent the values with locust bean gum, the bars represent the values with oligomers.

all WP mixtures showed an increase in the storage modulus and loss modulus upon hydrolysis of the locust bean gum. The loss modulus of the mixture without protein decreased after hydrolysis, suggesting that locust bean gum works as a lubricant in the mixture. The WP particle mixture showed a decrease in the storage modulus and loss modulus upon conversion of locust bean gum. This indicates that locust bean gum played a more active role in this system. Probably, locust bean gum influenced the WP particle network in an other way, e.g. through depletion interactions or as a coascervate. After hydrolysis of locust bean gum, most mixtures (except for the WP gel mixture) were too liquid to perform large deformation tests.

The large deformation test was only possible for these recipes that gave a (semi-) solid material. The material should be (semi-) solid to allow moulding of the sample. If the sample remains too liquid, it will break or flow during preparation or transport, making a tensile test impossible. This was the case for the reference mixture with 2.5 % gluten and the gluten-free mixture with WP aggregates. The other mixtures showed large differences in their behaviour upon large deformation. The stress at fracture is much higher for the Soissons mixture compared with the other mixtures. The most comparable mixture is the reference mixture containing 10 % vital gluten, but even this mixture has a stress at fracture that is only 23.2 % of that of the Soissons mixture. The results for the gluten-containing mixtures were in agreement with earlier results (e.g. [22]).

Table 1: Stability during mixing in a Farinograph (percentage of the consistency that remains after 3 min mixing, the peak consistency is set at 100 %), Stress at fracture, Strain at fracture, Strain hardening value of extensional tests in a Texture Analyzer of mixtures prepared of Soissons flour, prepared of starch and a protein source: gluten, whey protein aggregates, whey protein gel, whey protein particles, and without any protein added.

Protein source	Protein % (db)	state	stability	Stress at fracture (kN/m^2)	Henky strain at fracture (-)	Strain hardening (–)
Soissons flour	11	firm	96	37.5	1.4	1.2
Gluten	10	firm	60	8.7	1.5	2.0
Gluten	2.5	liquid	28	-	-	-
WP aggregates	2.5	liquid	18	-	-	-
WP gel	2.5	firm	62	0.7	0.5	0.8
WP particles	2.5	firm	83	2.7	0.7	1.2
No	0	liquid	62	-	-	-

The gluten-free mixture with the highest stress at fracture was the WP particle mixture. This value was 7.2 % of the value obtained for Soissons mixture. The Henky-strain at fracture was comparable for the Soissons mixture and the reference mixture containing 10 % vital gluten. The Henky-strain of the WP mixtures was about half of the gluten-containing mixtures. However, the strain hardening behaviour was rather similar. This is important because the strain hardening is often described as an important parameter to maintain air bubbles in the dough rather than the absolute values of strain and stress at fracture [8, 23, 24].

The fact that the mixture containing WP particles had a similar strain hardening behaviour as wheat dough suggests that these WP particles have resemble the required structural features in a dough used for bread-making. The reference mixture with 10 % vital gluten showed the strongest strain hardening. The Soissons mixture and the WP particle mixture showed comparable strain hardening behaviour. The WP gel mixture did not harden upon elongation. In other words, the mesoscopically structured WP system resulted in a mixture with strain hardening properties when combined with starch. By manipulating the structure of a whey protein suspension, it is possible to obtain a gluten-free mixture with dough-like properties, which are relevant for its bread baking performance.

5.4 Discussion

The main purpose of this study was to investigate whether gluten functionality could be replaced through a mesoscopically structured WP system. The unique properties of wheat dough stem from the presence of wheat gluten in the system, and more specifically the glutenin fraction. This fraction makes the greatest contribution to dough properties, although its amount is small, about 20 - 40 % of the total protein fraction present in wheat flour [25, 26]. This fraction can be made visually by SDS-extraction [26, 27], in which those proteins end up in a gel-layer (the so-called glutenin macropolymer, GMP, fraction). A further study reveals that this gel-layer contains protein particles having a size (d_{32}) of about 5 - 10 µm. Upon mixing, this particle size decreases. The existence of these particles implies the presence of a dispersed protein phase in the gluten network. It is hypothesised that the presence of those protein particles accounts for the specific properties of dough, such as viscoelasticity, strain hardening, and self-healing properties. In addition, the ability to (re-)form disulphide bonds is of importance [28]. Therefore, we proposed a mesoscopic protein particle suspension as gluten alternative. In addition, particles should be able to interact and have self-healing properties to a certain extent. Self-healing properties are generally explained by a mechanism in which initially physical interactions are formed that are subsequently stabilized by additional chemical crosslinks [29, 30]. That is why we selected WP particles. In suspension those particles showed a large extent of interaction, resulting in elastic properties. In addition, WP is able to form disulphide bonds. Here, we studied the effect of the addition of those particles to starch to obtain a gluten-free dough.

From all the results, it became clear that the systems containing a dispersed protein phase showed similarities in properties with wheat dough, with respect to strain hardening and recovery (as measured by Farinograph). Both aspects (strain hardening and recovery) are relevant properties for bread-making. Mixtures that did not contain a dispersed protein phase (i.e. the mixture containing only a hydrocolloid, and the mixture containing WP aggregates) remained liquid-like. It seems therefore that the protein particles could be responsible for the high mixing tolerance, the large storage modulus, and the strain hardening behaviour. Similar properties were obtained for the WP gel mixture, though the mixing tolerance and strain hardening were less than the WP particle mixture. Nevertheless, the fact that this gel system has those properties could be caused by the fact that the gel will be ruptured into small protein patches upon mixing leading to a dispersed protein phase. The fact that the gel patches form a less strong network could be related to the size and size distribution of the protein patches obtained [16]. These properties determine the particle network formation.

It is remarkable that the mixture containing a dispersed WP phase can form a coherent mixture, although their amount is only 2.5 %. It becomes even more remarkable when taking into account that mixture with gluten in the same concentration remained liquid-like. Both observations can be understood by considering that only 20 - 40 % of the wheat gluten comprises the main structuring protein, i.e. the glutenin. In the mixture with 2.5 % gluten, the total amount of glutenin might be too low to form a percolating network. Consequently, it seems that a large part of the WP contributes to the protein particle network, considering that its concentration is comparable with the percentage GMP normally present in wheat dough.

In addition to the dispersed protein phase, locust bean gum might play a role in the properties of a particle network as well. This can be caused by a viscosity increase of the aqueous phase and/or through depletion interactions. Both effects can be used to explain the effect of locust bean gum conversion on mixture rheology. In case of viscosity, the hydrolyses of locust beam gum will lead to a reduced viscosity. To explain the effect of depletion interaction, one has to consider that the depletion interaction caused by the presence of locust beam gum increases the strength of the particle network formed in the WP particle mixture and WP gel mixture. If the locust bean gum is hydrolysed, the depletion interactions in the WP particle mixture will decrease. Why this effect does not occur in case of the WP gel mixture is not clear. One possible explanation is that a mixture of small and large particle patches was obtained after mixing. In that situation, depletion interaction could be induced by the small gel patches thereby reducing the role of locust bean gum. The latter experiment also suggests that the viscosity effect of locust beam gum might be less important than the depletion effects. Further research is needed to completely unravel how the presence of locust bean gum influences the properties of the protein particle network in the mixtures.

Except for the fact that the gluten-free mixtures prepared with WP aggregates differ in the protein structure at meso-scale, they also differ in pH. The WP aggregate mixture had a higher pH because the WP aggregates were transformed into a gel upon pH-decrease. We checked the effect of pH by preparing protein-starch mixtures with WP suspensions in which the pH was adjusted to pH 7 with a 1 M NaOH solution (Merck, Germany). The consistency of the mixtures remained rather similar after pH adjustment. The fact that the effect of pH is small suggests that interactions at molecular scale have become less important due to the

structure formed at the mesoscopic scale. It confirms our idea that mesoscopic structuring is a tool to suppress differences at molecular scale, which opens up the possibility to replace gluten by other proteins.

This chapter describes the properties of a dough that only consists of 5 ingredients: WP particles, locus beam gum, starch, water and salt. In our composition, we mainly focussed on the structure forming properties of protein particles. This means that we have many opportunities to improve our dough. For example, a plasticiser comparable with the gliadins could be added. Besides, there are a lot of other ingredients added to a fully formulated wheat dough to improve its properties [13]. The WP-mixture in this study still lacks those additional ingredients. The use of additional ingredients in combination with the WP mixture opens up many new opportunities to create a mixture that has even more wheat dough-like properties.

5.5 Conclusions

The main conclusion of this study is that mesoscopic protein structuring is a promising new method to produce mixtures with viscoelastic and strain hardening properties.

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Chapter 6

Preparation of Gluten-Free Bread Using a Meso-Structured Whey Protein Particle System

This chapter presents a novel method for making gluten-free bread using mesoscopically structured whey protein. The use of the meso-structured protein is based on the hypothesis that the gluten structure present in a developed wheat dough features a particle structure on a mesoscopic length scale (100 nm – 100 μm). Whey protein particles were prepared by cold gelation of soluble whey protein aggregates during phase separation. The addition of a 2.4 % (w/w db) whey protein particle suspension to wheat starch resulted in a dough that could be baked into a leavened bread with a specific volume up to 3.7 ml/g and a bubble size comparable with a normal bread. The relevance for structuring the whey protein into mesoscopic particles was confirmed by tests in which only a homogeneous whey protein gel or a whey protein solution was used. The protein particle system gave better results after proving and baking compared with these systems.

This chapter was submitted as:
van Riemsdijk, L. E., van der Goot, A. J., Hamer, R. J. & Boom, R. M. Preparation of gluten-free bread using a meso-structured whey protein particle system. Journal of Cereal Science (2011)

6.1 Introduction

Gluten intolerance has become a well-known disorder affecting almost 1 percent of the population [1]. As a result, there is a demand for high-quality gluten-free products, because the only known remedy is a life-long gluten-free diet. This explains the industrial and scientific interest in methods to replace gluten in a broad range of products. Bread is the most important product example in which gluten has to be replaced. Regular bread is prepared from a leavened dough. Replacement of wheat dough by a gluten-free formulation requires a dough with good viscoelastic properties to allow handling and sufficient gas retention [2].

The first studies on gluten replacement go back to the 1960s. These studies described the use of ingredients such as glyceryl monostearate to replace gluten e.g. [3]. These first studies were followed by many other studies that further explored the use of ingredient addition (mostly hydrocolloids and emulsifiers) to gluten-free starches or flours e.g. [4-10]. Generally, gluten-free products were made starting from a batter instead of dough. In a bread prepared from a batter system, gas cell stabilization is not based on elasticity provided by a protein network as in wheat dough, but on stabilization through a high bulk viscosity obtained via hydrocolloid addition and starch gelatinization. A high bulk viscosity can provide some gas retention, but lack of elasticity generally gives rise to problems with gas cell stabilization [11, 12]. Others have studied the effect of changing the molecular protein properties, e.g. by protein crosslinking using enzymes [13] or by heat [14] or high pressure treatment [15].

In this chapter, we present a novel approach to making gluten-free breads. We use a formulation that contains only three main ingredients: wheat starch, whey protein and locust beam gum. The key characteristic of this mixture is that whey protein and locust bean gum are structured into a mesoscopic protein particle suspension. The idea of using mesoscopically structured whey protein is based on the hypothesis that the unique properties of the wheat dough originate, at least partially, from the protein properties at the mesoscopic length scale [16]. In the previous chapter, we showed that mesoscopically structured whey protein suspension gives the mixture dough-like properties, including strain hardening [17]. Strain hardening has not been reported in gluten-free mixtures before, even though strain hardening of dough is reported to be a good indicator for bread-making properties [18].

The aim of this study is therefore to investigate the bread-making potential of a mixture containing mesoscopically structured whey protein, locust bean gum and wheat starch. We have analysed whether the mixture can be used in a regular bread-making process that is based on dough as the starting material. The protein was transformed into three different mesoscopic structures to show the effects of the structure of the protein network on the final bread properties.

6.2 Experimental Section

6.2.1 Preparation of Starch Mixtures

The whey protein (WP) was transformed into three different protein structures, namely WP aggregates, a WP cold set gel, and WP particles. WP aggregates were prepared by heating a WP solution at 68 °C for 2.5 h. A WP cold set gel was prepared by gradually decreasing the pH of the WP aggregate suspension with glucono-delta-lacton (GDL, Sigma Chemicals, The Netherlands). WP particles were prepared similar to the gel, but here locust bean gum was added before gelation of WP to induce phase separation. A detailed description of the preparation method is described in the previous chapters [17, 19, 20].

Gluten-free mixtures consisted of 50 g wheat starch (Sigma Chemicals, The Netherlands), 38 ml of a WP (Davisco Foods International Inc., USA) locust bean gum (Danisco Holland BV, The Netherlands) suspension, 1.1 g salt (Merck, Germany), 0.9 g dried active bakery yeast (Algist Bruggeman Co., Belgium) and 0.5 g D-glucose (Sigma Chemicals, The Netherlands). This resulted in a final mixture with a protein concentration of 2.4 % (w/w db), a concentration of locust bean gum of 0.4 % (w/w db) and a moisture content of 46 % (w/w). The production method used for the protein particle solution restricted the protein concentration of the starch-protein mixtures to lower amounts than the protein content of normal wheat flour. The amount of water used was such that a coherent mass was obtained. One reference mixture was produced using 50 g wheat starch, 5.6 g vital gluten (Roquette, France), 38 ml water, 1.1 g salt, 0.9 g dried active bakery yeast and 0.5 g D-glucose. The final concentration of gluten and water in the mixture was equal to that of normal wheat dough.

Lowering the gluten content to 2.4% (w/w db) led to a mixture that was still too liquid to make a good bread. Consequently, the protein content in both formulations could not be made the same.

All mixtures were prepared by mixing the ingredients in a 50 g Farinograph bowl (Brabender OHG, Duisburg, Germany) for 3 min using a mixing rate of 63 rpm and a temperature of 30 °C. These mixing conditions were chosen such that a homogeneous mixture was obtained, and no over-mixing was observed. Wheat starch, gluten, salt, sugar and yeast were added before mixing; water and the protein suspensions were added during mixing within 30 s. Unless stated otherwise, each type of mixture was prepared in duplicate.

6.2.2 Analysis of Starch Mixtures

Structural Analysis

After protein structuring, the WP solutions (gel and particles) were transferred into a chambered cover glass (Nunc, Naperville, IL, USA). Rhodamine B was added before visualizing with a CLSM (LSM 510, Zeiss, Oberkochen, Germany). After mixture processing, the WP structure was liberated from the mixture by dissolving the starch present in the mixture. First, a ten times diluted solution of the mixture was heated at 80 ºC for 5 min. Then, the WP structure was separated by centrifugation at 1000×g for 3 min. The gel layer formed was diluted and transferred into a chambered cover glass, where it was stained with Rhodamine B for visualizing with the CLSM. The effect of the heat treatment and the effect of the starch solution were excluded with two tests. No effect of the heat treatment (at 80 ºC) immediately after particle formation was observed. No effect of converting all starch by incubation of the liberated particles with Amylase p500 (Gist-Brocades) was observed.

Proving Properties of the WP-Starch Mixtures

Immediately after preparation of the mixture, the aeration and the volume increase of the mixtures during proving were analyzed using two different methods. One method measured the total amount of gas produced during proving; the other method determined the decrease in density, and consequently the increase in volume during proving. Both measurements were carried out in duplicate at 35°C. The amount of gas produced during proving was measured using the method as described by Peighambardoust (2010) [21]. A mixture sample of 5 g was placed in a flask, that was connected to an inverted cylinder filled with an oxalic acid solution of pH 3. The liquid level in the cylinder went down due to the gas production. The amount of gas volume produced was measured every 5 min for 150 min.

The density decrease during proving was measured using the method described by Campbell (2001) [22]. A sample of 10 g was measured in air and in silicon oil at 35ºC (the density of silicon oil at this temperature was 0.95 kg/m^3). The sample was placed in a Sartorius Density Determination Kit (YDK 01 LP) with an anti-floating cap on a Sartorius ME 235S precision balance (Sartorius). The weight of the sample was measured every 10 s for 20 – 40 min, until the sample started to absorb the silicon oil. The density of the mixture was measured using a sample of the mixture without yeast (static dough density measurement) and sample with yeast (dynamic dough density measurement).

Bread Analysis

Two mixture balls of 30 g were placed in baking tins for proving in a climate chamber at 35 ºC and 85 % RH for 100 min. The dimensions of the tins were 18 cm^2 (top) / 15 cm^2 (bottom) x 3 cm high.

Two different proving methods were used. In the first method (with sheeting), the mixture was sheeted after 40 min proving, folded up and placed back into the tins to allow further proving for the remaining 60 min. In the second method (without sheeting) the mixtures were proved for 100 min, without any interruption. After proving, the mixtures were baked in a pre-heated automated kitchen bread machine at ~200 ºC for 35 min. Mixing, proving and baking were done in duplicate.

After baking, the breads were cooled to room temperature before further analyses. The bread volumes were analyzed using the rapeseed displacement method (AACC-2000 method 10-05). The bread structure was visualized by photographing the whole breads, section planes and 3 mm thick slices. The bread was sliced using a meat slicing machine (EH-170T, Graef). From each bread type a representative slice was used for photographic representation and for C-Cell analysis (Calibre Control International, Warrington, UK). C-Cell analyses were done with more slices if this was necessary and possible (only for the non-sheeted samples). The structure of the bread crumbs was evaluated using the C-Cell Bread Imaging System. The parameters used for crumb characterization were the average cell diameter (mm), and the area of holes (%). A smaller average cell diameter reflects a finer crumb structure. A larger area of holes reflects poor bread properties, often caused by a lack of elasticity of the original dough giving poor gas cell stabilization.

6.3 Results

Figure 1 depicts the Farinograph mixing characteristics of the reference mixture prepared with vital gluten and the three gluten-free mixtures. The Farinograph curves show an increase in the torque value during the first 30 s of mixing when water was added to all mixtures.

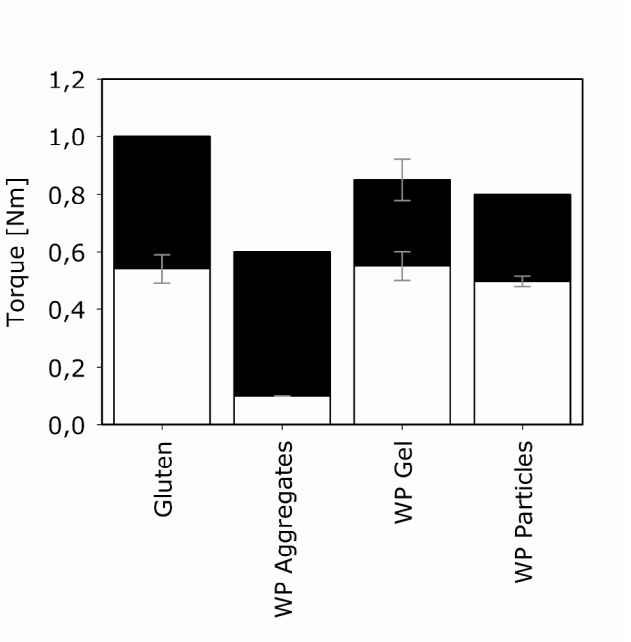


Figure 1: Farinograph values (50grams Farinograph) of mixtures prepared with gluten or whey protein structures. The white bars mark the torque values after preparation of the mixture; the black bars mark the torque values at the peak of the Farinograph curve.

The reference mixture with 10 % vital gluten had the highest peak consistency (1 Nm). The peak consistency of the mixtures prepared with a WP gel or WP particles was lower (0.8 Nm – 0.9 Nm), but the final consistencies of these last two mixtures were comparable with the final consistency of the wheat dough (0.5 Nm - 0.6 Nm).

The peak and final consistencies of the mixture prepared with WP aggregates were significantly lower (the peak consistency was 0.6 Nm, and the final consistency was 0.1 Nm). The low peak consistency and the abrupt decrease in torque value indicated that this mixture had hardly any ability to recover during mixing.

Figure 2 shows the microscopic images of the WP gel and the WP particles before mixture processing and after mixture processing and isolation. The WP gel was shred by the mixer into gel fragments. The WP particles showed no clear disruption due to mixing, the particles were only slightly deformed (less spherical). The similar behaviour of the WP gel and WP particles during mixing (Farinograph curve) can be explained by the similarities of the structures after mixing.

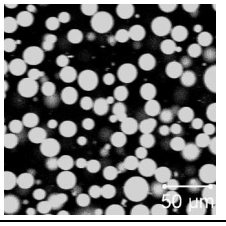
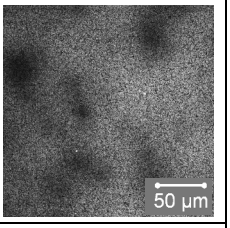
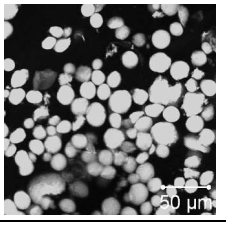
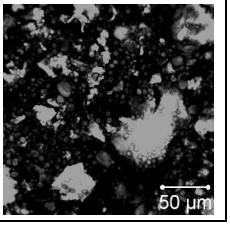
Microscopic image	Whey protein particles	Whey protein gel
Before mixture processing		
After mixture processing		

Figure 2: Microscopic images of protein particle structures before and after mixture processing

After mixing the WP gel fragments and the WP particles both formed a dispersed protein phase. The WP gel was shred by the mixer into gel fragments of a similar size as the WP particles.

Before comparing the mixtures on the baking properties, we checked the gas production by the yeast in the various mixtures. No differences in gas production were observed amongst the mixtures. Processing with WP instead of gluten had no influence on the gas production of the yeast. In all mixtures the gas was mainly produced during the first 50 min of proving, resulting in a final gas production of ~3.5 ml/g mixture. The gas production was low compared with the production in normal wheat dough (4 - 8 ml/g dough) [21]. This may be caused by a limitation in substrate availability, because the mixtures consisted of wheat starch, WP, locust beam gum, and only 0.5 g of D-glucose and did not contain any amylase for example. The low gas production implies that the specific volume of the bread in this study is limited. Because there was no difference between the gas production for the different mixtures, the gas production itself can be excluded as a basis for differences between the mixture volumes after proving and the resulting bread volumes.

The bread-making process resulted in differences in the bread volumes of the reference mixture and the gluten-free mixtures. Figure 3 depicts the specific volumes of the mixtures directly after mixing (in ml/g mixture), the volume of the mixtures after proving (in ml/g mixture) and the bread volumes after baking (in ml/g bread; this value is approximately 150 % of the specific volume in ml/g mixture caused by the lower mass of the bread due to moisture evaporation during baking). The differences between the volumes before baking (with and without proving) were small, but the differences in the volumes of the final breads (after baking, with and

without sheeting) were significant. Proving resulted in an increase in the bread volume for all samples to a final specific volume of 1.8 ± 0.1 ml/g mixture. The fact that the mixture volumes are not significantly different may indicate that all mixtures can incorporate a similar quantity of gas during proving.

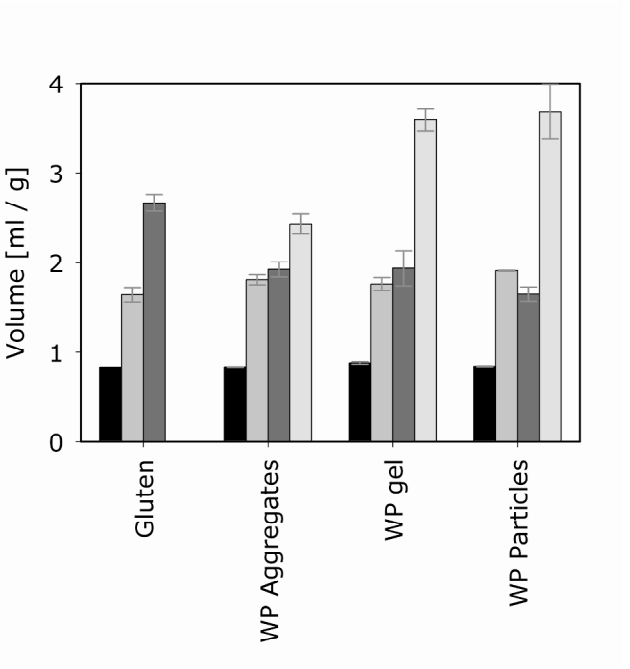


Figure 3: The specific mixture volume after mixing (black) and after proving (white) in ml/g mixture, and the specific bread volume with sheeting (dark grey) and without sheeting (light grey) in ml/g bread

The specific volume of the reference bread is not influenced by baking and remains 2.7 ml/g bread (which corresponds to 1.8 ml/g mixture). The volumes of the gluten-free breads depended on the sheeting step. If the gluten-free mixtures were sheeted, they showed a decrease in the bread-specific volume during baking, expressed as the volume per amount of mixture (the bread volume is 1.8 ± 0.1 ml/g bread and 1.3 ± 0.1 ml/g

mixture, the mixture volume before baking was 1.8 ml/g). The final specific bread volumes (with sheeting) of the gluten-free breads were low compared with the gluten-containing bread, and the specific volume of bread prepared from wheat flour. Omission the sheeting step had a large impact on the final bread volume. The final specific volumes of the gluten-free breads were 2.4 ml/g bread (1.6 ml/g mixture) for the WP aggregate bread, 3.6 ml/g bread (2.2 ml/g mixture) for the WP gel bread and 3.7 ml/g bread (2.3 ml/g mixture) for the WP particle bread. The specific volumes of those breads were larger than the specific volumes of the (sheeted) reference bread. These values are in the range of typical specific volumes for regular wheat breads: 3.5 – 4.0 ml/g bread [23]. These specific volumes suggest that the WP-gel and WP-particle mixtures captured more than 50 % of the gas produced by the yeast in the bread.

The impact of protein type and structure on the gas cell distribution in the bread is presented in figures 4 and 5. The reference bread had a uniform distribution of small and large gas cells over the whole bread. The C-Cell measurements, done to obtain a broad indication of the crumb structure, confirm the visual observations of the bread quality. C-Cell measurements show that the average gas cell diameter of this gluten-containing bread is 1.7 mm, and only 4 % of the bread volume is ruptured. The sheeted gluten-free breads showed a less uniform distribution; they contained more ruptured regions. The three gluten-free breads had different gas cell distributions, even though the overall volumes of the three gluten-free breads were similar. The bread with a WP cold set gel had the largest amount of ruptures, and the lowest amount of gas cells. The average gas cell diameter for this bread was 13 mm, indicating that the average gas cell has the size of a hole. The bread with WP aggregates and WP particles had smaller and more uniform gas cells with an average diameter of

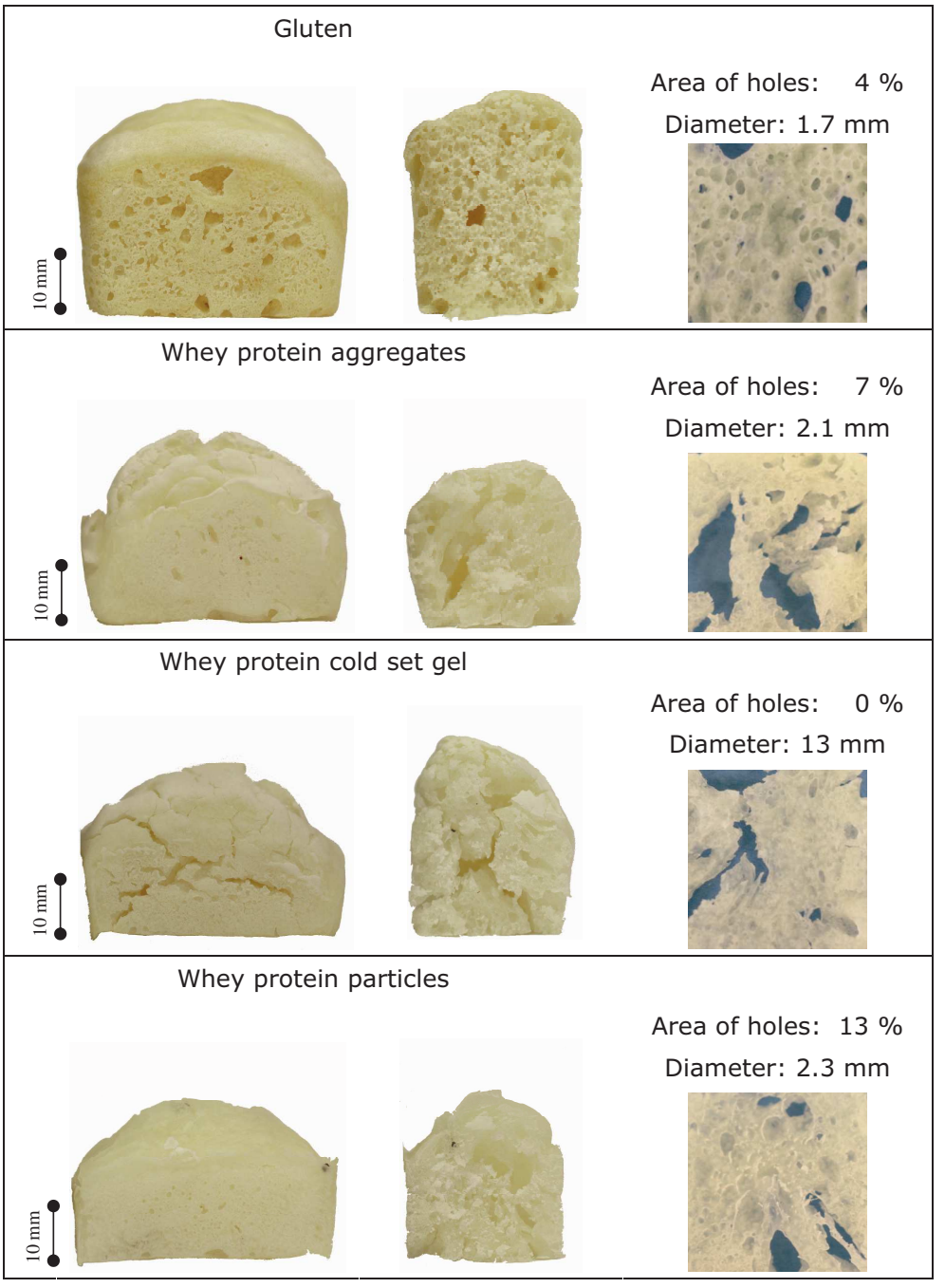


Figure 4: Photographic images of bread with sheeting

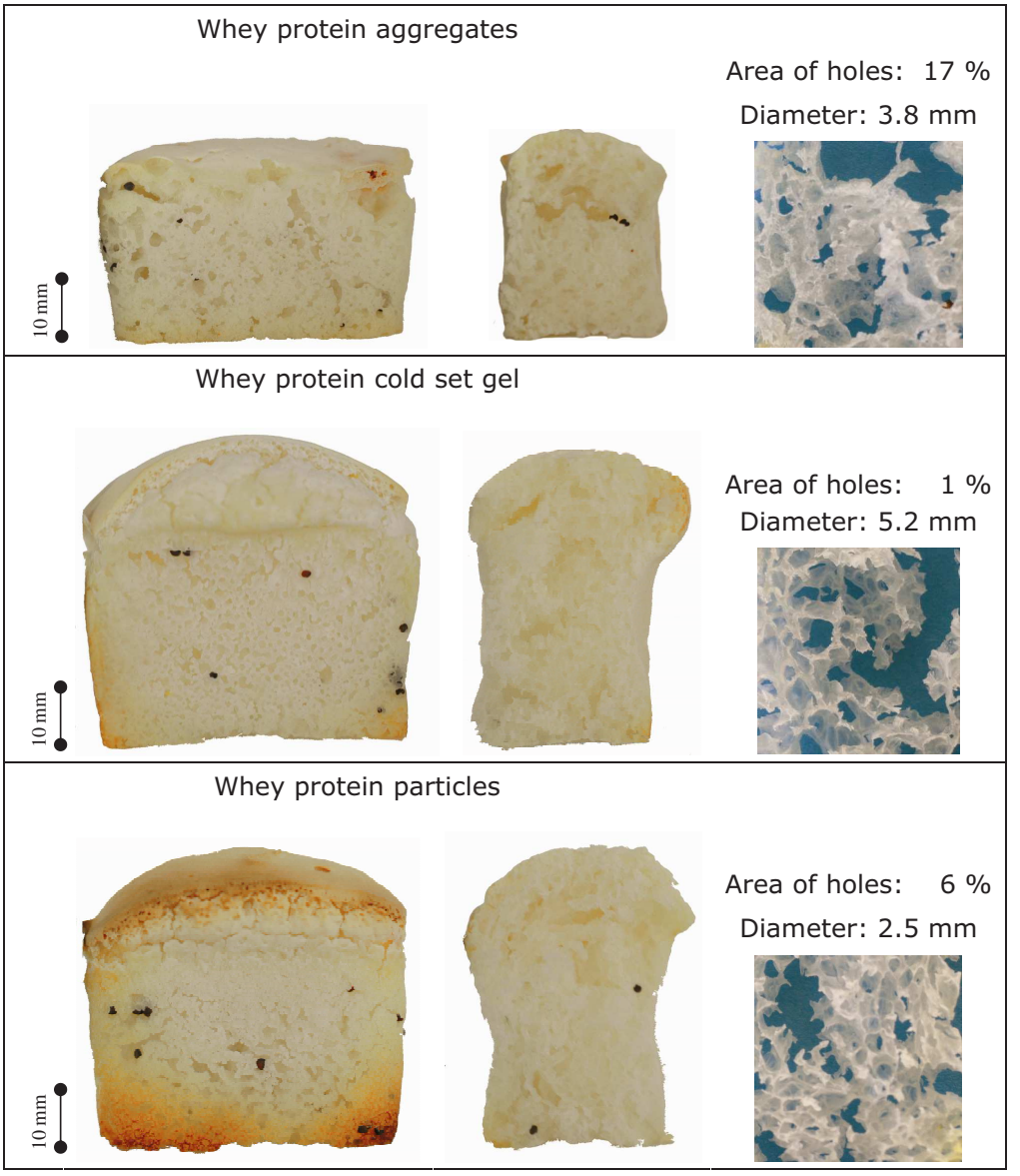


Figure 5: Photographic images of bread without sheeting

2.2 ± 0.1 mm, but still contained many large holes (7 % of the total volume of the WP aggregate bread and 13 % of the total volume of the WP particle bread is ruptured). Omission the sheeting step increased the volumes of the breads, but also influenced the structure of the breads. Omission the sheeting step decreased the average gas cell diameter for the bread prepared with a WP cold set gel to 5.2 mm (half of the value with sheeting). Omission the sheeting step had no influence on the average gas cell diameter for the bread prepared with WP particles (which remained 2.5 mm). For bread with no sheeting, the gas cell diameter for the bread prepared with WP aggregates increased to 3.8 mm. The bread prepared with WP aggregates also showed an increase in the bread volume that was ruptured (17 %). The ruptured bread volume decreased for the bread with WP particles (6 %).

6.4 Discussion

In this study, the bread-making potential of a mixture containing mesoscopic structured WP, locust bean gum and wheat starch is investigated. Even though the mixing tolerance is largely determined by the WP structure used (particles, gels or aggregates), all types of WP mixtures resulted in a comparable specific volume after mixing and proving. The fact that no large differences in air incorporation due to mixing were observed is in agreement with other studies done on strong and weak dough. These studies showed that weak dough could even result in a larger specific volume upon mixing than strong dough [22].

The dough volume obtained after proving is generally considered to be related to the strength of the gluten network, with a stronger gluten network giving a better gas retention [24]. The breads prepared with a WP gel and with WP particles showed the largest specific volumes. In both breads, a dispersed WP phase was present (figure 2). Although the breads with WP gel and WP particle had a comparable specific volume, the crumb structure showed large differences. These differences are probably related to the ability of gas cell stabilization of the mixture. Strain hardening is a property that is positively related to gas cell stability [25]. Only the gluten-free mixture prepared with WP particles showed strain hardening behaviour (the measured strain hardening coefficient was 1.2) [17], and indeed C-Cell experiments show that this bread had smaller gas cells than the other gluten-free breads (after omission the sheeting step the cell diameter is best comparable with a gluten-containing bread). Thus, the crumb structure of the gluten-free breads needs further improvement to become completely similar to a normal wheat bread. However, the WP-mixtures in this study have a very simple composition and a low protein

content. Moreover the mixtures lack some important ingredients for gas cell stabilization. The use of more or another protein and additional ingredients opens endless opportunities to improve the bread properties of the gluten-free formulations. The presence of bigger holes can be a result of the too large strain that was subjected to the system upon proving and baking. The WP particle mixture can handle a strain up to 0.7 [17]. The volume increase during proving did not exceed this strain limit, but the critical strain value was exceeded during baking and therefore gas cell stabilization could be compromised. The WP gel mixture can only handle a strain of about 0.5 [17], which could explain why larger gas cells were visible in those breads.

In gluten-based dough sheeting is necessary for dough development. Sheeting reduces the gas cells and develops the gluten network [26]. Most gluten-free breads are prepared starting with a batter system, and no sheeting step is included in the baking process [27]. All WP mixtures could be sheeted, which is a quite unique property for gluten-free formulations. Nevertheless sheeting decreased the bread volume after baking. The decrease in bread volume after sheeting can have different causes. It is possible that the decrease is caused by a total disruption of the network that can retain gas cells. Due to the network disruption, no gas cell stabilisation can occur after sheeting. According to this first scenario, the bread volume is independent of the proving time after sheeting. It is also possible that the low bread volume after sheeting is simply caused by the low amount of gas produced after 40 min. If sheeting decreases the gas volume of the mixture, gas production after sheeting is needed to increase the volume of the mixture. According to this second scenario the network formed after sheeting can still retain gas, but the low gas production after sheeting results in a low amount of

large gas cells. The predicted bread volume as a function of the proving time before sheeting is depicted in figure 6a. The dotted line in figure 6a represents the scenario of total network disruption, and the dashed line in figure 6a represents the scenario of low gas production.

To show the effect of sheeting, the WP particle bread was produced using different proving times before sheeting. After mixing, the mixture was allowed to prove for 10, 20 or 30 min, before sheeting, folding and proving for the remaining 90, 80 or 70 min, respectively. There were no changes in process or formulation and the total proving time was kept constant (100 min). The impact of proving time before sheeting on the volume of the final gluten-free breads is presented in figure 6b. The volume of the breads decreased when the proving time before sheeting increased. This result indicates that the low volume after sheeting could be due to the low amount of gas that is produced after 40 min, and sheeting gives no total disruption of the WP network. Nevertheless sheeting influences the WP network. The area of the bread crumb that is ruptured increases if the mixture is sheeted; this indicates that there are some changes in the WP network after sheeting. Modifying the formulation could solve this problem, but optimization of the recipe was outside the scope of this study.

It is remarkable that the breads with WP particle networks resulted in a larger volume than the reference bread prepared with vital gluten, while the amount of whey protein was only 2.4 %. The fact that this amount was sufficient to obtain breads with a large volume is probably related to the protein structure. It seems therefore that almost all WP was included in the protein network. From wheat dough it is known that only a small

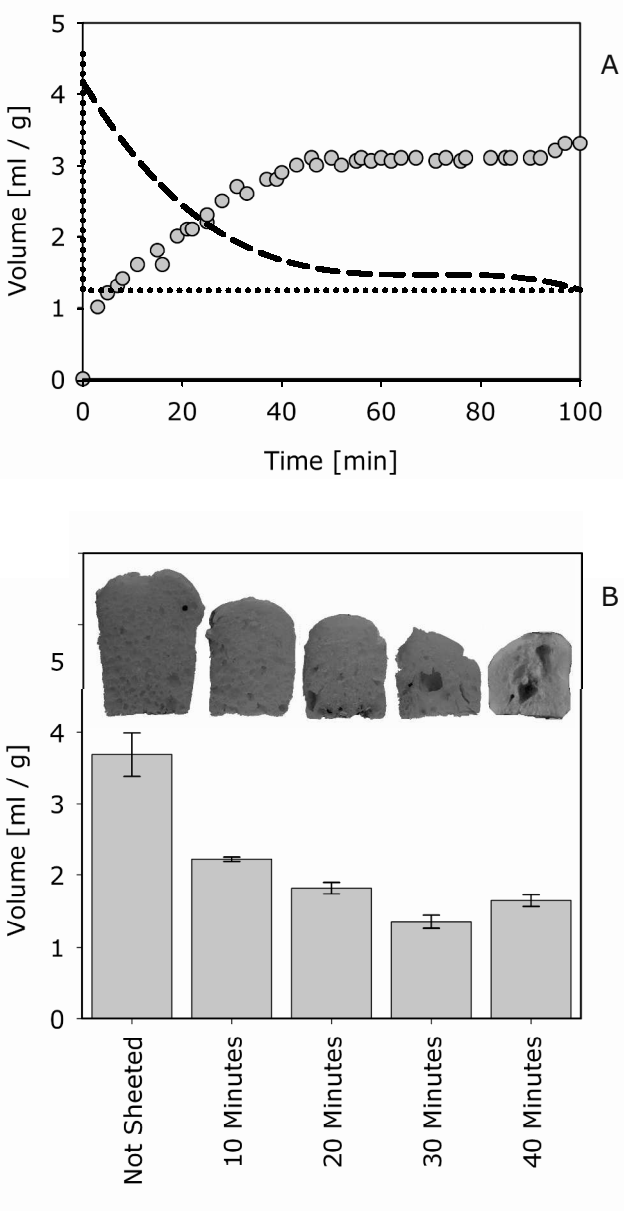


Figure 6: CO_2 production by the yeast as function of proving time (circles), and the predicted bread volume as a function of the moment of sheeting (dashed line and dotted line) (figure 6A). The final bread volume as a function of the moment of sheeting (figure 6B).

percentage (about 20 - 40 %) of the proteins build the network [28]. The results shown in this study were obtained with a gluten-free mixture that contained a limited number of ingredients. Further improvements of the bread properties can surely be obtained through the addition of more components often used as bread improvers in the baking industry. In addition, the breads lacked a sufficient amount of reducing sugars and had a low protein content, which explains the pale colour of the breads and the low yeast activity. The lack of surface active components could be a cause of gas cell coalescence during the final stage of proving [12, 24].

6.5 Conclusions

Nevertheless, it is important to consider the mesoscopic structure in the gluten-free mixture and not only focus on the functionality of the ingredients on the molecular scale. The use of a mesoscopic WP particle network is an illustration of this and represents a new and innovative approach to the development of next generation gluten-free products.

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Chapter 7

The Use of Whey Protein Particles in Gluten-Free Bread Production, the Effect of Particle Stability

Wheat dough has unique properties for bread-making due to its elastic and strain hardening behaviour. A mesoscopically structured whey protein particle system possesses those elastic and strain hardening properties when mixed with starch to a certain extent. However, the extensibility is lower and the particles are more stable than gluten particles upon kneading, probably due to a too high degree of internal crosslinking. This chapter describes the relation between the number of disulphide bonds of a mesoscopic whey protein particle suspension blocked by NEM treatment and the resulting properties of a dough and bread prepared with that suspension. This study shows that the properties of the particle network are influenced by the ability to form disulphide bonds. Our study shows that a certain amount of disulphide bonds is essential, but too many disulphide bonds can lead to too stiff dough and poorer bread properties.

This chapter was submitted as:
van Riemsdijk, L. E., van der Goot, A. J., & Hamer, R. J. The use of whey protein particles in gluten-free bread production, the effect of molecular properties. Food Hydrocolloids (2011)

7.1 Introduction

With the increasing numbers of people intolerant to gluten, the need is rising for high-quality gluten-free bread. Replacing or removing gluten is not trivial, because gluten has unique properties. Those properties of gluten are difficult to mimic with other components or cereals [1]. Gluten-free breads are typically made using a batter. However, the resulting breads often posses poor properties with respect to the bread volume and the crumb structure. Besides, gluten-free breads typically rapid stale after baking [2]. In many gluten-free recipes, ingredients such as polysaccharides are added to improve the properties of gluten-free bread through a high bulk viscosity [3]. A high bulk viscosity can improve the volume of the gluten-free breads, but due to a lack of elasticity, stability of gas cell against disproportionation remains limited [4, 5]. The ability of wheat dough to retain gas is related to the rheological properties, such as viscoelasticity, and strain hardening [6, 7]. The strain hardening behaviour of dough is often correlated with baking performance [8].

The viscoelastic and strain hardening properties of dough originates from the gluten network that give rise to elasticity. The gluten are able to recover after breakage upon deformation [9-12]. The glutenin macro polymer (GMP) fraction is generally accepted to be the gluten fraction that provides the greatest contribution to these elastic and strain hardening properties [13, 14]. Although it comprises only 2 – 4 % of the wheat flour, the GMP fraction is very important in bread-making [15, 16].

In previous chapters (chapters 2, 5 and 6), we showed some promising results to substitute gluten with a gluten-free protein source (whey protein) structured into mesoscopic (~20 μm) protein particles.

We demonstrated that a suspension containing those whey protein particles displays elastic properties [17]. Mixing these particles with starch and water gave rise to wheat-dough like properties including strain hardening behaviour [18]. Breads with a specific volume of 3.7 ml/g were obtained after baking this gluten-free dough [19].

Not withstanding the similarities, normal wheat dough and whey protein particle dough also differed. Compared to wheat dough, the particle dough showed a lower mixing tolerance (mixing tolerance was 96 % for wheat dough and 83 % for the particle dough, analyzed using a Farinograph) and showed less resistance to extension (strain at fracture was 1.4 for wheat dough and 0.7 for the particle dough - the stress at fracture was 37.5 kN/m^2 for wheat dough and 2.7 kN/m^2 for the particle dough, both analyzed with extensional tests in a Texture Analyzer) [18]. These differences in the rheological behaviour can (partly) explain why the breads prepared with whey protein particles have more ruptures than a dough with gluten (According to C-Cell experiments 4 % of the gluten rich bread is ruptured and 6% of the particle dough is ruptured) [19]. In addition, the particles used in the gluten-free recipe showed no signs of disruption after kneading. Previous research on glutenin particles showed that those particles are deformable and show a reduction in particle size upon dough mixing [11, 20]. Also, glutenin particles have a high ability to reform which is related with the viscoelastic behaviour of dough [11]. Thus, the particle network formed by the whey protein particles differs from the network present in wheat dough especially in a number of properties. Apparently, the whey protein particles are too rigid.

The strength of the particles is most likely related to the protein concentration in the particles, and to the number of disulphide bonds present in the particles. The protein concentration in GMP dispersions is ~1.2% (w/w) [11], which is 10 fold lower than the protein concentration in whey protein particles, which is ~12% (w/w). The amount of disulphide bonds per mol is higher for the glutenin proteins than for the whey proteins. Comparing the protein percentage in the particles and the amount of disulphide bonds present in gluten (~60 μM/g dry weight [21]) and in whey protein (~120 μM/g dry weight [22]), we conclude that the total amount of disulphide bonds/particle is much higher with whey protein particles. This high amount of disulphide bonds could be a cause for the fact that the whey protein particles are more rigid than gluten.

In this study we investigate the influences of the amount of disulphide bonds on dough and bread properties. The amount of disulphide bonds was controlled by blocking (part of) the reactive thiol groups of whey proteins with N-ethylmaleimide (NEM). The aim therefore is to provide a better insight in the similarities and differences between the whey protein particle network and the gluten network in dough.

7.2 Experimental Section

7.2.1 Preparation of Protein Structures

A whey protein (WP) solution was transformed into WP particles using a cold gelation method. The particles were prepared using a two step procedure. First, a 9 % (w/w) WP (Davisco Foods International Inc., USA) solution was heated at 68 °C for 2.5 h to form small WP aggregates. Then, the WP aggregates were mixed with locust bean gum (Danisco Holland BV, The Netherlands) and subsequently gelled with glucono-delta-lacton (GDL, Sigma Chemicals, The Netherlands).

To investigate the effect of disulphide bonds on the WP particle behaviour, the reactive thiol groups of the WP aggregates were blocked with N-ethylmaleimide (NEM). Analysis of the effect of the thiol-blocking with Ellman's reagent showed that treatment of a 9 % (w/w) WP aggregate solution with 2.25 mM NEM blocked 94 ± 2 % of the accessible thiol groups of the WP aggregates. Therefore, three different concentrations of NEM were selected 2.25 mM, 1.13 mM and 0.56 mM, and added to a 9 % (w/w) protein aggregate solution. The reaction with NEM was allowed to proceed at room temperature for at least 30 min. The preparation of particles was similar to the particle preparation without blocking of the reactive thiol groups. We also included a sample in which NEM was added after particle formation, but before dough processing. The amount of NEM added in this procedure was similar to the amount used to block 94 ± 2 % of the accessible thiol groups of the WP aggregates. In this case, the intact disulphide bonds in the WP particles will not be influenced by NEM, but disulphide bonds that break during dough mixing cannot be reformed.

7.2.2 Preparation of Dough Mixtures

Non-yeasted gluten-free dough mixtures were prepared by mixing wheat starch (Sigma Chemicals, The Netherlands), NaCl (Merck, Germany) and the WP locust bean gum suspensions in a Farinograph dough kneader for 3 min at a speed of 63 rpm and a temperature of 30 °C. The protein concentration in the mixture was 2.5 % (w/w db), the locust bean gum concentration was 0.4 % (w/w db), the salt concentration was 2.5 % (w/w db) and the moisture content was 47 % (w/w).

Yeasted gluten-free dough mixtures were prepared through mixing starch, salt, WP-locust bean gum suspension, dried active bakery yeast (Algist Bruggeman Co., Belgium) and D-glucose (Sigma Chemicals, The Netherlands) in a Farinograph dough kneader for 3 min using a mixing rate of 63 rpm and a temperature of 30 °C. The final protein concentration was 2.4 % (w/w db), the final locust bean gum concentration was 0.4 % (w/w db), the salt concentration was 2.4 % (w/w db), the glucose concentration was 1.1 % (w/w db), the yeast concentration was 1.9 % (w/w db) and the water percentage was 46 % (w/w). Two baking tins of 18 cm^2 (top) / 15 cm^2 (bottom) x 3 cm were filled with 30 g dough. The dough was proved in a climate chamber at 35 ºC and 85 % RH for 100 min. Addition of NEM had no influence on the CO_2 produced by the yeast. A dough ball (5 g) with 0 mM NEM and a dough ball (5 g) with 2.25 mM NEM produced both ~3.5 ml CO_2/g dough during proving. After proving, the dough mixtures were baked in a pre-heated automated kitchen bread machine at ~200 ºC for 35 min. The breads were produced in duplicate.

7.2.3 Analysis of Dough Mixtures

Structural Analysis

The WP suspensions were non-covalently labelled with Rhodamine B (Sigma Chemicals, The Netherlands) to visualize the protein structure before and after dough preparation with Confocal Laser Scanning Microscopy (CLSM - LSM 510, Zeiss, Oberkochen, Germany). After protein structuring, the WP suspensions were transferred into a chambered cover glass (Nunc, Naperville, IL, USA). Rhodamine B was added before visualizing.

Visualization after dough processing was done by separating the WP particles from the dough using the following procedure. First, the starch present in the dough was dissolved by heating a ten times diluted dough solution at 80 ºC for 5 min. Then, the WP particles were separated by centrifugation at 1000×g for 3 min. The gel layer formed was diluted and transferred into a chambered cover glass, where it was stained with Rhodamine B. To check if the separation procedure influenced the WP particle structure, we performed two additional experiments. The effect of the heat treatment on the protein structure was excluded by heating a WP particle sample at 80 ºC immediately after preparation. No differences in the structure were visible after heating. The effect of starch was excluded by including an extra separation step in a WP particle dough sample. After heating, the gluten-free dough was incubated with Amylase p500 (Gist-Brocades) for 3 h, and separated by centrifugation at 1000×g for 3 min. Full conversion of the starch was confirmed using iodine staining. For this purpose a 0.05 M iodine (Merck) solution was used. No difference in the structure was visible with and without amylase incubation.

The average particle diameter was calculated by measuring the mean diameter of eight particles.

Small Deformation Measurements, Strain Sweeps

The small deformation behaviour of dough was measured with a Paar Physica MCR 301 (Anton Paar, Austria) stress-controlled rheometer, equipped with a serrated plate/plate geometry (diameter 25 mm – gap 1 mm) and a solvent trap. Before the strain was logarithmically increased from 0.001 % to 400 %, samples were rested for 15 min to allow relaxation of the stresses induced during sample loading. The tests were done with a constant frequency of 1 Hz and a temperature of 25 °C.

Large Deformation Measurements, Uniaxial Extension Tests

The large deformation behaviour of dough was measured with a texture analyzer (Instron-5564Series-Table-Model-Systems-Twin-column-design, Canton USA), equipped with a Kieffer dough-and-gluten extensibility rig and a 50 N load cell. The dough was moulded into trapezium-shaped strips using a Kieffer mould coated with silicon oil. The samples were allowed to rest inside the mould at 25 °C and 90 % RH for 45 min before the sample strips (18 × 16 mm^2) were elongated using a deformation rate of 3.3 mm/s. At least three samples for each dough type were tested. The force–displacement curves were transformed into stress–strain data as described by Dunnewind et al. (2004) [23], taking into account that most of the samples had a negligible banding distance, and assuming a constant volume. The stress (σ) at fracture, the Henky-strain (ε) at fracture stress and the apparent strain hardening coefficient (n) were determined. The strain hardening coefficient was determined by applying an exponential fit on the σ – ε curve in the Henky strain ranging from 20 – 95 % of fracture strain.

Bread Analysis

After baking, the breads were cooled to room temperature before they were further analyzed. Bread volume was determined with the rapeseed displacement method (AACC-2000 method 10-05). The structure of bread was visualized by photographic imaging of the whole breads and bread slices. From each bread type a representative slice is both used as photographic representation and for C-Cell analysis. The structure of the bread crumbs were evaluated using the C-Cell Bread Imaging System. The parameters used for the crumb characterization are the average cell diameter (mm), and the area of holes (%). A smaller average cell diameter reflects a finer crumb structure. A larger area of holes reflects a lack of elasticity, and consequently a poor gas cell stabilization.

7.3 Results

The effect of NEM treatment on the particle shape and size was investigated using CLSM. Figure 1 shows the microscopic images of the WP particles before dough processing and after dough processing and isolation, using different NEM concentrations. The microscopic images of the WP particles before dough processing confirm that the WP particles with and without NEM treatment have a similar shape [24]. The size before dough processing is similar for the untreated particles (17 ± 4 μm), and the particles treated with 2.25 mM NEM (15 ± 3 μm). This similarity in size is in line with our earlier observation that NEM addition does not influence the particle formation process [24]. However, our data show that particle size is influenced by the NEM-treatment at intermediate concentrations. The average size before dough processing of the WP particles treated with 1.13 mM NEM or 0.56 mM NEM was larger (26 ± 5 μm and 31 ± 9 μm respectively) than the average size of the WP particles without NEM treatment.

The effects of mixing on the particle shape and size were also investigated using CLSM. When interpreting these results it is important to be aware of possible side effects of the separation procedure. Particles isolated from dough were subjected to heat (80 ºC), this was required to gelatinize and enzymatically remove the starch. Nevertheless some remarkable differences in the shape of the WP particles were observed after mixing. The untreated WP particles retained their size (16 ± 4 μm), and showed only slight particle deformation (less spherical) upon dough processing. Dough prepared with WP particles treated with 1.13 mM NEM or 0.56 mM NEM resulted in particle deformation and break-up, leading to the appearance of fragments and a decrease in particle size (to 21 ± 5 μm

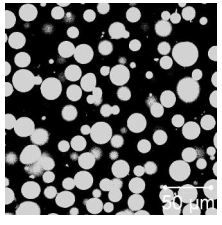
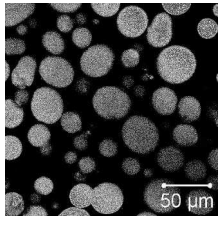
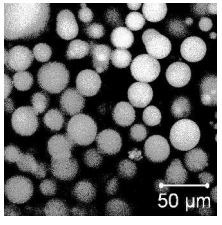
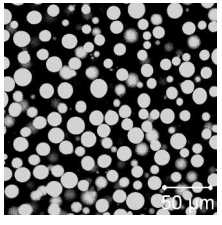
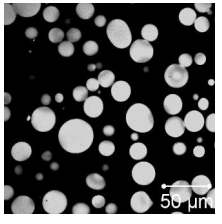
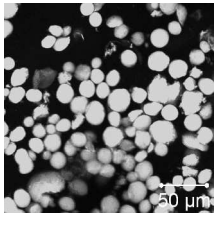
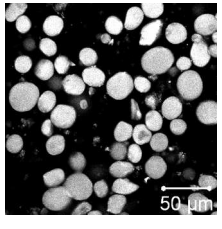
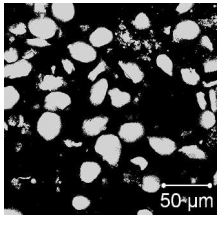
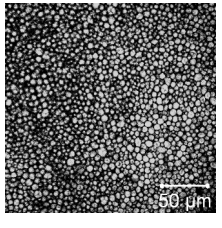
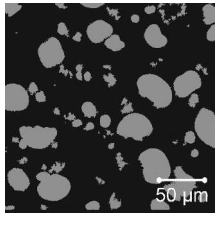
Microscopic Image	Untreated	NEM Treatment Before Processing			NEM Treatment During Processing
		0.56 mM NEM	1.13 mM NEM	2.25 mM NEM	2.25 mM NEM
Before Dough Processing 50 µm	 A1	 B1	 C1	 D1	 E1
After Dough Processing 50 µm	 A2	 B2	 C2	 D2	 E2

Figure 1: Overview of the structure of whey protein particles before and after dough processing. The particles vary in the NEM treatment. A 9 % (w/w) protein aggregate solution was untreated or treated with 0.56 mM NEM, 1.13 mM NEM or 2.25 mM NEM before particle formation.

and 16 ± 4 µm respectively) and irregularities in shape. Dough prepared with an even higher amount of NEM (2.25 mM NEM) resulted in a significant particle size reduction after dough processing. Here, the size decreased to 4 µm ± 1 µm.

When NEM was added after preparation, but during dough mixing, only disulphide bonds that break during processing will be affected. Our results reveal differences between NEM addition before WP particle formation and NEM addition during dough processing (compare figure 1 D2 and E2). The WP particles in which NEM was added during dough processing were clearly deformed and had lost their spherical shape. Besides, the mixing led to marked changes in particle size distribution, from a single distribution (19 µm ± 5 µm) to a bimodal distribution with both small (10 µm ± 4 µm) and large particles (36 µm ± 3 µm).

The mixing behaviour of a dough gives some information about the stability of the protein network. Figure 2 depicts the peak consistency and the consistency after 3 min mixing for the gluten-free dough mixtures. The more NEM was used the larger the value for the peak consistency of the dough mixture (3.3 Nm for untreated particles, 3.4 for particles treated with 0.56 mM NEM 3.6 for particles treated with 1.13 mM NEM and 4.0 Nm for particles treated with 2.25 mM NEM). Although the NEM treatment gave an increase in the peak consistency, the torque-value at the end of the mixing is lower for the dough mixture prepared with 2.25 mM NEM (1.9 Nm) than for the other dough mixtures (2.7 - 2.9 Nm).

The small deformation properties of the dough mixtures are depicted in table 1. The loss factor of all mixtures were between 0.1 - 0.2, indicating that the mixtures were firm. The strength of the mixtures differed however.

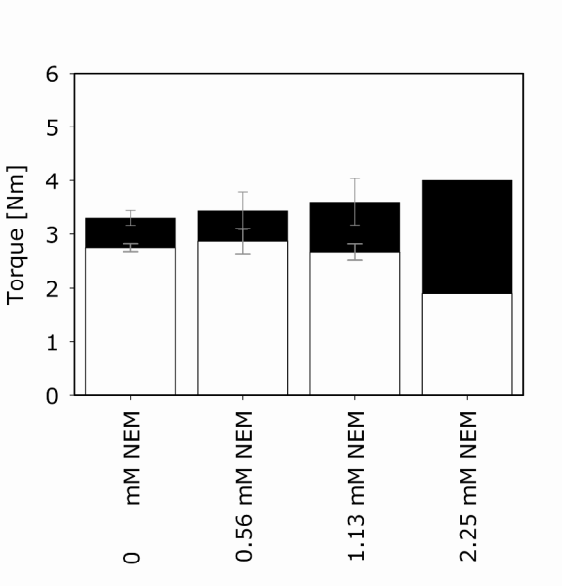


Figure 2: Torque values during mixing in a Farinograph of starch and whey protein particles. The particles vary in the NEM treatment. A 9 % (w/w) protein aggregate solution was untreated or treated with 0.56 mM NEM, 1.13 mM NEM or 2.25 mM NEM before particle formation. The white bars mark the torque values after mixing (3 minutes) dough, the black bars mark the torque values at the peak of the Farinograph curve. Different letters indicate statistically significant differences.

The storage and loss moduli of the dough mixture prepared with WP particles treated with 0.56 mM NEM or 1.13 mM NEM was higher (storage modulus was ~22 $\times 10^3$ Pa and the loss modulus was 4.3 $\times 10^3$ Pa) compared to dough prepared with untreated WP particles (15 $\times 10^3$ Pa and 4.3 $\times 10^3$ Pa respectively). The increase in the moduli can be a result of the higher phase volume of the particles. A dough prepared with WP particles treated with 2.25 mM NEM gave lower moduli (storage modulus was 8.8 $\times 10^3$ Pa and the loss modulus was 1.8 $\times 10^3$ Pa) than the other dough mixtures.

Table 1: Storage modulus, loss modulus and loss factor of dough samples under small deformation measurements in a rheometer. The dough mixtures are prepared of starch and whey protein particles. The dough mixtures are prepared with particles that vary in the NEM treatment. A 9 % (w/w) protein aggregate solution was untreated or treated with 0.56 mM NEM, 1.13 mM NEM or 2.25 mM NEM before particle formation.

NEM treatment	Storage Modulus (Pa)	Loss Modulus (Pa)	Loss factor (-)
0 mM NEM	$15.2 \times 10^3 \pm 0.0 \times 10^3$	$2.0 \times 10^3 \pm 0.0 \times 10^3$	0.13 ± 0.00
0.56 mM NEM	$23.4 \times 10^3 \pm 6.1 \times 10^3$	$4.3 \times 10^3 \pm 0.9 \times 10^3$	0.18 ± 0.01
1.13 mM NEM	$20.1 \times 10^3 \pm 0.7 \times 10^3$	$4.3 \times 10^3 \pm 0.1 \times 10^3$	0.22 ± 0.01
2.25 mM NEM	$8.8 \times 10^3 \pm 1.5 \times 10^3$	$1.8 \times 10^3 \pm 0.3 \times 10^3$	0.20 ± 0.00

The final bread properties can be often related with the large deformation behaviour of the dough. Especially, the strain hardening is related to the gas cell stabilization [25]. The large deformation properties of the dough mixtures are depicted in table 2. The results show that the NEM treatment of the WP particles had a relatively small impact on strain at fracture of the WP particle dough mixtures. There is a slight decrease visible in the strain at fracture when more NEM is used. Although there is no significant correlation observed. These results have to be interpreted carefully. The WP particle dough mixtures lack a component which increases the viscous behaviour such as gliadins. Adding gliadins increases the strain at fracture of a dough [26], hence the lack of gliadins can explain the low strain at fracture of the particle dough. The lack of gliadins resulted in a different behaviour upon deformation compared to normal wheat dough.

As a result, the strips of the mixtures broke at the hook rather than in the middle of the sample. Most likely, this effect will lead to an underestimation of the actual strain at break.

Table 2: Stress at fracture, strain at fracture and strain hardening value of dough samples under extensional tests in a Texture Analyzer. The dough mixtures are prepared of starch and whey protein particles. The dough mixtures are prepared with particles that vary in the NEM treatment. A 9 % (w/w) protein aggregate solution was untreated or treated with 0.56 mM NEM, 1.13 mM NEM or 2.25 mM NEM before particle formation.

NEM treatment	Henky strain at fracture (-)	Stress at fracture (KN/m2)	Strain hardening (–)
0 mM NEM	0.7 ± 0.1	2.7 ± 0.1	1.2 ± 0.2
0.56 mM NEM	0.6 ± 0.0	3.2 ± 0.3	2.3 ± 0.3
1.13 mM NEM	0.5 ± 0.0	1.1 ± 0.3	1.9 ± 0.2
2.25 mM NEM	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 1.0

We could observe that NEM treatment influenced the stress at fracture and the strain hardening behaviour significantly. The dough mixtures prepared with WP particles treated with 0.56 mM NEM showed the highest value for the stress at fracture (3.2 kN/m^2). A smaller or larger NEM concentration resulted in a lower stress at fracture (2.7 kN/m^2 for untreated WP particles and 1.1 kN/m^2 and 0.5 kN/m^2 for the dough mixtures prepared with WP particles treated with 1.13 mM NEM and 2.25 mM NEM respectively). An optimum was also visible for the strain hardening behaviour. Dough with untreated WP particles showed a limited strain hardening (1.2). The strain hardening has the highest value for the dough prepared with WP particles treated with 0.56 mM NEM (2.3). Strain hardening decreased again if more NEM was used (1.9). Dough prepared with WP particles treated with 2.25 mM NEM showed no strain hardening).

Besides mixing and rheological experiments, the gas holding ability of the mixtures is measured. First, we demonstrated that NEM treatment did not affect the activity of the yeast. Differences observed will therefore be due to differences in gas holding capacity. Under the conditions used (time, temperature, amount of yeast) the average gas production was ~3.5 ml/g dough. The dough mixtures were then used to bake breads. The bread prepared with 0.56 mM NEM gave the bread with the most attractive appearance (though we realise that this is a subjective visual observation). This bread had a nice cap, while other breads had a more cubic shape. Figure 3 depicts the volumes of the bread obtained by the different WP particle mixtures. Overall, the bread volume became smaller when the particles were treated with NEM. The specific volume of the untreated WP particle bread was 3.7 ml/g, the specific volume of the NEM treated WP particle breads was lower 2.8 - 3.0 ml/g.

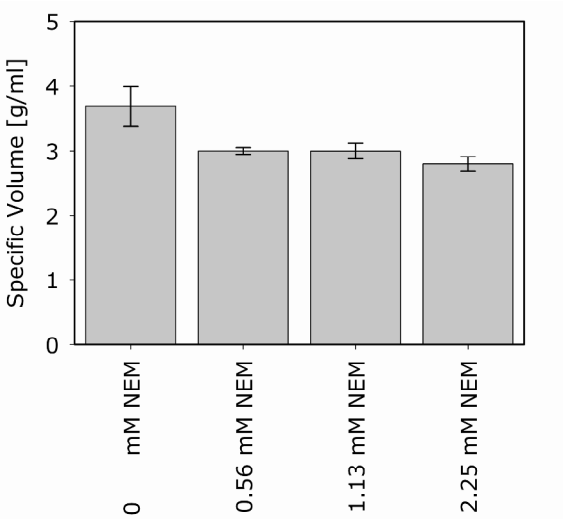


Figure 3: Specific volume (ml/g) of bread prepared of starch and whey protein particles. The breads are prepared with particles that vary in the NEM treatment. A 9 % (w/w) protein aggregate solution was untreated or treated with 0.56 mM NEM, 1.13 mM NEM or 2.25 mM NEM before particle formation.

Photographic images of the breads are depicted in figure 4. These images show that the height of the bread prepared with untreated WP particles was lower than the bread prepared with WP particles treated with 0.56 mM NEM. C-Cell experiments confirm that the maximum height is larger, but the average height is lower for the bread prepared with WP particles treated with 0.56 mM NEM. The bread prepared with untreated WP particles is more cubic, while the breads prepared with WP particles treated with 0.56 mM NEM had a cap.

Another difference between the breads is the colour. The bread prepared with untreated WP particles had a darker crust compared to the breads prepared with WP particles that are treated with NEM. The differences in bread shape and crust colour can have different causes (e.g. rate of water evaporation, relative humidity during baking [27, 28]).

The gas cell structures are visible in figure 4. In almost all breads, ruptures were visible. The bread with the lowest amount of ruptures is the bread prepared with WP particles treated with 0.56 mM NEM (3 % of the total bread volume). The bread with the highest amount of ruptures is the bread prepared with WP particles treated with 2.25 mM NEM (11 % of the total bread volume). The other two breads (untreated WP particles and WP particles treated with 1.13 mM NEM) had a comparable amount of ruptures (6 % and 7 % of the total bread volume respectively). The average diameter of the gas cells is the largest for the bread prepared with WP particles treated with 2.25 mM NEM (2.9 mm). The bread prepared with untreated WP particles had a slightly larger diameter (2.5 mm) than the breads prepared with WP particles treated with 0.56 mM NEM and 1.13 mM NEM (2.2 mm).

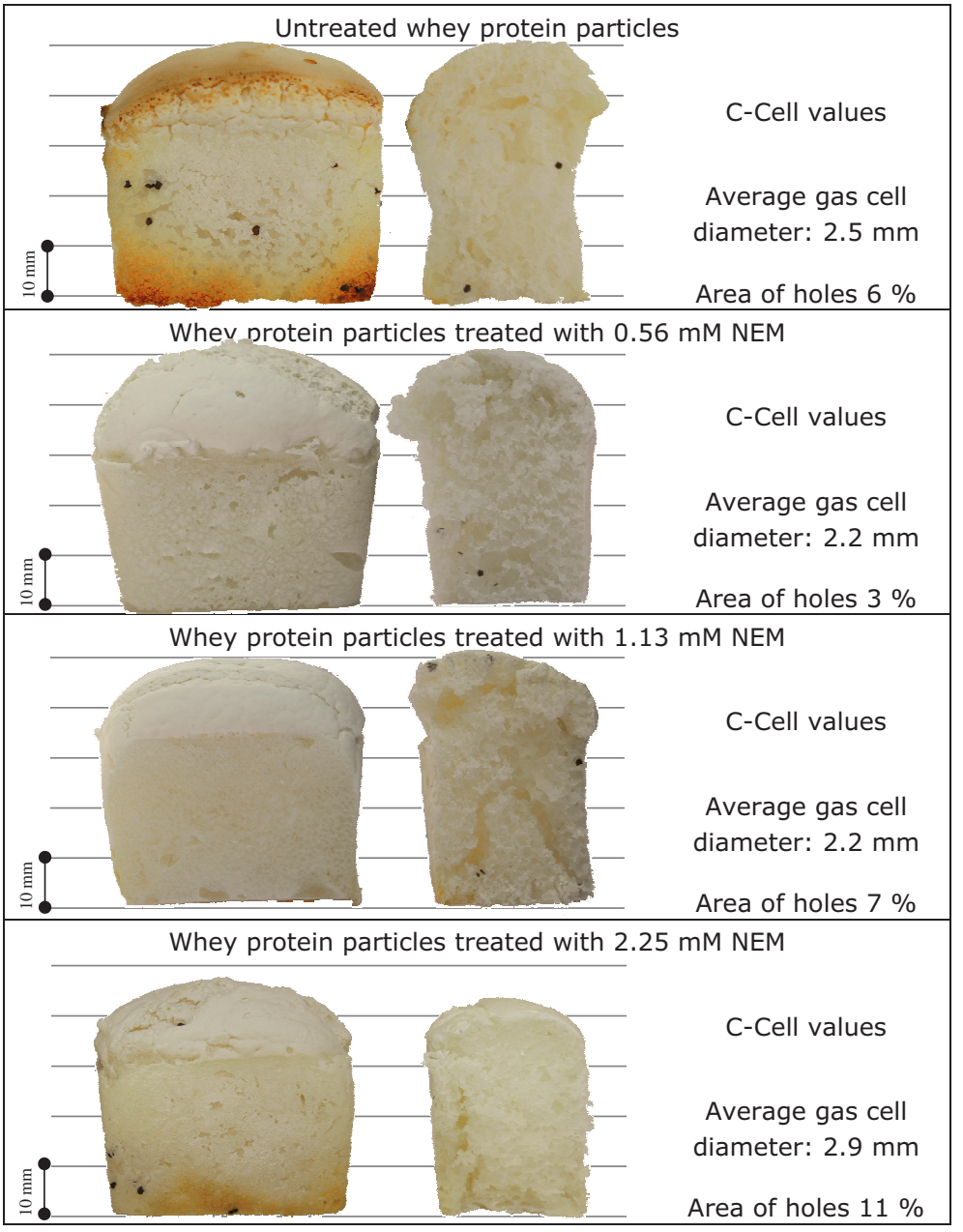


Figure 4: Photographic images of bread prepared of starch and whey protein particles. The breads are prepared with particles that vary in the NEM treatment. A 9 % (w/w) protein aggregate solution was untreated or treated with 0.56 mM NEM, 1.13 mM NEM or 2.25 mM NEM before particle formation.

7.4 Discussion

In the two previous chapters (chapters 5 and 6) we have demonstrated that a mesoscopically structured whey protein dispersion can be used as a substitute for gluten in the preparation of a dough and a leavened bread [18, 19]. We have demonstrated that the whey particles are quite stable, certainly in comparison with wheat glutenin particles, which are disrupted during mixing [11, 20].

The amount of disulphide bonds can have an effect on the phase volume of the WP particles. Too many cross-links prevent an increase in phase volume of the particles. Removing or blocking part of the reactive thiol groups will induce the phase volume. Consequently, the protein particles will behave more elastically. The increase in phase volume can have different causes e.g. swelling, or the formation of a more loosely packed particle structure. The volume increase is almost 8 times, which suggests that swelling can not be the only reason. Further research is needed to completely unravel how the NEM addition affects the particle size.

This study aims at clarifying the importance of disulphide bonds on the behaviour of whey particles in a dough system. Since the separation procedure can have side effects, we focussed on the main changes in the structure of the particles. The results presented here show that WP particles without NEM-treatment and WP particles treated with 0.56 mM NEM or 1.13 mM NEM can withstand the forces during dough mixing, although some deformation and break-up occurs. Only when the WP particles are treated with 2.25 mM NEM (blocking the ability to stabilize particles by the formation of disulphide bonds), the intra-particle interactions are not sufficient to prevent disruption of particles upon dough mixing. Nevertheless, these broken particles still form a cohesive

dough in combination with starch, although resulting dough and bread-making properties are deteriorated. The dough has a lower consistency after dough mixing (figure 2), the small deformation moduli are lower (table 1), the strain hardening behaviour has disappeared (table 2) and the gas bubble stability was reduced (as is clear from the lower volume and the large amount of ruptures and cracks, figures 4 and 5).

The effect of complete blocking of the reactive thiol groups via NEM addition has a similar effect on wheat gluten dough and the gluten-free dough mixtures studied here [20, 29]. In both materials, the final consistency and the mixing tolerance decreases. The initial high peak consistency of gluten after NEM treatment was related to the depolymerisation, which initially increases the water hydration capacity, and consequently the viscosity of the dough [20]. A decrease in stress at fracture, strain at fracture and the strain hardening behaviour upon NEM treatment was also observed in wheat dough [20]. A complete blocking of the reactive thiol groups weakened the gluten, and consequently weakened the dough [29]. All of the effects mentioned above were also found for WP particle dough in case of complete blocking of the reactive thiol groups, suggesting that the WP particle system creates dough-like properties in a similar manner as gluten does in wheat dough.

A main difference between dough and the gluten-free mixtures is related to the apparent absence of breakage of particles when no NEM was added. The fact that the particles kept their original size if the thiol groups were not blocked can have two causes. First, the fact that the amount of disulphide bonds in whey protein particles is high compared to the amount in a GMP dispersion results in a high mechanical strength of the particles. Second, the high concentration of thiol groups will allow fast

(re-)formation of disulphide bonds in case these are broken due to shear forces onto the particles. We performed an experiment in which NEM was added during dough mixing to provide further understanding of the effect of dough mixing on the particles.

Our observations show that after dough mixing the WP particles with NEM addition during dough mixing, were not identical to particles present in the dough without NEM addition (figure 1 A2 and E2). After mixing, small particles as well as larger particles were observed. The small particles suggesting particle break-up. The large particles suggesting an increase in phase volume. From the increased phase volume, we conclude that dough mixing results in rupture of (part of) the disulphide bonds, NEM prevent reformation of these bonds, and as a result the number of bonds will decrease. The reduced number of disulphide bonds in the particles will weaken the particles, which could explain the particle break-up observed.

The additional volume fraction of the particles treated with 0.56 mM NEM might explain why the dough prepared with these WP particles has a mixing consistency, strain hardening behaviour and stress at fracture that approaches wheat dough better than those of the other WP particle dough mixtures. Also the increased elasticity of the particles might play a role.

7.5 Conclusions

The present study confirms the potential of mesoscopic protein particle networks to imitate gluten properties. Despite the simple composition and low protein concentration the dough already showed important similarities (e.g. strain hardening behaviour) to wheat dough. The present study focuses on the role of the mechanical stability of particles as affected by internal cross-linking.

By chemically affecting disulfide bond formation, we demonstrated the role of disulphide bonds, not only in the formation of such particles, but also in determining their mechanical stability, phase volume and ability to form a viscoelastic network. This phenomenon can be used to further improve dough and bread-making properties of mesoscopically structured non-gluten proteins.

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Abbreviations

Formula

ε	Henky-strain
σ	stress
G’	storage modulus
G”	loss modulus
n	strain hardening coefficient
tan δ	loss tangent / loss factor

Text

CLSM	confocal laser scanning microscope
db	dry basis
GDL	glucono-delta-lacton
GMP	glutenin macro polymer
NEM	N-ethylmaleimide
RH	relative humidity
SDS	sodium dodecyl Sulphate
S-S	disulfide
WP	whey protein

Part III
In Conclusion

Chapter 8

General Discussion

This chapter summarizes the main findings of the project on "The formation and deformation of protein structures with viscoelastic properties". The findings support the initial hypothesis that the functionality of gluten can be mimicked using a meso-structured protein system. Nevertheless some questions and points of discussion remain. In this chapter some of those questions will be discussed. The chapter finishes with a discussion on the potential of this new methodology to develop a next generation of gluten-free breads.

8.1 Introduction

Wheat flour is used in many different products such as bread, cake, spaghetti, beer, salad dressings, sauces and even toothpaste. Unfortunately, ca 1 % of the population has a predisposition to develop celiac disease [1-3]. Celiac disease is a serious disorder related to an intolerance to wheat gluten for which no medical treatment exists yet. It therefore requires the complete and lifelong avoidance of gluten in any food product. Consequently, a need exists for a broad range of gluten-free alternatives to replace existing wheat-based products. This need explains the development of a range of gluten-free products. Unfortunately, it is difficult to replace gluten and to find alternative ingredients that provide the proper functional properties. As a consequence, the quality of current gluten-free products is poor compared to the original. Breads produced without wheat flour have a lower specific volume, a very compact crumb, and a short shelf life compared to breads produced with wheat flour [4-7]. It is for this reason that recent research tried to improve the quality of gluten-free breads by using gluten-free cereals e.g. oat, corn and buckwheat [8-12] or by adding ingredients (mostly hydrocolloids and emulsifiers) to gluten-free starches or flours e.g. [13-21]. Others change the molecular properties, e.g. by protein crosslinking using enzymes, heat or high pressure treatment [8, 10, 12, 19]. In these approaches, improvement of the bread volume is mainly provided through a high bulk viscosity obtained through hydrocolloid addition and starch gelatinization [22]. Those ingredients can improve the volume of the breads, but lack of elasticity generally gives rise to problems with gas cell stability due to coalescence and disproportionation [23, 24]. The lack of gas cell stabilization leads the inferior structure (holes and cracks) that characterise current day gluten-free breads.

In this study we followed a different approach. We aimed at creating gluten functionality through mesoscopic structuring. The objective of this study was to develop a protein based system that resembles the key features of gluten network proteins. The hypothesis guiding this work was that the functionality of gluten is, at least partly, due to the presence of a particle network (see chapter 1). The key features of the particles that form the viscoelastic network are: their mesoscopic size (1 - 100 μm), their soft deformable behaviour, and their interparticle interactions (they can form a network) [25-27]. This is why we developed protein particles that could be mixed with starch to produce a viscoelastic dough and breads with good volume. These practical results, the considerations of the used approach, and the scientific significance of the results will be discussed in the following sections. The chapter finishes with a discussion on the potential of this new methodology to develop a next generation of gluten-free breads.

8.2 Main Findings

This thesis describes the use of mesoscopic whey protein particles as a gluten substitute. The thesis consists of two parts. Part I (chapters 2 - 4) deals with the formation and characterisation of the properties of the protein particle suspensions. Part II (chapters 5 - 7) focuses on the application of the protein particle system in a gluten-free formulation. Overall, the results show that mesoscopic protein particles can be used to develop a next generation gluten-free products. This conclusion is based on the main conclusions found on the formation (1), properties (2) and application (3) of the protein particle suspension.

Formation: Protein particles can be prepared by a versatile method based on the gelation of a phase separating protein-polysaccharide mixture. The method was proven to be suitable for different types of proteins (gelatin and whey protein) that differ in their molecular properties, **(chapter 2)**. The formation of mesoscopically structured protein particles depends on the rate and onset of phase separation and gelation. The process conditions, including well-defined shear flow, can be used to tune the particle sizes and, as a result, the properties of the suspension **(chapters 3 and 4)**. Interestingly, the ability of the protein to form disulphide bonds has no influence on the particle formation process **(chapter 4)**.

Properties: A suspension of mesoscopic protein particles (gelatin and whey protein) forms an elastic particle network. Properties similar to model systems of synthetic colloidal particles were obtained. The behaviour of the particle suspension mainly depends on the mesoscopic structure rather than on the specific chemical nature of the constituent material **(chapter 2)**. The elastic properties are a result of the interactions present between the protein particles **(chapters 2, 3 and 4)**. The particle interaction can be further increased by reducing the particle size **(chapters 3 and 4)**. Although the ability to form disulphide bonds has no influence on the particle formation process itself **(chapter 4),** disulphide bonds influence the properties of the resulting network. **(chapters 2 and 4)**.

Application: The addition of a dispersed protein phase (whey protein) can be used to transform a starch slurry into a cohesive dough. This dough shows viscoelastic and strain hardening properties and has a high gas retention during proving and baking **(chapters 5, 6 and 7)**.

The dough and leavened gluten-free bread are obtained with a gluten-free mixture that contains three basic ingredients: starch, protein particles and polysaccharide. Due to constraints in the particle production process, the amounts of whey protein and polysaccharide are low (2.4 % and 0.4 % (w/w db), respectively) compared to the amount of gluten in wheat dough (10 - 14 % (w/w db)) **(chapters 5 and 6)**. Remarkably, no cohesive mass can be formed, when vital gluten are added in the same amount (i.e. 2.4 % (w/w db)) **(chapter 5)**.

Our results show that the mesoscopic structure of the dispersed phase is important, but that molecular aspects can not be neglected. If the protein particles have no ability to form disulphide bonds, no strain hardening behaviour was observed **(chapter 7)**. In addition, the gas holding capacity of this dough was less than that of a starch mixture with protein particles that do have the ability to form disulphide bonds **(chapter 7)**. However, the ability to form disulphide bonds can also be too high. Partial blocking of the reactive thiol groups, to reduce the disulphide bonds formation, results in breads with better properties, especially crumb structure.

8.3 Main Considerations

The findings support the initial hypothesis that the functionality of gluten can be mimicked using a meso-structured protein system. The objective, which is to develop a protein based ingredient that resembles the key features of gluten network proteins, was therefore achieved. The developed protein particles are able to form a protein network that is resistant to stretching and can recover after deformation. Dough prepared with these protein particles shows viscoelastic and strain hardening properties and has high gas retention. Even at the simple formulation

used here, the bread volumes obtained were significantly higher compared to volumes reported to other gluten-free breads (3.7 ml/g vs 1 - 3 ml/g) [9, 12, 16-19, 21, 28-33]. Nevertheless, several questions regarding the formation (1), properties (2) and application (3) of the protein particle suspension remain. These questions will be discussed below.

1: Is liquid-liquid phase separation (including the use of simple shear flow) a feasible process for the formation of protein particles

We have demonstrated that a protein particle system is necessary to obtain a dough with strain hardening properties and high gas retention. A comparable system of protein gel patches, where first a gel was formed that was shred by the mixer, gives a dough without strain hardening properties and bread that has more cracks in the structure. Apparently the protein particles formed are superior to gel fragments of a similar size. However, the protein particle formation process that was applied in this study has some limitations. Although the process is versatile, the particle formation is critically dependent on the rate and onset of phase separation and gelation [34, 35]. Small variations can result in unwanted changes such as phase inversion, gel formation or macroscopic phase separation. Therefore, a small change in the process or the protein concentration requires adjusting the production procedure. Besides, the formation process requires the use of a high molecular polysaccharide to induce phase separation, which is difficult to completely eliminate after particle preparation. Despite the fact that the procedure is rather laborious, liquid-liquid phase separation remains the only method to obtain the protein particles used in this study. The protein particles obtained with other processes often show little interaction [36, 37].

The liquid-liquid phase separation process requires that the protein concentration and production procedure match in such a way that particles are formed. If the right match is found, the size and shape of the protein particles in the suspensions can be altered by using simple shear flow. Simple shear flow is proven to be an effective method to control the protein structure without altering other process parameters.

2: How can information about the properties of (different types of) protein particle suspensions give guidance for a gluten substitute

Most scientific studies on gluten-free bread analyse the properties of the dough/batter (e.g. mixing tolerance and small deformation properties) and the properties of the final bread (e.g. specific volume, gas cell stabilization) [9-11, 16-20]. Dough/batter and bread analysis were indeed necessary to understand the impact of the protein particles as gluten substitute. In this thesis, a significant part focuses on the properties of protein particles. In the first chapter, the properties of particles prepared with two different proteins (gelatin and whey protein) are analysed (chapter 2). The next two chapters extend this work: chapter 3 focuses on gelatin particles and chapter 4 focuses on whey protein particles. The studies on the particle properties are important, because these properties can be related to previous research on glutenin and to the recent insights on the behaviour of glutenin particles. Previous research showed that glutenin forms soft, swollen protein particles [26, 38]. Dough mixing leads to a disruption of these protein particles. The resulting fragments have a high tendency to re-aggregate into larger structures [25].

Comparison of the properties of gelatin particles and whey protein particles showed that the different nature of the particles (e.g. the ability to form physical interactions and the ability to form disulphide bonds) influences the properties of the particle networks. Gelatin particles have no ability to form disulphide bonds, but they do have the ability to form physical interactions. Those physical interactions are relevant for the elastic behaviour of the gelatin particle suspension. Whey protein particles have a lower ability to form physical interactions; probably as a consequence the whey protein particles are smaller. In contrast to gelatin, the whey protein particles have the ability to form disulphide bonds. Those disulphide bonds are relevant for the strength of the whey protein particle network. A gelatin particle network shows a higher deformability than the whey protein particle network. The whey protein particle network is more brittle and less elastic than the gelatin particle network.

The high elasticity of the gelatin network makes this network more comparable with the glutenin network than a network of whey protein particles. Unfortunately, due to melting of gelatin particles, they can not be used in actual baking tests. This is the reason why we selected whey protein as a possible gluten substitute, and the reason why it is tried to adjust the behaviour of the whey protein particles to become more like the glutenin particles (soft and elastic). To do so, whey protein particles without the ability to form disulphide bonds are prepared by blocking the reactive thiol groups. The results obtained (chapters 2, 3 and 4 - summarized in figure 1) show that the ability to form disulphide bonds can not (totally) explain the differences between gelatin and whey protein particles. The gelatin particle suspension shows more cluster formation and is more elastic than the whey protein particles without disulphide bonds (probably this is related with the higher ability of gelatin to form

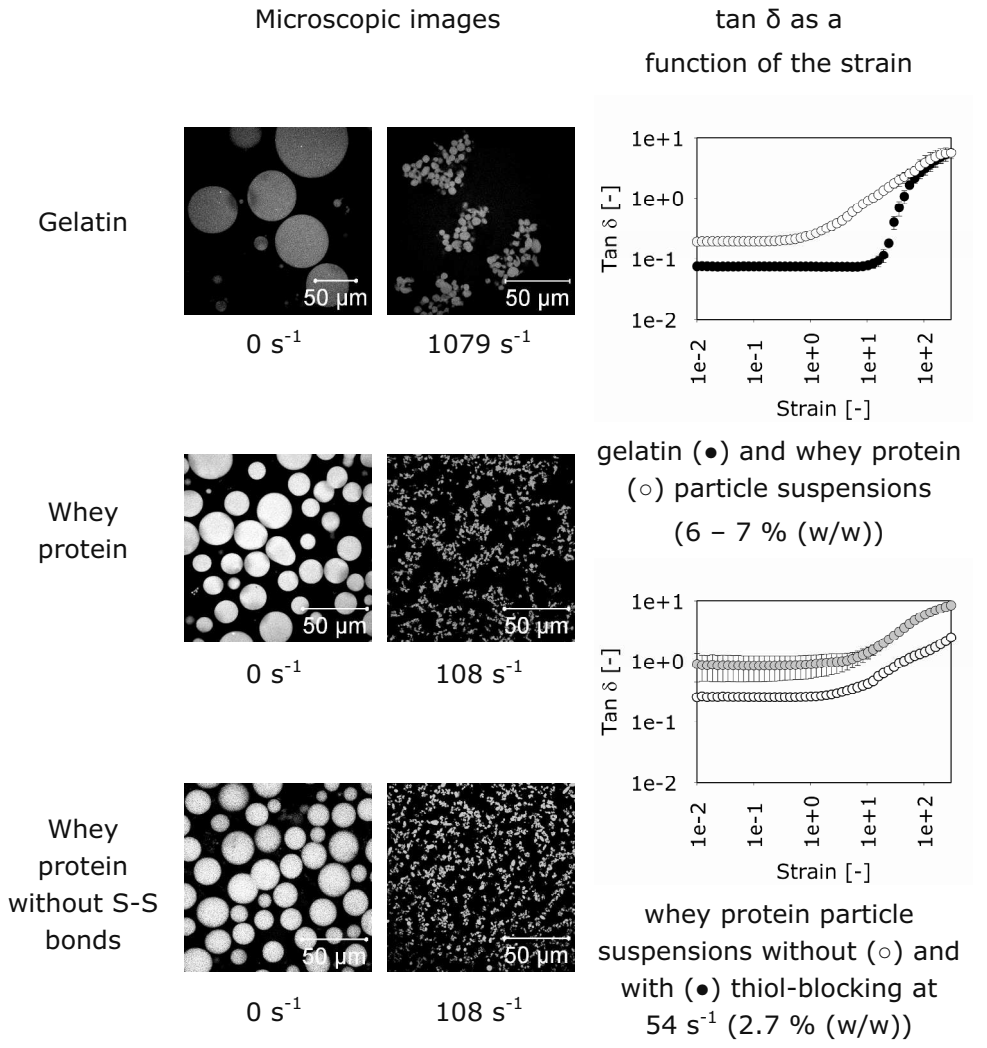


Figure 1: Overview of the structures and small deformation behaviour of gelatin and whey protein particle suspensions without and with thiol-blocking. The particles were prepared using different shear rates (0, 54, 108 and 1079 s^{-1}) during processing.

physical interactions). Future research could focus on clarifying the relation between intrinsic molecular properties and resulting particle suspension properties. Addition of crosslinking agents (e.g. Glutaraldehyde) to gelatin can help to increase the understanding of covalent interactions in protein particles. The outcome of this work can be compared to recent insights obtained for glutenin particles.

3: Why studying the application using a model dough

A model dough was prepared that consisted of a limited number of ingredients (starch, protein-polysaccharide suspension, salt, sugar and yeast). The breads obtained (chapters 5 and 6) lacked reducing sugars and had a low amount of proteins, which explains the pale colour of the breads, and a low yeast activity. Improvements of the breads might be obtained through the addition of more components. In this study only the structure forming glutenin fraction of wheat flour was replaced. The other gluten fraction, the gliadins - which act as a plasticizer, are not replaced. Besides these gluten proteins also non-gluten proteins, arabinoxylans and lipids influence the properties of wheat flour dough and bread [39]. The use of a gluten-free flour (e.g. rice flour, oat flour, Amaranth flour) can be an option to obtain a better performing system. Then, it is also possible to use recent scientific studies on gluten-free bread, which make use of more complex systems of different flours e.g. [8, 9].

Nevertheless, we have chosen to start with a simple model system, because it also has advantages. First of all, the model limits the phenomena that may occur, making it easier to interpret the physical behaviour. It therefore allows a better and faster comparison of different systems. A pitfall of model systems is that the specific behaviour or characteristic may be the result of the simplification, rather than of the

fundamental properties of the system. Therefore, to eliminate this pitfall of an oversimplified model system, we compared the behaviour of our protein systems with the system to be replaced (gluten) at two different concentrations.

From the studies with the model dough and bread, we know which mesoscopic structure and microscopic structure is necessary to obtain strain-hardening behaviour and high gas retention. There are many ingredients present in fully formulated wheat dough which all affect its properties to some degree [39]. Even the starch used in this model system has some limitations. The starch used only consists of type A-starch (large granules), while wheat flower contains both type A and type B (small granules) starch. This will probably influence the final dough and bread properties. Future research aimed at improving the gluten substitute that was developed here should focus on the impact of the ingredients that were not included in the starch–protein particle system used here. It is relevant to investigate what happens when the protein particle suspension is added to a gluten-free flour that has a normal starch composition. Addition of the protein particles to a gluten-free flour (e.g. rice flour, oat flour, Amaranth flour) also gives a more complete system.

8.4 Methodology

As mentioned in the introduction, the route followed was a step-by-step approach. Rather than concentrating on the analysis of gluten network properties, and only finally attempting to develop a gluten substitute, we chose for compiling the existing insights into the design of protein ingredients, and comparing their properties with those of gluten. By following this route, the design process itself leads to the refinement of

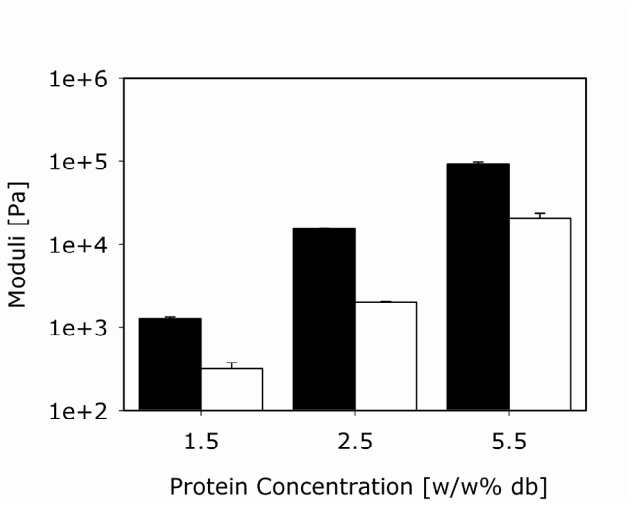
insights on those aspects that need improvement. While the final gluten replacing ingredient is important for helping patients of celiac disease, the insight that is created in parallel, may well open routes into structuring of other types of products.

We found that this route indeed led to better insight. A route that is only focused on analysis of the working of gluten may be difficult in complex systems such as the current ones, with many different components and many interactions (even when using a model dough system). On the other hand, a pure process of designing will lead to a trial-and-error approach, and may drown as well in the complexity of the system. It also bears the risk of finding sub-optimal solutions. Combining the design process with the understanding of the product at that moment gives faster improvements. Especially if each new design step is based on at least partial understanding and the outcomes is used to refine the insight.

8.5 Scientific Implications and Future Research

8.5.1 A State Diagram

In this study protein particles were created that showed a high tendency for interparticle interactions. Even though the percentage of protein in the final product is low (particle weight percentage 2.5 % (w/w db) and volume percentage ~10 %), these amounts are shown to be crucial for the macroscopic properties of a product. It would be interesting to further investigate other aspects of these protein particles e.g. the effect of the particle concentration, and the effect of different types of molecular interactions. Some preliminary results of experiments in which the protein concentration was increased are shown in figure 2.



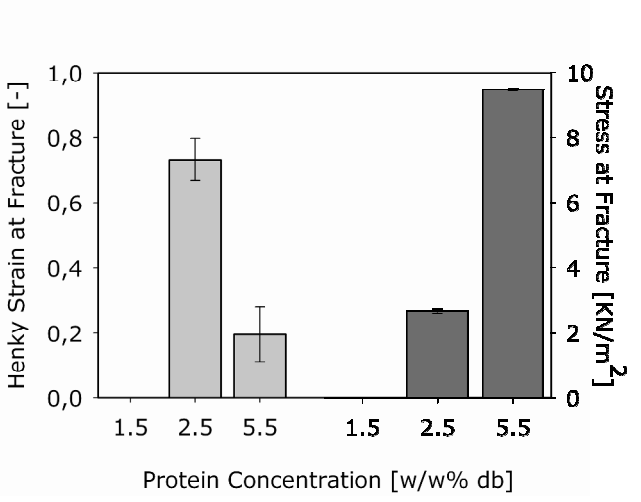


Figure 2: Storage modulus (black) and loss modulus (white) of dough samples under small deformation measurements in a rheometer. Strain at fracture and stress at fracture of dough samples under extensional tests in a Texture Analyzer. The dough mixtures are prepared with different protein concentration.

Rheological characterisation of dough with different whey-protein particle concentrations shows that the material behaves more fluid-like at low concentration (large deformation tests are not possible). Increasing the concentration gives a viscoelastic material, but the elasticity of the dough reduces upon further increasing the concentration. The material becomes stronger, but less stretchable; i.e. the behaviour changes towards more solid-like.

The experiments with different concentrations and interactions show that the behaviour can be captured in a state diagram of these dough/particle systems that resembles the state diagram of a simple colloidal system [40]. Changes in the particle concentration give: A) fluid behaviour at low concentration, B) viscoelastic behaviour at intermediate concentration, C) solid behaviour at high concentration. If these changes in concentration are repeated with particles having less interaction, the transition from fluid behaviour to viscoelastic behaviour (from a to b) and from viscoelastic behaviour to solid behaviour (from b to c) occurs at higher concentrations. The state diagram is depicted in figure 3.

However, rheological characterisation of dough prepared with whey protein particles with different amounts of disulphide bonds (chapter 7 - summarized in figure 4) showed that the impact of interactions in dough systems is less straight forward than those in colloidal suspension. When the amount of disulphide bonds is increased, the dough becomes stronger as was expected from the state diagram. But a further increase in the amount of disulphide bonds gives a softer dough. This softening of dough with increasing disulphide bonds can be explained by the changes in the intra-particle interactions. Without disulphide bonds, the particles are unstable and break by dough processing leading to small protein

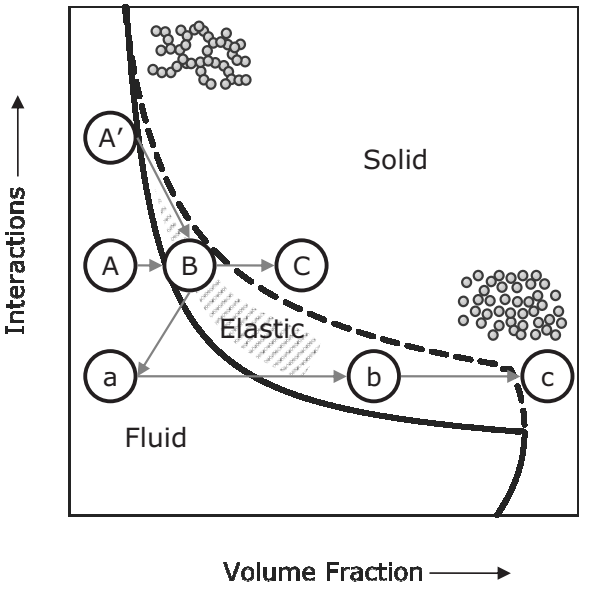
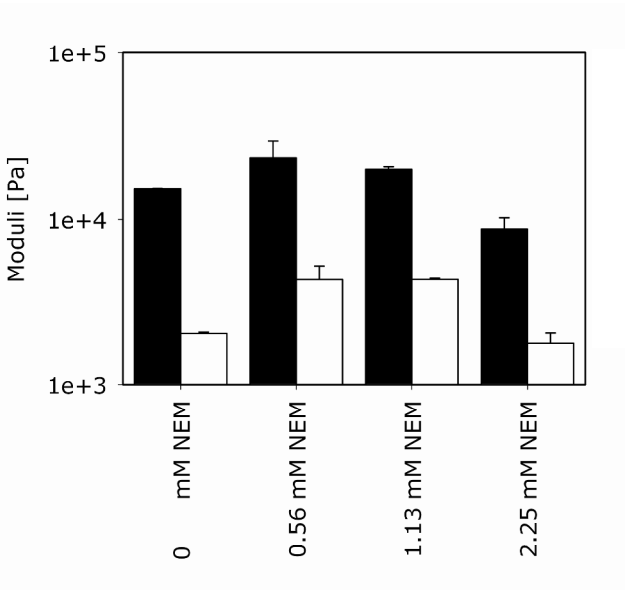


Figure 3: Schematic state diagram of whey protein particles. The solid line represents the boundary of the fluid state, the dashed line the boundary of the solid state. The grey area is the region with the required viscoelastic behaviour.

patches (a). Increasing the amount of disulphide bonds gives stable protein particles. These protein particles do not break by dough processing, but they are deformable and soft. If the amount of disulphide bonds is further increased, the protein particles lose this soft behaviour, and are less deformable. This interplay of deformability by the sparsity of the (sticky) inter-particle contact points, and the solidity of the particles, gives the viscoelastic properties. In addition, highly crosslinked particles have a lower possibility to increase their phase volume (A'). Since the number of contact points between the individual particles is low; the number of contact points increases strongly if the volume fraction of the particles is increased (B).



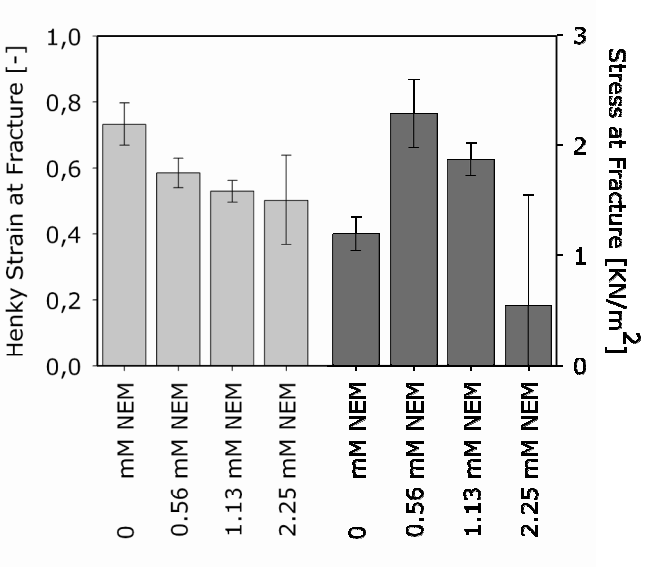


Figure 4: Storage modulus (black) and loss modulus (white) of dough samples under small deformation measurements in a rheometer. Strain at fracture and stress at fracture of dough samples under extensional tests in a Texture Analyzer. The dough mixtures are prepared with particles that vary in the NEM treatment. A 9 % (w/w) protein aggregate solution was untreated or treated with 0.56 mM NEM, 1.13 mM NEM or 2.25 mM NEM before particle formation.

The state diagram indicates a region in which the required viscoelastic behaviour can be obtained (the dashed area). From that it becomes clear that both particle interaction and volume occupied by particles are important. The latter is determined by the ability to swell, which is most likely determined by the number of crosslinks due to disulphide bonds.

The amount of disulphide bonds in whey protein particles is high compared to the amount in a GMP dispersion. It is possible that this high amount of disulphide bonds makes the whey protein particles too rigid. In analogy, heavily crosslinked rubber is also not elastic anymore [41]. Whey proteins that have no ability to form disulphide bonds are not a solution, since disulphide bonds are necessary for particle stabilization. Blocking all reactive thiol groups has a negative effect on dough and bread properties. The behaviour of the protein particles should be made less rigid, without a total loss of disulphide bonds formation. Chapter 7 indeed confirms that partial blocking of the reactive thiol groups leads to improved dough properties and bread structure. Physical interactions can be used to further improve the dough properties. Physical interactions are known to relevant for self healing structures [42].

8.5.2 A particle network in dough

The results from this study also have implications for the understanding of the gluten protein structure-function relationship. Most of the gluten models concern the molecular length scale since they focus on molecular interactions, like the chemical structure of the glutenin polymer network or the ability to form entanglements (see chapter 1). The work reported in this study showed the relevance of the mesoscopic length scale. The relevance of the mesoscopic structure of the gluten network was proposed earlier by Hamer and van Vliet [25, 27].

In figure 5, the relation between the mesoscopic structure and its mechanical function is schematically depicted for a particle based system on the one hand, and the molecular model of MacRitchie on the other hand. The simplicity of the depicted particle network should not be misinterpreted. We do not state that only the mesoscopic scale is important in the gluten network or that the gluten network only consists of glutenin particles. For example, our system does not take other important gluten fractions (e.g. gliadins) into account. The picture serves to present the general concept.

Structure: The structures of the so-called 'physical' gluten models use both physical and chemical interactions. The models suppose that the gluten network is formed by polymers having heavily connected regions. In between, there are regions where no polymer-polymer interaction is present [43-46].

In contrast, the structure of the hyper-aggregation model shows the importance of the mesoscopic length scale. This model uses two different length scales. According to this model, the disulphide bonds between glutenin subunits are dominant at small length scales, while the physical interactions are dominant at larger length scales. If not stabilized by chemical interactions, the glutenin particles fall apart during dough processing, and have no ability to reform [47]. The network formation of glutenin particles is related with the break-up during mixing. It is stated that when the disulphide bonds are present the glutenin particles will reform giving the required network properties [25].

The structure formed by our whey protein particles follows the hyper-aggregation model quite closely. On the microscopic length scale there are protein aggregates that form the building blocks for the protein particles.

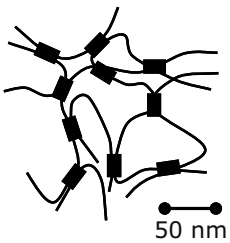
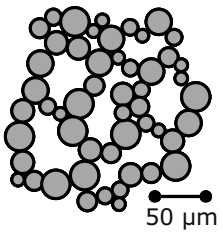
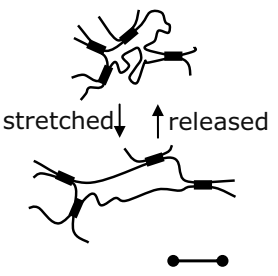
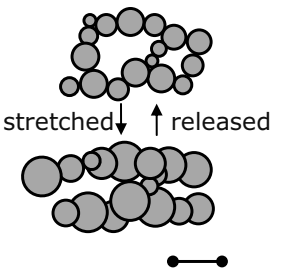
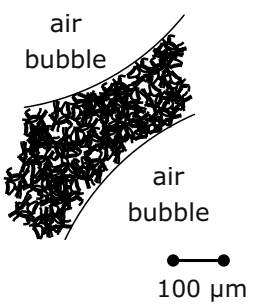
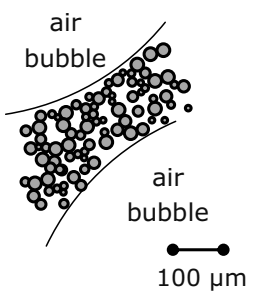
	gluten	Physical model (MacRitchie)	Mesoscopic Particle model
Structure	Unknown		
Properties upon stretching	Elastic Strain hardening		
Application	Air bubble stabilization in dough and bread		

Figure 5: Schematic representation of a gluten model and the protein particles that are suggested to explain the gluten protein structure-function relationship

Both physical and chemical interactions are important in the formation of protein particles by the aggregates. Chemical interactions are not directly involved in the initial formation process, but are important for the stabilization of the structures once formed (for a detailed description see chapters 4 and 7). In similarity with glutenin particles, the whey protein particles fall apart during dough processing if they are not stabilized by chemical interactions. The type and amount of interactions between the aggregates determine the final properties of the particles. If part of the chemical interactions are broken, the WP particles become softer (the phase volume increases) and more deformable. This softening of the particles makes the behaviour more comparable with gluten.

Our studies confirm the suggestion that in addition to the microscopic structure, the mesoscopic structure is important. Studies done with a suspension of protein particles showed that the particles are linked through physical and chemical interactions. The physical interactions are important for the self healing properties of the particle network. The chemical interactions are important for strengthening the particle network (for a detailed description see chapters 2 and 4). Probably those interactions between the particles are also relevant if the particle suspension is mixed with starch, as the behaviour of a starch–protein mixture is comparable with the behaviour of a starch–gluten mixture (chapter 5).

There is one remarkable difference between the glutenin particles and the whey protein particles. A total disruption and reformation was not observed for the protein particles used in this study. Nevertheless a partly disruption of the disulphide bonds gives a more gluten like behaviour. We consider these observations a first step towards systematically unravelling

gluten structure-function relationships. The mesoscopic particle system represents a unique approach to unravel the importance of different aspects of glutenin particles, such as their break-up and reformation behaviour.

Properties: The properties of the physical models during stretching are explained with the deformation of the non-connected regions and the heavily connected regions [16, 43]. Upon stretching, the deformation of the unconnected regions is easier than stretching the heavily connected regions. This explains the strain hardening [16].

The hyper-aggregation model explains the behaviour during stretching by the different interactions present in the network. Breakage of the disulphide bonds weakens the network, but due to a large amount of physical interactions reformation or healing of the network takes place. The different packaging after reformation strengthens the network.

Upon stretching the whey protein particle network shows a strong elastic behaviour, which will still be apparent in dough. As with the glutenin particles, the strain hardening behaviour of the whey protein particles network is not only related to the mesoscopic structure, but also to the microscopic structure. This study suggests that (partly) breakage of the particles is important for the strain hardening behaviour. We propose that some intra-particle bonds are broken due to deformation. Breakage of the intra-particle bonds during dough processing gives an increase in the phase volume of the particles, and the breakage of the intra-particle bonds makes the protein particles behave more elastically. The increase in phase volume results in an increase in the volume fraction of the particles and consequently the number of inter-particle interactions can increase (for a detailed description see chapter 7).

Application: The gas retention properties of the gluten network are not often considered in the gluten models. It is generally accepted that gas retention is related to the strain hardening behaviour and elastic properties of the protein network [48]. Therefore the gluten models consider the strain hardening behaviour and the elastic properties, and in this way explain the gas retention properties.

The fact that the particle network provides the dough with strain hardening behaviour is important for gas retention. The network that is present in the dough is deformed by the growing gas cells. Due to its strain hardening behaviour, this deformation strengthens the network and therefore the gas cells are stabilized.

Although strain hardening behaviour and elastic properties are necessary for good gas retention, there is another important aspect. The included gas should not lead to a weakening of the network. The effect of air bubble incorporation on the strength of the network depends on: the ratio of the size of the air bubbles and the network pore size, the volume fraction of air bubbles, and the interaction between the air bubbles and the network. When the air bubbles are small compared to the building blocks of the network, the air bubbles can strengthen the network, while air bubbles that are large compared to the building blocks of the network will disrupt the network, and weaken it [49]. Strengthening of the network during gas cell formation is important, which suggest that the network should not be made of too small building blocks.

The insight that is created in this study may help to further improve the current gluten models.

8.6 Potential Applications

Meso-structured protein particles represent an important first step towards high quality gluten-free products. A protein particle system can be used as a gluten substitute in products that are normally based on the use of wheat flour or vital gluten. Gluten-containing flours are used in many different products such as bread, salad dressings, sauces and even toothpaste. Vital gluten is often used as an additive e.g. to fortify flours that have a low or poorly functional gluten content, and to improve the properties of cereal products such as the crispiness of breakfast cereals and tortilla chips [50]. In the products where gluten are required for their network properties, they can be replaced by the meso-structured whey protein particles.

To replace (part of) the gluten in those products with a protein particle system, the system should fulfil some requirements. The cost of a product with a gluten substitute should be comparable or lower than the cost of the current gluten-free products. There should be a good and stable supply of the gluten substitute. Finally, the gluten substitute should be easy to handle. At this stage, the protein particle systems that were described in this thesis do not fulfil all criteria. Even though whey protein is readily available, the current process and the state of the protein particle suspension raise the cost. The main points of attention are: (1) the protein suspension is not efficient with respect to cost (e.g. storage and transport cost); (2) the preparation process should be scaled-up towards a larger scale and an end-product with a higher protein concentration.

The current protein suspension has a low protein concentration. This is not efficient with respect to storage and transport cost. The protein particle formation process is based on liquid-liquid phase separation between two biopolymers, which occurs at low biopolymer concentrations generally. In addition, a suspension is not easy to store and handle, as it is (understandably) susceptible to aggregation and microbial spoilage. It would be more beneficial to have a protein particle powder. Unfortunately it was not possible to make a protein particle powder by freeze drying the protein particle suspension, since freeze drying influences the particle properties. The protein particles are suitable for application as long as the protein suspension is made and used immediately and a low protein concentration is required. For those applications a large scale production process is possible with the current preparation process.

To use the protein particles in applications that requires a high protein concentration (concentrated dough or non-cereal applications), a different preparation method should be developed. The use of a whey protein gel (chapters 5 and 6) gives a less elastic dough and bread that have more cracks. But, the method has a number of important advantages. The preparation process of gel patches is more flexible. For example, the protein concentration can be easily adjusted. [51, 52]. A cold set whey protein gel can be prepared at higher concentration (limited to ~12 % (w/w) [53]) than whey protein particles (limited to ~3 % (w/w)). In contrast to particles, gel patches can be prepared without polysaccharide. The influence of a polysaccharide can be further investigated and optimised in a gel system. The size (and size distribution) of the gel patches can be altered by the exact milling or grinding conditions. After some adjustments, the use of gel patches as gluten substitute may be a suitable successor for the particle system.

Beside the application as a gluten replacer, the whey protein particles can also be used as model systems for glutenin particles. The system developed in this study can be used to study the structure-function relationship of glutenin particles (e.g. viscoelasticity and strain hardening). In this way the gluten model proposed by Hamer and van Vliet can be further improved.

8.7 Summary of Key Findings

- Mesoscopic structuring is promising to develop high quality gluten-free bread products
- Mesoscopic structuring is a new and promising tool in product development. Altering the mesoscopic properties of a product can be done without changing the composition.
- Mesoscopic properties of products are relevant with respect to final product properties and should be more often taken into account.
- Soft protein particle networks can be used to create viscoelastic properties.
- The step-by-step approach used in this thesis to develop a gluten substitute turned out to be an effective way of working, because this approach combines the generation of insight and practical solutions at the same time
- The change in formulation should be accompanied by an adaptation in the production process (omission of sheeting)

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Summary

Samenvatting

Summary

The prevalence of gluten intolerance has increased dramatically in the last 20 years. As a result, there is an increased demand for a gluten-free alternative for wheat-flour containing products such as bread, cookies etc. The difficulty is that gluten, which triggers the inflammatory reaction, is often crucial for the final structure of a product. Bread is the most important cereal product, because it is used and consumed during the regular meals on daily basis. That is why the aim of this thesis is to develop a novel technology to making gluten-free breads.

Till now, recipe modification and ingredients additions were the common methods to develop high-quality gluten-free breads. Generally research on gluten-free breads explored the use of a batter consisting of hydrocolloids, emulsifiers, and gluten-free starches or flours. In this thesis, we present an alternative approach for the design of a gluten-free bread. In this approach the gluten will be replaced by a protein suspension structured at the mesoscopic scale. The idea of using mesoscopically structured whey protein is based on the hypothesis that the unique properties of the wheat dough originate, at least partially, from the protein properties at the mesoscopic length scale **(chapter 1).**

The thesis consists of two big parts.

In the first part (chapters 2 - 4), the preparation method and the resulting properties of a mesoscopically structured protein particle system are investigated. The main focus was on the network forming properties of the protein particles. The protein particles were prepared by a versatile method based on the gelation of a phase-separating protein–polysaccharide mixture. Two proteins were selected: gelatin and whey protein. Even though the intrinsic properties of those proteins are

different, both gelatin particles and whey protein particles form an elastic particle network. The behaviour of the protein particles is comparable with model systems containing synthetic colloidal particles. This implies that the protein particles show a remarkably high degree of interaction, given the fact that the protein particles are much larger than the particles used in the model studies. The main difference in the network properties of gelatin and whey protein particles was the strength of the network. Gelatin particles formed a loose network that can easily be reformed. The whey protein particle network showed a higher degree of structure that can withstand a small deformation even if it is partly disrupted **(chapter 2)**.

The experimental method described above was extended by introducing well-defined shear flow as a process parameter during preparation of the particles. This method allowed control over the size of the protein particles formed. Both proteins (gelatin and whey protein) showed a decrease in particle size with increasing shear rates. The application of shear-flow resulted in a more homogeneous size distribution in case of gelatin. The whey protein particles prepared under shear possessed a non-spherical shape. The rheological properties of the resulting suspensions were strongly influenced by the effect of particle clustering. The sizes of the clusters depended on the primary protein particle size, and on the ability to form disulphide bonds. Suspensions containing clusters originating from small particles could resist more deformation than suspensions containing the larger particles **(chapters 3 and 4)**.

The mechanism involved in the particle formation process can explain the effect of shear applied during processing. The gelatin particles are formed before gelation, and their size is therefore depending on the properties of

the continuous phase. Upon gelation the properties of the protein phase changes, hence the particles start to cluster and form a gel. The whey protein particle formation is triggered by gelation, implying that the actual particle formation is fast, if not instantaneous once protein aggregates starts to form a gel. This abrupt formation can be responsible for the non-spherical shape of the particles formed **(chapters 3 and 4)**.

Besides the effect of the particle size, also the effect of disulphide bond formation is investigated. The ability to form disulphide bonds of whey protein particles is steered by blocking the reactive thiol groups of the whey proteins with N-ethylmaleimide. The complete blocking of the disulphide bonds did not result in significant changes in the particles sizes, most likely due to the fact that the formation process is fast compared to the rate of disulphide bond formation. However, the ability to form disulphide bonds influenced the cluster formation and consequently the rheological properties of the particle suspension **(chapter 4)**.

The whey protein particle suspension with blocked thiol groups showed less cluster formation than the gelatine particle suspension and was less elastic. This is probably related to the other intrinsic properties (e.g. the physical interactions), but also other factors such as the polysaccharide used **(chapters 3 and 4)**.

In the second part of this thesis (chapters 5 - 7), the protein particles were used as a gluten-alternative in a gluten-free formulation. Mixtures were prepared using a Farinograph dough mixer in which the suspension containing the particles and wheat starch were combined, leading to a mixture having 2.4% (w/w db) protein. In case proving or baking tests were performed, sugar and yeast were added to the mixture as well.

The properties of those mixtures were compared with mixtures in which the protein was structured in different ways **(chapters 5, 6 and 7).**

Chapter 5 describes the rheological and mechanical properties that are caused by the addition of whey protein particle suspensions to starch mixtures. The addition of whey protein particles transformed the starch from a liquid substance to a material with dough-like properties. The gluten-free mixtures showed the strain hardening properties that were previously considered to be the unique property of gluten containing dough. The addition of whey protein gel or whey protein aggregates to a starch mixture did not result in those strain hardening properties. The mixture prepared with whey protein aggregates remained a liquid **(chapter 5)**.

Chapter 6 describes the results form the baking tests with the dough described in the previous chapter. Baking the dough mixture prepared with whey protein suspensions gave breads with a good volume and an attractive crumb structure. All WP dough mixtures could be sheeted, which is a quite unique property for gluten-free formulations. Most gluten-free breads are prepared starting with a batter system, and no sheeting step is included in the baking process. The breads prepared with protein particles could be produced using a common production process.

The importance of the mesoscopic protein structures was demonstrated by testing two other protein structures (whey protein gel or whey protein aggregates). The volume and texture of those breads were less good than the whey protein particle bread, even though the bread volumes were still comparable or even higher than that of the gluten-starch bread **(chapter 6)**.

Finally the impact of the particle stability on the dough and bread properties is investigated. The ability to form disulphide bonds is steered by blocking (part of) the reactive thiol groups of the whey proteins with N-ethylmaleimide. If the protein particles added to a starch mixture had no ability to form disulphide bonds (i.e. all thiol groups were blocked), the dough showed no strain hardening behaviour. After baking breads with lower volumes and more cracks were obtained suggesting reduced gas holding capacity. Therefore, different amounts of the thiol groups were blocked to investigate the effect of disulphide bonds. Remarkably, the amount of disulphide bonds of the protein particles added has an optimum with respect to strain hardening and bread properties **(chapter 7)**.

When all the results in the thesis are considered, it can be concluded that the use of a mesoscopic whey protein-particle network is a new and innovative approach to develop a next generation gluten-free products. The results are unique, taken into account that we only used 2.4% (w/w db) protein. Further improvements can still be obtained through the use of a gluten-free flour instead of starch.

The current protein particle production process is suitable for large-scale production, as long as the protein suspension is made and used immediately and a low protein concentration is sufficient **(chapter 8)**.

Apart from the relevance for application, this study gives more insight in the behaviour of protein particle suspensions. In addition the understanding of the gluten protein structure-function relationship might benefit from this study as well.

Samenvatting

Er is de laatste 20 jaar een grote toename van het aantal patiënten met coeliakie (glutenintolerantie). Omdat de enige therapie tot nu toe een volledig glutenvrij dieet is, neemt de vraag naar glutenvrije producten (brood, koek etc.) sterk toe. Helaas is de kwaliteit van glutenvrije producten nog niet optimaal. Met name het ontwikkelen van glutenvrije levensmiddelen met de juiste textuur is moeilijk. Dit komt omdat gluteneiwitten (die de allergische reactie veroorzaken) cruciaal zijn voor de textuur van een product. Brood is een belangrijk basisproduct dat veel gluten bevat. Goede glutenvrije alternatieven voor brood kunnen bijdragen aan het welzijn van patiënten met een glutenintolerantie. Het doel van deze studie is daarom het ontwikkelen van een nieuwe methode om een glutenvrij brood te produceren.

Glutenvrije broden van een goede kwaliteit werden tot nu toe bereid uit de receptuur die als basiscomponenten een glutenvrije bloem of zetmeel bevat. Een beslag wordt gemaakt door emulgatoren of hydrocolloïden toe te voegen en te mengen met water. In deze studie gebruikten wij een alternatieve procedure voor het ontwikkelen van een glutenvrij brood. Gluten werd vervangen door een eiwitsuspensie, die is gestructureerd op de mesoscopische schaal (10 – 100 µm). Het idee om het eiwit op de mesoscopische schaal te structureren is gebaseerd op de hypothese dat de unieke eigenschappen van tarwedeeg worden bepaald door het gedrag van gluteneiwitten op deze schaal **(hoofdstuk 1)**.

Deze studie bestaat uit twee hoofddelen.

In het eerste deel (hoofdstukken 2 - 4) is onderzocht hoe mesoscopische eiwitdeeltjes gemaakt kunnen worden en wat de eigenschappen zijn van deze mesoscopische eiwitdeeltjes. De eiwitdeeltjes zijn gemaakt met een methode die voor verschillende eiwitten toepasbaar is. Deze methode is

gebaseerd op gelering van een faseschеidend eiwit-polysacharide mengsel. Twee verschillende eiwitten (gelatine en wei-eiwit) zijn gebruikt om deeltjes te vormen. Ondanks dat deze twee eiwitten verschillen in hun intrinsieke eigenschappen, vormen zowel gelatinedeeltjes als wei-eiwitdeeltjes een elastisch deeltjesnetwerk. Het gedrag van deze eiwitdeeltjes is vergelijkbaar met modelsystemen van synthetische colloïdale-deeltjes. Aangezien de eiwitdeeltjes veel groter zijn dan de colloïdale-deeltjes die gebruikt zijn in deze modelstudies, moeten de eiwitdeeltjes een opmerkelijk grote aantrekkingskracht voor andere deeltjes hebben. Het belangrijkste verschil in de netwerkeigenschappen van gelatinedeeltjes en wei-eiwitdeeltjes is de sterkte van het netwerk. Het netwerk van gelatinedeeltjes is losjes en kan gemakkelijk vervormen. Het netwerk van wei-eiwitdeeltjes is stugger en is niet gemakkelijk te vervormen, zelfs niet wanneer de netwerkstructuur al deels is verstoord **(hoofdstuk 2)**.

Aan de hierboven beschreven productiemethode werd een extra procesparameter, afschuifstroming tijdens de vorming van eiwitdeeltjes, toegevoegd. Deze methode maakt het mogelijk om de grootte van de eiwitdeeltjes te controleren. Beide eiwitten (gelatine en wei-eiwit) vormen kleinere deeltjes wanneer de afschuifstroming toeneemt. In het geval van gelatine zorgde de toepassing van afschuifstroming voor een homogenere verdeling van de deeltjesgrootte. De wei-eiwitdeeltjes werden onregelmatig van vorm door afschuifstroming. De reologische eigenschappen van de deeltjessuspensies werden sterk beïnvloed door het feit dat de deeltjes clusters vormden. De grootte van de clusters waren afhankelijk van de grootte van de eiwitdeeltjes en van de mogelijkheid om zwavelbruggen te vormen. Suspensies die clusters bevatten van kleine deeltjes kunnen meer deformatie aan dan suspensies die grote deeltjes bevatten **(hoofdstukken 3 en 4)**.

Het vormingsproces van eiwitdeeltjes kan het effect van de afschuifstroming verklaren. De gelatinedeeltjes worden gevormd voor gelering en hun grootte is daarom afhankelijk van de eigenschappen van de continue fase. Tijdens gelering veranderen de eigenschappen van de eiwitfase, waardoor de eiwitdeeltjes clusteren. De vorming van wei-eiwitdeeltjes wordt veroorzaakt door gelering. Dit houdt in dat de werkelijke vorming snel of zelfs instantaan plaatsvindt wanneer de eiwitaggregaatjes een gel vormen. Deze abrupte vorming kan verantwoordelijk zijn voor de onregelmatige vorm van de eiwitdeeltjes **(hoofdstukken 3 en 4)**.

Naast het effect van de grootte van de deeltjes is ook de rol van zwavelbruggen onderzocht. De mogelijkheid van wei-eiwit om zwavelbruggen te vormen werd gecontroleerd door de reactieve thiolgroepen van wei-eiwit te blokkeren met N-ethylmaleimide. Een complete blokkering van de zwavelbruggen zorgde niet voor grote veranderingen in de deeltjesgrootte. Dit komt waarschijnlijk doordat de vorming van zwavelbruggen veel meer tijd kost dan vorming van de eiwitdeeltjes zelf. De mogelijkheid om zwavelbruggen te vormen beïnvloedt echter wel de clustervorming en daarmee het reologisch gedrag van de deeltjessuspensie **(hoofdstuk 4)**.

Een suspensie met wei-eiwitdeeltjes met geblokkeerde thiolgroepen vormde minder clusters en was minder elastisch dan een suspensie met gelatinedeeltjes. Dit is waarschijnlijk gerelateerd aan de verschillen in de intrinsieke eigenschappen van de eiwitten (bijvoorbeeld de fysische interactie), maar ook aan andere factoren zoals de polysacharide die is gebruikt **(hoofdstukken 3 en 4)**.

In het tweede gedeelte van deze studie (hoofdstukken 5 – 7) zijn de eiwitdeeltjes gebruikt als glutenvervanger in glutenvrije toepassingen. In een Farinograaf deegkneder zijn mengsels van een suspensie van eiwitdeeltjes en tarwezetmeel gemaakt. De mengsels bevatten 2.4% (w/w) eiwit. Bij baktesten werd ook suiker en gist toegevoegd. **(hoofdstukken 5, 6 en 7)**.

Hoofdstuk 5 beschrijft het reologisch gedrag en de mechanische eigenschappen van mengsels met wei-eiwitdeeltjes en zetmeel. Het toevoegen van wei-eiwitdeeltjes veranderde de vloeibare zetmeelsubstantie in een materiaal met deegachtige eigenschappen. Het is bijzonder dat een glutenvrij mengsel een deeg vormt en geen vloeibare substantie blijft. De glutenvrije degen vertoonden koudeversteviging, een eigenschap die karakteristiek is voor glutenbevattende degen. Het belang van de mesoscopische eiwitstructuur is onderzocht door naast de wei-eiwitdeeltjes twee andere eiwitstructuren te testen (een wei-eiwit gel en wei-eiwit aggregaatjes). Het toevoegen van een wei-eiwit gel of van wei-eiwit aggregaatjes aan een zetmeelmengsel gaf deeg waarin geen koudeversteviging optreedt. Een mengsel waaraan wei-eiwit aggregaatjes waren toegevoegd bleef zelfs een vloeibare substantie **(hoofdstuk 5)**.

De resultaten van de baktesten van de nieuwe glutenvrije mengsels staan beschreven in hoofdstuk 6. Het tot brood bakken van de deegmengsels die gemaakt zijn met wei-eiwit suspensies gaf een brood met een goed volume en een aantrekkelijke kruimstructuur. Alle degen gemaakt met wei-eiwit konden worden uitgerold met een deegroller, wat een zeer unieke eigenschap is voor glutenvrije mengsels. De meeste glutenvrije mengsels zijn een beslag, waardoor er geen sprake kan zijn van uitrollen met een deegroller. De broden die zijn gemaakt met wei-eiwitdeeltjes konden worden geproduceerd met een normaal broodbak proces.

Het belang van de mesoscopische eiwitstructuur is onderzocht door naast broden gemaakt met wei-eiwitdeeltjes, ook broden te testen die gemaakt zijn met twee andere eiwitstructuren (een wei-eiwit gel en wei-eiwit aggregaatjes). Het volume en de textuur van de broden met deze andere wei-eiwitstructuren was minder goed dan die van de broden met wei-eiwitdeeltjes. Toch waren de volumes van deze broden vergelijkbaar of beter dan de volumes van broden gemaakt met gluten en zetmeel **(hoofdstuk 6)**.

Tenslotte is het belang van de eiwitstabiliteit op deegeigenschappen en broodeigenschappen onderzocht. De mogelijkheid om zwavelbruggen te vormen is gecontroleerd door (een deel van) de reactieve thiolgroepen te blokkeren met N-ethylmaleimide. Wanneer de eiwitdeeltjes in het zetmeelmengsel geen mogelijkheid hadden om zwavelbruggen te vormen (alle thiolgroepen zijn geblokkeerd), vertoonde het deeg geen koudeversteviging. Na bakken hadden de broden een kleiner volume en meer scheuren wat een lagere gashoudende capaciteit suggereerde. Om het effect van zwavelbruggen verder te onderzoeken zijn verschillende hoeveelheden zwavelbruggen geblokkeerd. Het bleek dat blokkeren van een deel van de thiolgroepen het beste brood opleverde. Blijkbaar is er een optimum in de hoeveelheid zwavelbruggen die nodig zijn voor de koudeversteviging en broodeigenschappen **(hoofdstuk 7)**.

Op basis van alle resultaten in deze studie kunnen we concluderen dat een mesoscopsisch netwerk van wei-eiwitdeeltjes een veelbelovende nieuwe aanpak is om een toekomstige generatie glutenvrije producten te maken. Het resultaat is uniek, helemaal als men bedenkt dat slechts 2.4% (w/w) eiwit is gebruikt. Bovendien zijn verbeteringen mogelijk door een glutenvrij bloem te gebruiken in plaats van zetmeel en broodverbeteraars toe te voegen.

Het huidige productieproces van de eiwitdeeltjes is geschikt voor gebruik op industriële schaal wanneer de eiwitsuspensie direct na productie verder wordt verwerkt en wanneer een lage eiwitconcentratie kan worden gebruikt **(hoofdstuk 8)**.

Naast de relevantie voor de applicatie geeft deze studie ook wetenschappelijk inzicht in het gedrag van een suspensie van eiwitdeeltjes. Zo kan deze studie inzicht geven in de relatie tussen structuur en functie van het gluteneiwit.

Dankwoord

Acknowledgement

Dit is het dan ……, het resultaat van vier jaar werk in Wageningen. Een resultaat dat hier niet zonder slag of stoot voor je ligt. Net zo trots als ik ben op ons zelf verbouwde huis, ben ik dat ook op mijn zelf vervaardigde proefschrift. Naja zelf ……, voor beide waren mensen nodig die meer ervaring en deskundigheid hebben.

Remko en Atze Jan, jullie hebben me (samen met Julita) enthousiast gemaakt voor een AIO-baan. Bedankt voor jullie vertrouwen in mij. Atze Jan, ik heb onze samenwerking erg gewaardeerd en heb veel van je geleerd. Van experimentele ideeën en acute fase-scheiding tot time-management en begeleidingstips. Na onze discussies kon ik altijd weer vooruit. Remko, de bijeenkomsten met jou hebben me vaak gemotiveerd om op een andere manier naar mijn resultaten te kijken. Je creatieve ideeën en positieve houding hebben een belangrijke bijdrage geleverd aan dit proefschrift en mijn promotie. Rob en Harry, de inbreng van chemie was voor mij vaak verrassend, bedankt voor jullie goede ideeën. Rob, bedankt voor de vele discussies. Ik denk dat maar weinig promotoren zo betrokken zijn bij de artikelen van hun AIO's.

Meedenkende studenten hebben een positieve invloed op mijn promotie gehad. Het was opbouwend om samen met jullie verder te zoeken naar de oplossing. Xiao Li, Jeske, Nienke, Rikkert, Pascalle, hartelijk dank voor jullie enthousiaste inzet. Xiao Li, you was the first student who helped me in this project. You started your Bsc.-thesis a few months after I had started my Ph.D. Xiao Li investigated the effect of gluten in dough (from literature) and did some experiments with gluten and dough in our lab, to find a good experimental set-up. Jeske en Nienke, jullie hebben voor een hoop gezelligheid in het lab gezorgd. Twee studenten tegelijk begeleiden was voor mij dubbel inspannend, maar ook dubbel zo gezellig. Jullie hebben twee totaal andere opdrachten uitgevoerd binnen hetzelfde

eindproject. Jeske heeft onderzocht hoe afschuifstroming en zwavelbruggen de eiwitdeeltjes beïnvloeden. Nienke werkte aan de eerste deegjes met eiwitbollen. Lastig werk met dat kleefspul, maar met verrassende resultaten. Rikkert heeft gewerkt aan verdieping van het inzicht over faseschеidende eiwit-polysacharide systemen. Rikkert, succes met je Ph.D. project in Spanje. Pascalle heeft als eerste echt brood van eiwitbollen gebakken en het effect van wei-eiwit (in alle soorten) op deeg en brood onderzocht. Pascalle, ik vind het erg leuk dat je mijn collega bent geworden, succes met je Ph.D. project.

Ik wil mijn collega's van de food structuring groep bedanken voor de gezellige sfeer en de discussies. Jos, Julita, Cynthia, Edwin, Elsbeth, Nanik, Kasia, Vukasin, Nicolas, Lena and Atze Jan, thank you for all the good meetings. Julita, Edwin, Elsbeth, Nanik, Kasia, Vukasin and Atze Jan, thank you for the pleasant time in Zurich. Julita, jij hebt me laten zien dat AIO zijn echt niet zo saai is als gedacht wordt. Je was een super thesis begeleider, ik heb veel van je geleerd. Ook alle collega's van de gluten meetings en de protein meetings, bedankt voor jullie ideeën.

Natuurlijk wil ik mijn paranimfen, Elsbeth en Kasia bedanken. Elsbeth, in 2001 begonnen we allebei aan de opleiding levensmiddelentechnologie. We hebben allebei ons Bsc. project gedaan over friet bij PDQ en ons Msc. project bij Julita. En toen werd gevraagd of we AIO wilden worden … na heel veel mailtjes heen en weer besloten we allebei om AIO te worden … en nu is voor ons het einde inzicht. Gelukkig ben jij ook AIO geworden. Het was fijn om samen naar evenementen in Wageningen, Gent, Veldhoven, Leuven, Zurich en de US te gaan en onze frustraties en goede momenten te delen. Kasia, je bent al een aantal jaren mijn collega en sinds kort heb je ook je eigen AIO-project. Het is altijd plezierig om met jou samen te werken en je bent altijd even behulpzaam.

Dan zijn er nog heel veel onmisbare mensen voor een AIO, de deskundigen van chemicaliën, apparatuur en administratieve rompslomp. Jos, Maurice, Kasia, Gerrit, Sebastiaan, Fred, Rouke, Joyce, Miranda, Jolanda, Martin, Jan, Peter, Jolan, René, Edwin, Harry, Hans, Hans, Jan, Mees, Derk, Mark en Margaret, bedankt voor jullie hulp met de apparatuur en jullie suggesties tijdens discussies. Ook wil ik de experts van proceskunde, chemie en natuurkunde die mij geholpen hebben (Karin, Henk, Elke, Leonard, Paul en Yvonne) bedanken. De koffiepauzes, activiteiten, Aio-reizen en de meetings met de collega's waren altijd erg gezellig en goed. Ik wil daarom alle collega's van Food Chemistry en van Food Engineering bedanken voor de goede werksfeer. In het bijzonder wil ik mijn kamergenoten bedanken (Rudy, Yvonne, Koen, Evelien, Laura, Sayam, Hassan, Petra, Norhan, Daan, Jun en Maaike).

Ik ben blij dat mijn leven de afgelopen vier jaar niet alleen uit promoveren bestond. De vriendengroep uit Huizen, de oud-Ichthianen, de bijbel studiekring, de themacommissie en de kringgenootjes hebben voor de broodnodige momenten van ontspanning gezorgd. Al is het met sommige vrienden bijna onmogelijk om een datum te prikken, als we bij elkaar zijn, is het altijd ouderwets gezellig.

Natuurlijk wil ik ook mijn familie en schoonfamilie bedanken voor hun interesse en steun. Marloes, Gert en Elja, bij jullie was het altijd goed ontspannen, een wijntje een spelletje en lekker uitblazen. Elja, bedankt voor het lenen van je kralenframe voor de omslag van dit proefschrift. Pap en mam, bedankt dat jullie er altijd voor me zijn en dat ik altijd mijn verhaal bij jullie kwijt kan. Lieve Aart, jij bent er altijd voor mij. Ik ben enorm blij dat jij mijn metgezel voor het leven wilt zijn. Dank je dat je me altijd helpt met wat ik doe. Dank je voor je liefde.

Lieke

Training Activities

Curriculum Vitae

List of Publications

Overview of Completed Training Activities

Discipline specific courses

Industrial Proteins, VLAG	2006
Numerical Methods for Chemical Engineers, OSPT	2007
A Unified Approach to Mass Transfer, OSPT	2007
Rheological Measurements, KU-Leuven	2007
Polysaccharides as Food Colloids and Biomaterials: Fundamentals and Applications, VLAG	2007
Structure and Rheology of Cereal-Based Foods, AACC**	2010

General courses

VLAG-PhD week, VLAG	2007
The Art of Writing, Wageningen University	2007
Techniques for Writing and Presenting a Scientific Paper, WGS	2008
Organising and Supervising MSc Thesis Projects, Wageningen University	2008
Philosophy and Ethics of Food Science & Technology, WGS	2009
Scientific Writing, Wageningen University	2009
Career Perspectives, WGS	2010

Conferences

Food Texture and Rheology, NIZO food research, Ede	2007
Netherlands Process Technology Symposium, Veldhoven*	2008
The International Symposium on Food Rheology and Structure, Zurich**	2009
Delivery of Functionality in Complex Food Systems, Wageningen*	2009
AACC International Annual Meeting, Savannah**	2010

Other activities

Preparation PhD Research Proposal	2006
PhD-trip Food Chemistry (Belgium France and UK)	2006
PhD-trip Food & Bioprocess Engineering (Japan)	2008

Teaching

Practicum Assistant - Food Ingredients Functionality	2008-2009
Group Supervision - Case Studies Product Quality	2007
Preparation Case Study - Process Engineering	2007
Supervision BSc. Thesis (Xiao Li)	2007
Supervision MSc. Thesis (Jeske, Nienke, Rikkert, Pascalle)	2008-2010

* Poster Presentation

** Oral Presentation

Curriculum Vitae

Lieke Elizabeth van Riemsdijk was born in Huizen, The Netherlands, on December 6th 1981. She attended secondary school at the Erfgooiers College Huizen. She received a diploma for the Higher General Secondary Education in 1999 and a diploma for the Pre-university education in 2001. In the same year Lieke enrolled in the Food Science and Technology program at Wageningen University. She completed a Bsc.-thesis in Product Design and Quality Management (2004), and a Msc.-thesis in Food Process Engineering (2006). In the same year she conducted an internship at TNO, Zeist. In 2006 Lieke graduated from Wageningen University with a Msc. degree. From 2006 – 2010 she worked as a Ph.D. student in a combined project of the Laboratory of Food Chemistry and the Laboratory of Food Process Engineering.

List of Publications

van Riemsdijk, L. E., van der Goot, A. J. & Hamer, R. J. On the stability of whey protein particles during production of gluten-free bread. Food Hydrocolloids 2011 – submitted

van Riemsdijk, L. E., van der Goot, A. J., Boom, R. M. & Hamer, R. J. Preparation of gluten free bread using a meso-structured whey protein particle system. Journal of Cereal Science 2010 – submitted

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Research presented in this PhD dissertation was financially supported by the Graduate School VLAG.