

**Enzyme-induced aggregation of whey proteins with
Bacillus licheniformis protease**

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Nathalie Creusot

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ABSTRACT

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Keywords: whey proteins; *Bacillus licheniformis* protease; aggregation; hydrolysate; peptide identification; solubility

This study aimed at understanding the underlying mechanism of peptide-induced aggregation of whey protein hydrolysates made with *Bacillus licheniformis* protease (BLP). Peptide aggregation was shown to increase with the degree of hydrolysis up to the practical end point of hydrolysis (degree of hydrolysis –DH– of 6.8 %). The dominant aggregating peptide was identified as β -lg AB [f1-45]. In addition, the peptides β -lg AB [f90-108]-S-S- α -la [f50-113], α -la [f12-49]-S-S- α -la [f50-113], β -lg AB [f90-108]-S-S- β -lg AB [f90-108], β -lg A [f90-157] and β -lg AB [f135-157/158] were also identified as main aggregating peptides. To explain peptide aggregation, it was hypothesized that BLP breaks down hydrophilic segments in the substrate and, therefore, preserves hydrophobic segments that aggregate once exposed to the solvent. Aggregation during hydrolysis prevented further degradation of the substrate, explaining that aggregating peptides are larger than the non-aggregating ones. Solubility experiments performed on fractionated aggregating peptides showed that peptide co-aggregation is an important factor in the aggregation process.

In order to evaluate if aggregating peptides could aggregate intact proteins, hydrolysis was uncoupled from aggregation and aggregation was induced in the presence of added intact proteins. In this way the proportions of intact protein and peptides were not interrelated as it is in the case with total hydrolysates. Our results clearly showed that the hydrolysates are able to aggregate added parental protein. The additional amount of aggregated material increased with increasing DH. The aggregating peptides could form a network in which the presence of both insoluble and partly insoluble peptides was required for the aggregation of intact protein. In addition, it was shown that the aggregating peptides acted in a generic manner since the same peptides were involved in the aggregation of whey proteins, β -casein and bovine serum albumin.

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CHAPTER 1

General introduction

1.1 Introduction

Proteins in food products contribute to nutritional value, to sensory properties and to texture. Texture includes all rheological and structural attributes of a food product perceptible by means of mechanical, tactile, visual and auditive receptors. Texture comprises the formation of interfacial films, to stabilize foams and emulsions, and the structural networks present in gels, fibrils and edible films. Bovine whey proteins are widely used as ingredients to exert the properties mentioned above in foods such as drinks, desserts, meat and bakery products. One of the important functional properties of whey proteins is gelation. Gelation refers to the formation of a structural network that maintains shape, has mechanical strength, viscoelasticity and retains entrapped water with minimum syneresis (Kinsella and Whitehead, 1989). It is, therefore, important to be able to modulate the gelation properties of whey proteins to further extend their application as ingredients in foods. Proteolysis is a way to modulate the aggregation and thereby the gelation properties of whey proteins (see section 1.4). The present study aimed at investigating the mode of action of enzyme-induced aggregation of whey proteins.

1.2 Whey proteins

Whey proteins represent about 20% of the protein content in bovine milk and are defined as the proteins that stay soluble after acid casein precipitation (acid whey) or after rennet casein precipitation (sweet whey).

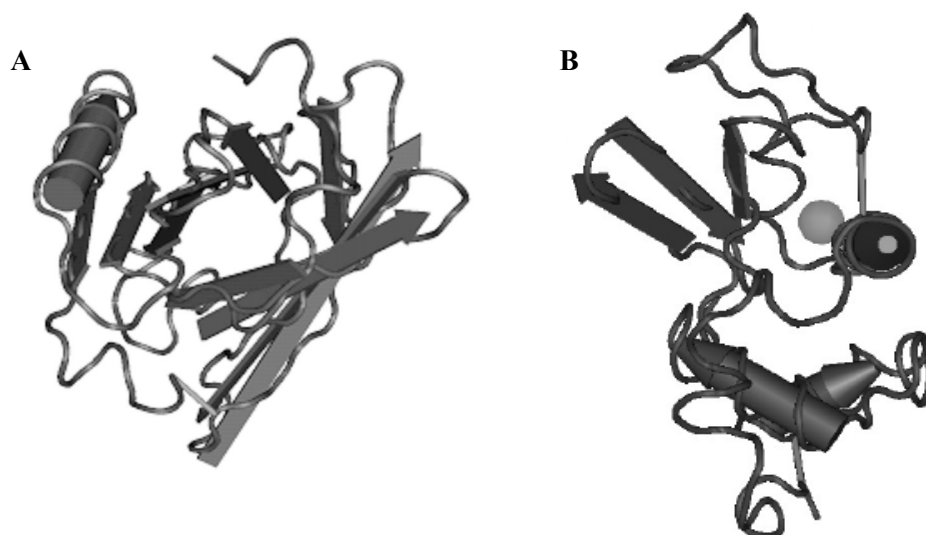


Figure 1.1. Ribbon drawings of the tertiary structures of A) bovine β -lactoglobulin (Protein Data Bank: 1BEB) and B) bovine α -lactalbumin (Protein Data Bank: 1F6S), as solved by X-ray crystallography. Arrows and cylinders represent sheets and helices, respectively. Structuring calcium in bovine α -lactalbumin is represented as a ball.

β -Lactoglobulin (β -lg) and α -lactalbumin (α -la) represent 70 to 80% of the protein content of bovine whey. β -Lg, the major protein in bovine whey, is a globular protein constituted by 162 amino acid residues resulting in a molar mass of 18.3 kDa and a pI of 5.2. It belongs to the lipocalin protein superfamily and contains nine strands of antiparallel β -sheets (**Figure 1.1A**). The protein contains two stabilizing disulfide bridges and a free thiol group. The denaturation temperature of β -lg at pH 7.0 is 70-73°C (Hoffmann et al., 1997). α -La is a globular protein constituted by 123 amino acid residues resulting in a molar mass of 14.4 kDa and a pI of 4.8. α -La has less secondary structure than β -lg, but has a tertiary structure (**Figure 1.1B**), due to presence of calcium ion and four disulfide bridges. The denaturation temperature of α -la at pH 7.0 is 64°C (Boye and Alli, 2000).

1.3 Whey proteins aggregation and gelation

The thermal induction of protein gels has been extensively described in literature. The mechanism of heat-induced protein gelation involves an initial unfolding of protein molecules, resulting in an exposure of hydrophobic amino acid residues. Subsequently, the unfolded molecules aggregate irreversibly via disulfide bridges, hydrogen bonds, hydrophobic and van der Waals interactions. Finally, aggregation continues with association of protein particles, and if the protein concentration is sufficiently high, a three dimensional network is created (Aguilera, 1995; Lefèvre and Subirade, 2000). β -Lg dominates the overall heat-induced aggregation and gelation behavior of whey proteins preparations (Mulvihill and Donavan, 1987).

Whey proteins gelation can be obtained not only by heat treatment. Actually, both heat and cold gelation processes of globular proteins exist. The cold gelation process refers to gelation occurring at ambient temperature (Alting, 2003). In addition, gelation can be obtained not only after an one-step process, but also after a two-step process (**Figure 1.2**). In an one-step process, whey proteins gelation can be obtained by heat, as described earlier, as well as by enzymatic treatment such as cross-linking with transglutaminase (Færgemand et al., 1997), by high hydrostatic pressure treatment (van Camp and Huyghebaert, 1995) and by enzymatic proteolysis (see after). In a two-step process, the first step consists of producing activated particles or pre-aggregates, usually by a heat treatment at a concentration lower than the gelation concentration threshold, but also by cross-linking proteins with transglutaminase, by proteolysis (see after) or by high hydrostatic pressure treatment (Dumay et al., 1994). Gelation is subsequently obtained in the second step upon a change of conditions such as acidification, addition of salts, addition of an enzyme, addition of a cross-linker, high hydrostatic pressure or heat treatment (Tang et al., 1995; Vardhanabhuti et al., 2001; McClements and Koegh, 1995; Alting, 2003). It should be noted that in situations where aggregation or gelation is induced by either protein cross-linking with transglutaminase or by proteolysis, the term “protein unfolding” cannot be used.

As described above and shown in **Figure 1.2**, whey proteins gelation can be obtained by a number of different process combinations. As a consequence this offers many possibilities to adapt the gelation process to all kinds of food products.

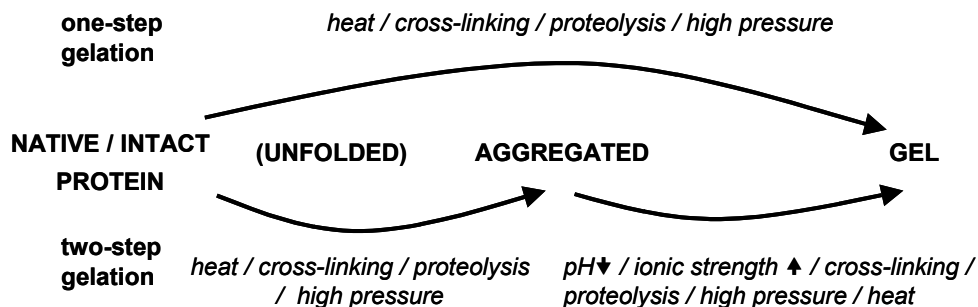


Figure 1.2. Conversion from native protein to network protein (gel) according to an one-step gelation process or a two-step gelation process.

In general, changes in aggregation and gelation conditions (pH, ionic strength, temperature, protein concentration) alter protein-protein and protein-solvent interactions by shifting the balance of attractive and repulsive forces (electrostatic, hydrophobic, and H-bonding). This ultimately affects the rates of (unfolding and) aggregation, resulting in formation of different gel structures (Huang et al., 1999). For example, coagulation (or clotting) occurs when the (unfolded) proteins randomly aggregate via non-specific protein-protein interactions (Campbell et al., 2003), whereas a fine-stranded gel is obtained when the (unfolded) proteins aggregate orderly via balanced protein-protein and protein-solvent interactions.

Proteolysis is a way to modulate gelation properties of globular proteins, since it was reported to have both gelation inhibiting and enhancing effects. The next section reports the effects of enzymatic hydrolysis on globular protein gelation.

1.4 Effects of enzymatic hydrolysis on globular protein gelation

Enzymatic modification of globular proteins has been of interest to the food industry for decades (Whitaker and Puigserver, 1982; Adler-Nissen, 1986; Panyam and Kilara, 1996; Foegeding et al., 2002) as proteolysis can be a tool to enhance their functional and nutritional properties. Depending on the extent of hydrolysis, environmental conditions, specificity and selectivity of the enzyme, a wide variety of peptides is usually generated.

One of the key parameters describing a protein hydrolysate is the degree of hydrolysis (DH). The DH is the extent to which peptide bonds are cleaved in the protein. The parameter expresses the number of peptide bonds cleaved (h) as a percentage of the total number of peptide bonds present in the intact protein (h_{tot}):

$$DH = \frac{h}{h_{tot}} \times 100\% \quad (h \text{ and } h_{tot} \text{ are expressed in meq g}^{-1} \text{ protein; Adler-Nissen, 1986}).$$

Specificity is a characteristic of enzyme catalysis since the reaction takes place in a particular region of the enzyme, the active site. The active site is able to recognize and bind to the substrate due to structural complementarities between amino acid residues in the substrate and amino acid residues in the vicinity of the active site. Proteases with high substrate specificity have sharp interaction requirements and, therefore, only recognize a narrow range of peptide bonds. Proteases with broad substrate specificity have flexible interaction requirements and, as a consequence, recognize a wider range of peptide bonds. In the protein substrate, structural complementarities with the enzyme is ensured by the amino acid residues that are at the carboxyl side of the cleaved peptide bond, denoted P_n , and these at the amino side of the cleaved peptide bond, denoted P'_n (**Figure 1.3**; Barrett et al., 1998).

In addition to protease specificity, protease selectivity is another important characteristic of enzyme catalysis since among all susceptible cleavable bonds some are preferentially split. This has to do again with structural complementarities between amino acid residues in the vicinity of the active site and amino acid residues in the surrounding of the cleavable peptide bonds.

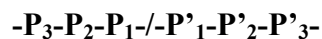


Figure 1.3. Denotation of amino acid residues at carboxyl (P_n) and amino (P'_n) sides of the cleaved amide bond (Barrett et al., 1998).

In most cases, hydrolysis leads to a decreased gelation ability or decreased gel strength because it reduces the molecular weight and increases the hydrophilicity of the proteinaceous material (Mahmoud, 1994; Nielsen, 1997). Indeed, hydrolysis generally improves protein solubility by increasing protein-water interaction as the cleavage of peptide bonds produces charged groups ($-\text{COO}^-$ and $-\text{NH}_3^+$). However, the effective hydrophobicity of certain globular proteins could be increased through exposure of buried apolar residues upon limited hydrolysis (Kester and Richardson, 1984).

Both inhibiting and enhancing effects of proteolysis on protein gelation have been reported in literature. The main substrates investigated were whey proteins and soy proteins. In soy protein studies, the term “coagulation” is often used to refer to enzyme-induced gelation. The following section, as well as **Table 1.1**, reports some examples of the effects of hydrolysis on protein gelation. Examples are classified as a function of the specificity, broad or narrow, of the proteases described.

Table1.1 Examples of effects of hydrolysis on protein gelation.

Source	Name	Organism	Specificity	DH (%)	Effect on gelation	Reference
Plant	Ficin	Ficus latex	Broad	c	Gelation soybean milk	Ayoama et al., 2000
Plant	Bromelain	Pineapple stem	Broad	c	Gelation soybean milk	Fuke et al., 1985
Plant	Papain	Papaya latex	P2: Large hydrophobic a.a. ^a	DH 23%	Gelation of heat denatured whey proteins	Sato et al., 1995
Bacterial	Alkaline protease	<i>Bacillus amyloliquefaciens</i>	P ¹ : Leu, Val, Phe	c	Gelation soy proteins	Murata et al., 1987
Bacterial	Alkaline protease	<i>Bacillus subtilis</i>	b	c	Gelation soy proteins	Murata et al., 1987
Bacterial	Alkaline protease	<i>Aspergillus oryzae</i>	b	c	Gelation soy proteins	Murata et al., 1987
Bacterial	Alkaline protease	<i>Aspergillus sojae</i>	b	c	Gelation soy proteins	Murata et al., 1987
Bacterial	Neutral protease	<i>Bacillus thermoproteolyticus</i>	P ¹ : Ile, Leu, Val, Phe	c	Gelation soy proteins	Murata et al., 1987
Bacterial	Alkaline protease	<i>Bacillus pumilus</i>	Broad	c	Gelation soy proteins	Ayoama et al., 2000
Bacterial	Subtilisin Carlsberg	<i>Bacillus licheniformis</i>	P1: Phe, Trp, Tyr, Met, Leu, Ala, Ser, Lys	c	Gelation soy proteins No gelation whey proteins	Inouye et al., 2002 Spellman et al., 2005
Bacterial	Alcalase 2.4L ®	<i>Bacillus licheniformis</i>	P1: Phe, Trp, Tyr, Glu, Met, Leu, Ala, Ser, Lys	DH>18%	Gelation whey proteins Gelation β-Ig	Doucet et al., 2001 Doucet et al., 2005
Bacterial	<i>Bacillus licheniformis</i> protease	<i>Bacillus licheniformis</i>	P1: Glu, Asp	DH 2.2% low	Gelation whey proteins Gelation α-Ia	Otte et al., 1996 Ipsen et al., 2001b
				low	Gelation β-Ig	Otte et al., 1997
				DH<1.8% low	Gelation whey proteins after heating Gelation β-Ig after heating	Ju et al., 1995 Otte et al., 2000b
Animal	Trypsin	Porcine, bovine	P1: Lys, Arg	c	No gelation whey proteins after heating	Ju et al., 1995, Hidalgo and Gamper, 1977
				low	Gelation β-Ig after heating	Chen et al., 1994
				DH 27%	Gelation of heat denatured whey proteins	Sato et al., 1995
				c	Gelation sunflower proteins after heating	Sanchez and Burgos, 1996

a Amino acid residue. b Not specified. c Not known.

Proteases with broad specificity

Proteases with a broad specificity like plant proteases such as ficin (Ayoama et al., 2000) and bromelain (Fuke et al., 1985) are capable of gelling soybean milk. Papain (preference for a residue bearing a large hydrophobic side chain in P₂) hydrolysis can also induce gelation of whey proteins, but only after they are partially heat denatured and during extensive hydrolysis up to DH 23 % (Sato et al., 1995).

Alkaline and neutral proteases from micro organisms such as *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Aspergillus oryzae*, *Aspergillus sojae*, *Bacillus thermoproteolyticus* (Murata et al., 1987) and *Bacillus pumillus* (Ayoama et al., 2000) could induce gelation of soy proteins. As well, Subtilisin Carlsberg (preference for a large uncharged residue in P₁), from *Bacillus licheniformis*, can induce gelation of soy proteins at pH 8.0 (Inouye et al., 2002). Kuipers and co-workers (Kuipers et al., 2005) further showed that acid-induced gelation of these hydrolysates was as well possible, after inhibiting the enzyme in order to uncouple the hydrolysis from the subsequent gelation.

Unlike soy proteins, enzyme-induced gelation of whey proteins with proteases from microorganisms was observed in only a few situations. Doucet and co-workers (Doucet et al., 2001) found that gelation occurred during extensive hydrolysis (DH>18%) of whey proteins with Alcalase 2.4L ®, a *Bacillus licheniformis* protease preparation, with Subtilisin Carlsberg as main proteolytic component. Gelation occurred at high concentration (20% w/v) and at pH 6.0. Doucet and co-workers (Doucet et al., 2003a) identified 130 peptides released when the gel was formed. These peptides had an average chain length of 4.3 residues, an average hydrophobicity of 1.0-1.5 kcal per residue, and a net charge of 0 at the pH at which gel formed (Doucet et al., 2003a). Gelation occurred by physical aggregation, mainly via hydrophobic interactions, with H-bonds and electrostatic interactions playing a minor role (Doucet et al., 2003b). Mainly the peptides released from β -lg associated into aggregates, and if the concentration was high enough (15% w/v), they formed a gel network (Doucet et al., 2005).

Proteases with narrow specificity

After partial hydrolysis of whey proteins with another enzyme preparation from *Bacillus licheniformis*, *Bacillus licheniformis* protease (BLP), that has a narrow specificity (specific for Glu and Asp residues in P₁), it has been shown that gels formed by heat treatment at 80°C of the hydrolysate, pH 7.0, are much stronger (until ten times) than gels made using intact whey proteins (Ju et al., 1995). In addition, whey proteins could form aggregates during proteolysis at temperatures (20 to 45°C) far below the thermal denaturation temperature of the intact proteins (Otte et al., 1996; Ju and Kilara, 1998). In fact, already only the process of hydrolysis of whey proteins by BLP, at neutral pH and 40°C, led to formation of a soft gel with a particulate microstructure (Otte et al., 1996).

The work of Spellman and co-workers (Spellman et al., 2005) further emphasized the contribution of enzyme specificity on enzyme-induced aggregation of whey proteins. As reported by Doucet and co-workers (Doucet et al., 2003a), glutamyl endopeptidase activity was present in Alcalase 2.4L®. After isolating subtilisin and glutamyl endopeptidase activities from Alcalase 2.4L®, Spellman and co-workers (Spellman et al., 2005) demonstrated that the glutamyl endopeptidase activity was responsible for the enzyme-induced peptide aggregation and that subtilisin activity did not induce aggregation of whey proteins. This work, therefore, linked the observations on enzyme-induced aggregation of Otte and co-workers and that of Doucet and co-workers, despite the fact that gelation of whey proteins occurred in one situation upon limited hydrolysis (with BLP) and in the other situation upon extensive hydrolysis (with Alcalase 2.4L®).

In addition, trypsin, another protease with narrow specificity, has been widely used to modify protein gelation properties. Trypsin (specific for Lys and Arg residues in P₁) hydrolysis usually improves the solubility of whey proteins (Hidalgo and Gamper, 1977) and inhibits their heat-induced gelation (Ju et al., 1995). However, it was possible to improve the heat-induced gelation of β -lg by the use of trypsin (Chen et al., 1994), since the resulting limited hydrolysate formed stronger gels at low gelling temperature (60°C) than intact β -lg. This was attributed to the weaker thermal stability of tryptic fragments in comparison to the intact protein. Sato and co-workers (Sato et al., 1995) have also shown that enzyme-induced gelation of partially heat-denatured whey proteins (at pH 7.0) occurred during extensive hydrolysis with trypsin. The DH at which gelation occurred was 27 %. However, as this value is not possible considering the amino acid sequence of the whey proteins and the specificity of trypsin (maximum DH~11%), presumably non-specific cleavage occurred. It was reported that hydrolysis of soy proteins with trypsin does not result in gelation (Murata et al., 1987). In a last example, trypsin proteolysis was successful in improving sunflower proteins gelation, for intact sunflower proteins do not gel during or after heating. Gelation was only possible at alkaline pH, where, if not hydrolyzed, sunflower proteins precipitated, due to strong protein-protein interactions, while the trypsin hydrolysate was more soluble (Sanchez and Burgos, 1996).

From the examples cited above and as schematically depicted in **Figure 1.4**, the enhancing or inhibiting effect of proteolysis on cold- and heat-induced gelation is a specific property of a hydrolysate since it is only observed within specific combinations of substrates, enzymes and degrees of hydrolysis. In addition, depending on the fact whether hydrolysis is limited or extensive and depending whether the substrate is native or not, it is assumed that different enhancing mechanisms are involved. It is clear that on the one hand, enzyme-induced aggregation and gelation of soy proteins can be obtained with many proteases derived from plant and micro organism sources, most of them non-specific. On the other hand, enzyme-induced aggregation and gelation of native whey proteins was, to our knowledge, only obtained with glutamyl endopeptidase activity. Gelation of whey proteins

was also obtained during extensive hydrolysis with non-specific proteases but after the substrate was partially heat-denatured.

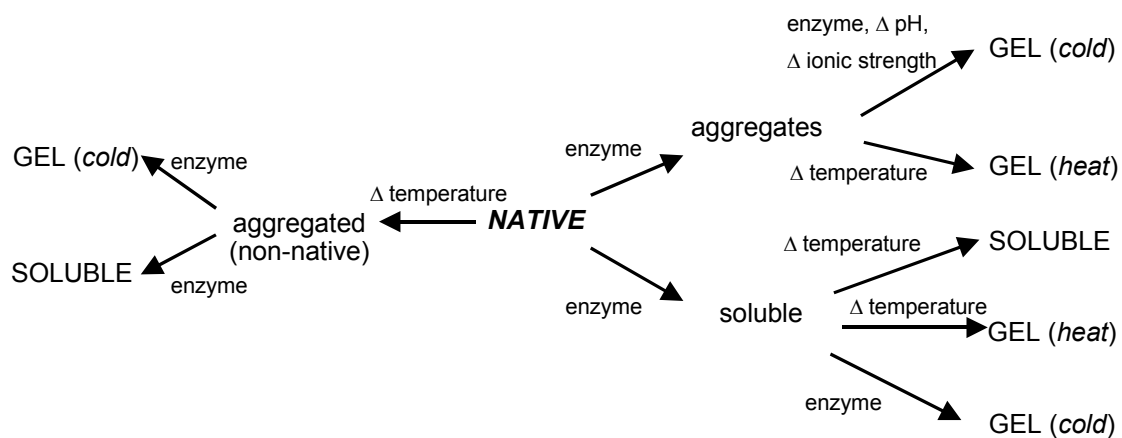


Figure 1.4 Enhancement and inhibition of cold- and heat-induced globular protein gelation using enzymatic hydrolysis. (We chose not to take the degree of hydrolysis into consideration for it would make the scheme more complex).

As enzyme-induced aggregation and gelation of whey proteins is rather specific, it is of interest to investigate the mechanism(s) behind this process. Our goal is to investigate the mode of action of the enhancement of whey proteins aggregation by hydrolysis with *Bacillus licheniformis* protease. The next section describes what is already known from investigations on this system.

1.4 Enzyme-induced aggregation and gelation of whey proteins with *Bacillus licheniformis* protease

1.4.1 *Bacillus licheniformis* protease

Bacillus licheniformis is a Gram-positive rod, belonging to the *B. subtilis* group of *Bacilli*. It is an apathogenic organism that can be isolated from soils and plant materials (Sneath, 1986). It is used for large-scale industrial production of enzymes (proteases and amylases) and the antibiotic bacitracin.

Bacillus licheniformis protease (BLP) is a glutamyl endopeptidase (EC-number 3.4.21.19) that belongs to the serine proteases class. It has narrow substrate specificity. BLP cleaves the peptide bonds formed by α -carboxyl groups of Glu and Asp residues, but is 100 to 1000 times more selective for the bonds formed by Glu residues than by Asp residues (Svendsen and Breddam, 1992; Breddam and Meldal, 1992). However, other cleavages were observed after prolonged hydrolysis at high enzyme concentrations (Svendsen

and Breddam, 1992), possibly caused by side activities. Glutamyl endopeptidases were isolated not only from *Bacillus licheniformis*, but also from *Staphylococcus aureus* V8 (Drapeau et al., 1972, 1977, 1978; Houmard and Drapeau, 1972), *Actinomyces* sp. (Mosolova et al., 1987), *Streptomyces thermovulgaris* (Khaidarova et al., 1989), *Streptomyces griseus* (Yoshida et al., 1988; Svendsen et al., 1991), *Bacillus intermedius* (Leshchinskaya et al., 1997), etc. Other names for glutamyl endopeptidases are BIEP, Blase, endoproteinase Glu-C, glutamic acid specific enzyme, GSE, protease V8, V8-GSE, etc (cf. BRENDA Database, <http://brenda.bc.uni-koeln.de/index.php4>).

The complete amino acid sequence of BLP is known and was established on the basis of the analysis of the nucleotide sequence of the cloned gene (Kakudo et al., 1992). It contains 222 amino acids (Kakudo et al., 1992) and has a calculated molecular mass of 23589 Da (Svendsen and Breddam, 1992). The presence of Ca^{2+} is necessary to stabilize the enzyme (Svendsen and Breddam, 1992). BLP is stable in the pH range 4.0-10.0 and exhibits an optimal activity at pH 7.5-8.0 (Svendsen and Breddam, 1992) and at a temperature of 50°C (Birktoft and Breddam, 1994). BLP is irreversibly inhibited by diisopropylfluorophosphate, which is inherent to the serine proteases, and is reversibly inhibited by EDTA.

1.4.2 BLP-induced aggregation of whey proteins

Otte and co-workers (Otte et al., 1996) showed that hydrolysis of whey proteins with BLP, at neutral pH and 40°C, led to formation of peptide aggregates, which ultimately formed a gel (**Figure 1.5**). Upon hydrolysis, aggregates of increasing size and/or concentration were formed, as shown with turbidimetry measurements (Otte et al., 1996). A similar type of aggregates was formed from a β -lg solution upon incubation with BLP (Otte et al., 1997), which further made a whitish and opaque gel. Madsen and co-workers (Madsen et al., 1997) reported that the sensitive bonds involving Asp and Glu residues in native β -lg were available for BLP. The resulting peptides had molecular masses between 2000 and 6000 Da and pI's ranging from 5.0 to 8.0 (Otte et al., 1997). As the aggregates were solubilized in sodium dodecyl sulphate, urea or extreme pH values, non-covalent interactions, mainly electrostatic and hydrophobic, were reported to be the major interacting forces (Otte et al., 1997). The aggregates formed upon hydrolysis of β -lg consisted of 6 to 7 major peptides, of which 4 were identified (β -lg [f135-157], β -lg [f135-158], β -lg [f75-108], and β -lg [f75-89]; Otte et al., 2000a). The peptides were positively charged at neutral pH and had a high charge-to-mass ratio at low pH. Otte and co-workers (Otte et al., 2000a) proposed the fragment β -lg [f135-158] to be the initiator of aggregation. This fragment contains several basic and acidic amino acid residues alternating with hydrophobic residues, which would be in accordance with formation of non-covalently linked aggregates.

1.4.3 Cold-induced gelation of BLP hydrolysates

According to Otte and co-workers (Otte et al., 1999), a whey proteins solution with a concentration as low as 2% was able to gel upon hydrolysis at pH 7.0 and at 50°C. Increasing the substrate concentration led to a higher rate of gelation and a higher final gel strength. The hardness of the gels induced by BLP did not vary with the size of the aggregates (Ju and Kilara, 1998). A soft gel was formed at temperatures between 40 and 60°C. Increasing the temperature reduced the time for gelation (Otte et al., 1999) and the higher gel strength observed at high hydrolysis temperature could be due to more SS/S_H interchange reactions taking place at high temperature.

Cold gelation of the hydrolysates could also be induced by salt addition or acidification (**Figure 1.5**; Ju and Kilara 1998). Addition of 200 mM NaCl or 40 mM CaCl₂ or glucono- δ -lactone (GDL) to a hydrolysate with a protein/peptide concentration of 18%, at DH 2.0%, led to gels that were 1.5, 2 and 3 times stronger than those without additives, respectively.

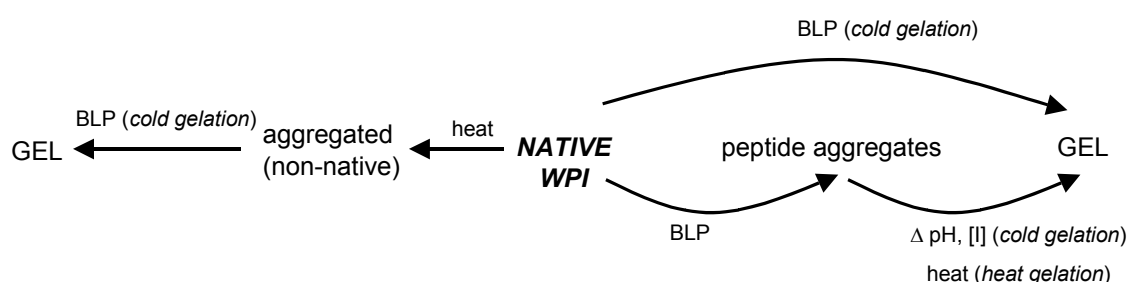


Figure 1.5. Cold- and heat-induced gelation of whey proteins hydrolysates made with BLP.

Despite changes in protein concentration, gelation always took place at a constant extent of proteolysis, namely DH 2.2% (Ju and Kilara, 1998). But, as reported by Ipsen and co-workers (Ipsen et al., 2000), gelation took also place at a higher DH when the enzyme concentration was increased, indicating that gelation was not a function of DH alone, but both DH and aggregation influenced the rate of gelation. The latter authors also suggested that aggregation of the peptides was the rate-limiting step for gelation. A proposed mechanism for the BLP-induced gelation was that hydrophobic peptides released from β -lg hydrolysis had more possibilities for hydrophobic interactions than the intact protein, causing them to aggregate into particles that finally constitute a gel network (Ipsen et al., 2000).

Subjecting solutions of whey proteins to heating before adding the BLP (**Figure 1.5**) had a strong influence on gelation since it led to a lower gel time, a higher rate of gelation and a major increase in the gel strength (Otte et al., 1999). Furthermore, it changed the microstructure and the appearance of the gel (Ju et al., 1997). Gels formed from the non-denatured whey proteins consisted of small aggregates ($\approx 0.1\mu\text{m}$) formed during hydrolysis, whereas gels from the denatured whey proteins were fine-stranded and thus similar to heat-set gels obtained from intact whey proteins or β -lg (Ju et al., 1997). The gelation mechanism

could be described by assuming the enzyme could shave off stabilizing peptide segments from the thermally induced aggregates, resulting in increased attraction between aggregates that led to gelation (Otte et al., 1999, Ipsen et al., 2000).

As β -lg is the main protein in whey and because it formed aggregates upon hydrolysis with BLP, it was first thought that β -lg was responsible for what was observed with whey proteins. But when α -la (around 20 % of the whey proteins) was hydrolyzed with BLP, aggregation and gelation were also observed. Gels made with α -la during hydrolysis with BLP were almost transparent and were more than 20 times stiffer than gels made from β -lg at the same (10 %, w/v) concentration (Ipsen et al., 2001b). The gels consisted of strands of a uniform diameter (around 20 nm) similar to that of microtubules. The presence of Ca^{2+} was necessary to induce gel formation and influenced the gel microstructure. The gelation mechanism could involve the self-assembly of partially hydrolyzed α -la into tubes via ionic bonds between Ca^{2+} and carboxylic acid groups at the C-terminal position of the peptides created by the action of BLP (Ipsen et al., 2001b).

1.4.4 Heat-induced gelation of BLP hydrolysates

The minimum protein concentration for heat-induced gelation at pH 7.0 and without salts added (80°C, 30 min, pH 7.0) is about 9% (w/v) for whey proteins (Ju et al., 1997). Formation of enzyme-induced aggregates improved the thermal gelation ability (at 80°C) of whey proteins, since it lowered the critical gelling protein concentration from 9 to 1% (Ju and Kilara, 1998). The gels formed at a concentration of 12%, at pH 7.0 and at low DH (~1%) were around ten times harder than those of intact whey proteins, (Ju et al., 1995; **Figure 1.5**). In addition, the gel hardness continuously decreased with increased aggregate sizes and the amount of aggregates (Ju and Kilara, 1998). Indeed, the hardest thermal gels were formed when the solutions were the least hydrolyzed (Ju and Kilara, 1998).

The protein composition and/or the conditions of hydrolysis and gelation can influence the gel strengthening effect of partial hydrolysis (Otte et al., 2000b) since the maximal (heat) gel stiffening effect obtained after partial hydrolysis on β -lg with BLP was 1.5-fold that of gels made with intact β -lg (Otte et al., 2000b). This was much lower than the stiffening effect obtained with a mixture of whey proteins as substrate.

Otte and co-workers (Otte et al., 2000b) presented a mechanism to explain the observed effects of hydrolysis on heat-induced gelation of β -lg and gel properties. The hydrophobic peptides created upon hydrolysis associated into aggregates with or without the hydrophobic parts of intact β -lg molecules exposed during heating to form the protein strands that ultimately formed the gel network. Disulfide bonds could help such mixed strands, since thiol-disulfide exchange reactions are favored at pH 7.5 (Caessens et al., 1999) and elevated temperature during gelation (Otte et al., 2000b). The negatively charged hydrophilic peptides that are created would either associate with oppositely charged areas of the β -lg molecules via electrostatic interactions or be released as soluble peptides.

1.6 Aim of the study

It was shown above that enzyme-induced aggregation of whey proteins with *Bacillus licheniformis* protease is rather specific. The underlying mechanisms of the increased functionality of partial hydrolyzed whey proteins preparations are, however, still not understood, although several attempts have been done to provide mechanism(s) behind this process. The role of intact protein in aggregation is still not clear. At the DH at which gelation occurs, there are still intact protein molecules present in the hydrolysates (**Figure 1.6**). It is hypothesized that intact proteins participate in aggregation, so that protein-peptide interactions further contribute to a homogeneous gel network.

This study aims at understanding the mechanism of peptide-induced aggregation of whey proteins hydrolysates made with *Bacillus licheniformis* protease. Next, in order to evaluate if aggregating peptides could aggregate intact proteins, it was decided to uncouple hydrolysis from aggregation and to induce aggregation in presence of added intact proteins (**Figure 1.6**).

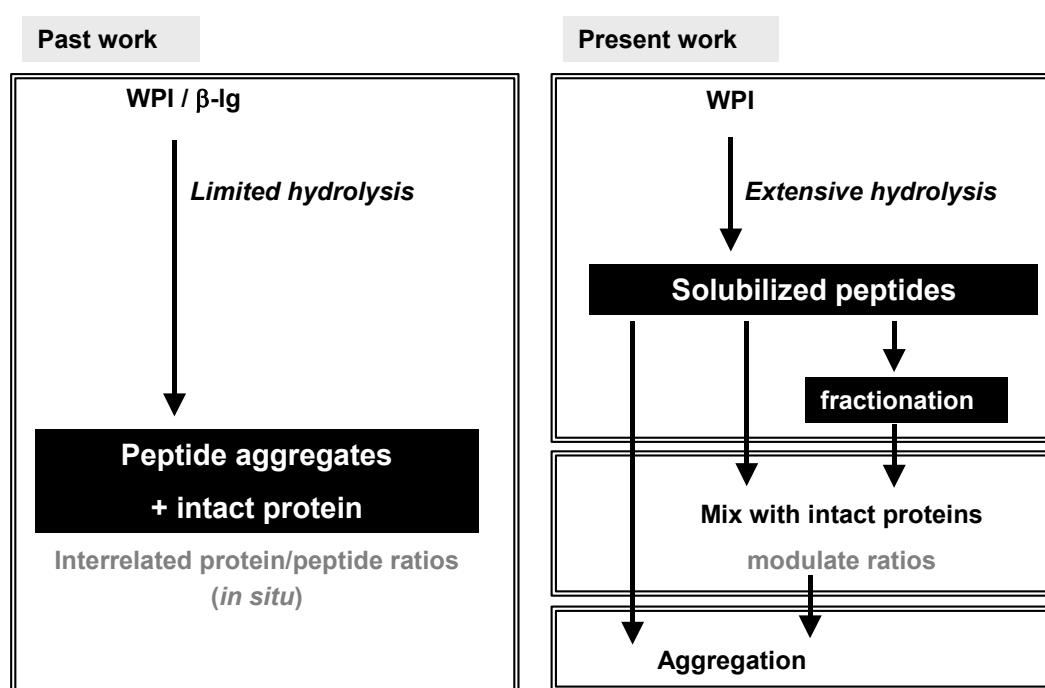


Figure 1.6. Approaches to study enzyme-induced aggregation of whey proteins with BLP.

1.7 Outline of the thesis

In **Chapter 2** the extent of aggregation in hydrolysates of whey protein isolate induced by *Bacillus licheniformis* protease (BLP) was quantified as a function of degree of

hydrolysis, temperature and ionic strength. In addition, the capacity of the hydrolysates to aggregate added intact protein was studied.

In **Chapter 3**, the dominant peptides that have the capability to aggregate intact whey proteins were identified. The underlying protein-peptide interactions leading to aggregation in mixtures of whey protein hydrolysate, made with *Bacillus licheniformis* protease, and whey protein isolate were investigated.

Chapter 4 describes the fractionation and the identification of the dominant aggregating peptides from a whey protein hydrolysate (degree of hydrolysis of 6.8%) obtained with *Bacillus licheniformis* protease.

The solubility behavior of the fractionated aggregating peptides, as a function of concentration, and their aggregating capacities towards added intact proteins was studied on **Chapter 5**.

Finally, a general discussion on the work presented in this thesis is given in **Chapter 6**.

CHAPTER 2

Peptide-peptide and protein-peptide interactions in mixtures of whey protein isolate and whey protein isolate hydrolysates

Based on:

Creusot, N., H. Gruppen, G. A. van Koningsveld, C. G. de Kruif, and A. G. J. Voragen. Peptide-peptide and protein-peptide interactions in mixtures of whey protein isolate and whey protein isolate hydrolysate. *International Dairy Journal*. **2006**, 16, 840-849.

Abstract

The extent of aggregation in hydrolysates of whey protein isolate (WPI) induced by *Bacillus licheniformis* protease (BLP) was quantified as a function of degree of hydrolysis (DH), temperature and ionic strength. In addition, the capacity of the hydrolysates to aggregate added intact protein was studied. The amount of aggregated material and the size of the aggregating peptides were measured by nitrogen content and size exclusion chromatography, respectively.

Aggregation was shown to increase with DH up to the practical end point of hydrolysis (DH 6.8 %). The aggregates formed at the various conditions consisted of peptides with masses ranging from 1.4 to 7.5 kDa. The hydrolysates were also able to aggregate added WPI. The additional amount of aggregated material increased with increasing DH. Peptides involved in peptide-peptide interactions were also involved in protein-peptide interactions. It is hypothesized that hydrophobic interactions dominated peptide-peptide interactions, while protein-peptide interactions depended on the balance between hydrophobic attraction and electrostatic repulsions.

Keywords: *Bacillus licheniformis* protease; aggregation; whey proteins; peptides

2.1 Introduction

Whey proteins are widely used to improve the texture of food products. The ability to form gels is one of the important functional properties of whey proteins. The general mechanism of protein gelation involves an initial unfolding of protein molecules that subsequently leads to aggregation via various interaction forces (disulfide bridges, electrostatic attractions, hydrogen bonds, hydrophobic and/or Van der Waals interactions). Aggregation then proceeds with further association among protein particles thus creating a three-dimensional network.

The gelling properties of proteins can be modified in various ways, chemically as well as physically, but also via enzymatic hydrolysis. Enzymatic hydrolysis of proteins causes a decrease in molecular weight, an increase in the number of ionisable groups, and an increased exposure of hydrophobic groups (Panyam and Kilara, 1996). In most cases, the propensity of the hydrolyzed protein to form a gel is less than that of the intact protein (Kester and Richardson, 1984).

Some cases of improved enzyme-induced gelation of whey proteins have been reported in literature. Doucet and co-workers (Doucet et al., 2001) have shown that enzyme-induced aggregation and gelation occurs during extensive hydrolysis of whey protein isolate (WPI) with Alcalase 2.4L[®] (an enzyme preparation derived from *Bacillus licheniformis*). Hydrophobic interactions between peptides, with a molecular mass less than 2 kDa, were observed to be involved in the formation of aggregates that associated further to form a gel (Doucet et al., 2003a). Also, it has been shown that partial hydrolysis of WPI with a seryl protease from *Bacillus licheniformis* (BLP) may lead to the formation of enzyme-induced aggregates that eventually form a gel (Otte et al., 1996). A similar type of aggregate is formed when β -lactoglobulin (β -lg), the most abundant protein in bovine whey, is hydrolyzed with BLP (Otte et al., 1997), suggesting that β -lg is mainly responsible for the effects observed with WPI. Aggregates formed during hydrolysis of β -lg with BLP consist of peptides of intermediate size (2 – 6 kDa) held together by mainly hydrophobic interactions (Otte et al., 1997). The aggregates are made up of 6 to 7 major peptides, of which 4 have been identified (Otte et al., 2000). Hydrolysis of α -lactalbumin, the second major protein in bovine whey, with BLP also leads to the formation of aggregates and under certain conditions to so-called nanotubules (Otte et al., 2004; Graveland-Bikker et al., 2004).

The conditions (degree of hydrolysis -DH-, pH, ionic strength, temperature) that favor peptide-peptide interactions, or even more interestingly, the interactions between peptides and intact proteins, have not been studied in detail. Information on this could help understanding gelation mechanism in protein hydrolysates. In addition, previous studies have shown the ability of β -lg to interact with peptides (Barbeau et al., 1996; Noiseux et al., 2002).

In the present work, the extent of aggregation in hydrolysates of WPI made with BLP was quantified as a function of DH, temperature and ionic strength. The aggregates formed were analyzed with respect to peptide composition. Furthermore, the capacity of the hydrolysates to aggregate added intact protein was studied using mixing experiments with intact WPI. The composition of the resulting aggregates (peptides and intact protein) was also investigated.

2.2 Materials and methods

2.2.1. Materials

A commercial whey protein isolate (WPI) powder (trade name Bipro, Davisco Foods International Inc., Le Sueur, MN, USA) was used for the experiments. According to the manufacturer, it consisted of 74.0 % (w/w) β -lactoglobulin (β -lg), 12.5 % (w/w) α -lactalbumin (α -la), 5.5 % (w/w) bovine serum albumin, and 5.5 % (w/w) immunoglobulins. The protein content of the powder was 93.4 % (w/w) and it contained 0.12 % (w/w) calcium. The enzyme used was a seryl proteinase from *Bacillus licheniformis* (BLP, product name NS-46007, batch PPA 6219; E.C. 3.4.21.19), specific for Glu-X bonds and to a lesser extent for Asp-X bonds (Breddam and Meldal, 1992). The enzyme was kindly provided by Novozymes (Novozymes A/S, Bagsvaerd, Denmark).

All reagents were of analytical grade and purchased from Sigma (Sigma Chemical CO, St Louis, USA), Merck (Darmstadt, Germany) or Roche (Roche Diagnostics, Almere, The Netherlands).

2.2.2. Hydrolysis of WPI

WPI was dispersed in Millipore water at a concentration of 120 mg g⁻¹ and stirred overnight at 4°C. The suspension was centrifuged for 30 min (19000 x g, 20°C) and the supernatant was subsequently filtered (Rezist 30GF92 and PVDF 0.45 μ m, Schleicher and Schuell GmbH, Dassel, Germany). The supernatant was diluted to 50 mg g⁻¹, preheated at 40°C for 15 min and the pH was adjusted to pH 8.0, 5 min before addition of the enzyme solution. The enzyme/substrate ratios (E/S) v/v used were 1/1250, 1/500, 1/192 and 1/100 to reach DH values of 1.8, 3.2, 5.3 and 6.8 %, respectively. During hydrolysis, the reaction mixture, incubated at 40°C, was maintained at pH 8.0 by addition of a 0.4 M NaOH solution in a pH-STAT (719 S Titrino, Metrohm Ion Analysis, Metrohm Ltd., Herisau, Switzerland). Incubation times were between 50 and 180 min. To permanently inactivate the enzymatic reaction of BLP (as checked with size exclusion chromatography), the pH was adjusted to pH 2.0 with a 6 M HCl solution. A solution of WPI incubated under the same conditions as

during the longest hydrolysis incubation and containing heat-inactivated (15 min, 95°C) enzyme (E/S 1/100, v/v) served as control.

Nitrogen contents of the hydrolysates were measured using the combustion or Dumas method (AOAC, 1995) with a NA 2100 Protein nitrogen analyzer (CE Instruments, Milan, Italy). A 6.38 x N conversion factor was used to convert nitrogen content to protein content. The hydrolysates were stored at 4°C before further analysis.

2.2.3. Aggregate formation in the WPI hydrolysates

WPI hydrolysates (DH 3.2 %, 5.3 % and 6.8 %) and the WPI control solution, containing 10 mg of proteinaceous material were adjusted to pH 7.0 with 0.25-2 M NaOH solutions and to ionic strengths of 0.020, 0.075 and 0.200 M with a 2 M NaCl solution. The total mass of each sample was set to 1 g with Millipore water. Each sample was divided into two parts. After incubation for 1 h at 20, 40 or 60°C, one part was centrifuged (20 min at 19000 x g, at 20°C) while the other part was not centrifuged. In order to determine the extent of aggregation, the nitrogen content (N) of the supernatant and the non-centrifuged hydrolysate (N_0) were determined using the Dumas method. The proportion of aggregation was defined as $(1 - N/N_0) \times 100$ %. Aggregated material was defined as the material removed using the centrifugation and solvent conditions applied. The experiment was performed in duplicate.

The pellets, containing the aggregates, were washed twice with 0.020, 0.075 or 0.200 M NaCl solutions at 20, 40 or 60°C and analyzed by size exclusion chromatography.

2.2.4. Mixing of hydrolysates with intact whey protein isolate

For mixing of hydrolysates with intact whey proteins, the procedure was essentially identical to that described above for analysis of aggregate formation, with the exception that the samples (DH 3.2%, 5.3% and 6.8 %) and the WPI control solution were first mixed with samples containing 0, 5, 10, 20, 30, 40 and 50 mg of intact WPI solution (pH 7.0). After pH and ionic strength adjustment and mass setting to 1 g with Millipore water (as described in the previous section), a final hydrolysate concentration of 10 mg g⁻¹ and WPI concentrations of 0, 5, 10, 20, 30, 40 and 50 mg g⁻¹ were obtained. All further processing was as described in the previous section.

2.2.5. Size exclusion chromatography

Experiments were performed with an Äkta Purifier System (Amersham, Pharmacia Biotech, Uppsala, Sweden). Hydrolysis products were separated with an analytical Shodex Protein KW-802.5 column (300 x 8 mm; particle size: 7 µm; pore size: 500 Å; Showa Denko K. K., Kanagawa, Japan). The column was equilibrated with 6 M urea containing 30 % (v/v) acetonitrile and 0.1 % (v/v) trifluoroacetic acid (TFA). The flow rate was 0.5 mL min⁻¹ and

the column temperature 20°C. A volume of 20 µL sample was injected onto the column. Detection was performed at 220 nm.

Sample preparation for analysis was as follows. Hydrolysates were mixed with 0.05 M Tris-HCl buffer containing 0.1 M dithiothreitol (DTT) and 8 M guanidinium hydrochloride (pH 8.0), in order to reach a protein concentration of 4.3 mg mL⁻¹ and a guanidinium hydrochloride concentration of 7.2 M. After 2 h of incubation at room temperature, TFA and acetonitrile were added to reach final concentrations of 0.7 % (v/v) and 30 % (v/v), respectively. Final protein concentrations were 3 mg mL⁻¹ and final guanidinium hydrochloride concentration was 5 M. Hydrolysate samples were also prepared under non-reducing conditions using the same sample preparation as above, without using Tris-HCl buffer and DTT.

Washed pellets were dissolved in 500 µL 8 M guanidinium hydrochloride. Then, a volume of 150 µL of dissolved pellet was mixed with 370 µL of 0.05 M Tris-HCl buffer containing 0.1 M DTT, 8 M guanidinium hydrochloride, pH 8.0. After 2 h of incubation at room temperature, 6 µL of TFA and 225 µL of acetonitrile were added. The pellets were also dissolved under non-reducing conditions using the same sample preparation as above, without using Tris-HCl buffer and DTT.

The areas of the peaks of the peptide fractions present in the DH 6.8 % hydrolysate, under reducing conditions, were calculated using the program Peak Fit (SPSS Inc., Chicago, IL, USA). The masses of the peptides were estimated using a calibration curve.

The column was calibrated using a low molecular weight gel filtration calibration kit (Amersham). The kit contained blue dextran (2000 kDa), aldolase (42 kDa), ribonuclease A (13.7 kDa). In addition, aprotinin (6.5 kDa), insulin chain B (3.5 kDa), angiotensin I (1.296 kDa), bradykinin (1.06 kDa) (Sigma, product numbers A-6012, I-6383, A-9650 and B-3259, respectively) and leupeptin (0.426 kDa, Roche, product number 1017101) were used for calibration purposes.

2.3 Results

2.3.1. Hydrolysis of whey protein isolate

WPI hydrolysates made with BLP were prepared to various degrees of hydrolysis (DH 0 – 6.8 %). The size distribution in these hydrolysates, as analyzed with size exclusion chromatography under reducing conditions, is shown in **Figure 2.1A**. From the peak areas of intact β-Ig and α-La as a function of DH, it can be seen that β-Ig was readily digested by BLP; only 8 % of intact β-Ig was left at DH 3.2 %. α-La was not degraded below DH 1.8 %, while at DH 6.8 % its intact form was still present. Therefore, BLP had preference for certain proteins present in WPI, as was already observed by Ipsen and co-workers (Ipsen et al.,

2001a). At low DH (DH 1.8 %), the fragments eluted at 7.8, 8.8 and 10.5 mL. These fragments presumably originated from β -lg, since α -la was hardly digested at DH 1.8 %. From DH 1.8 to 6.8 %, an increase in the amount of peptides eluting between 9.0 and 10.8 mL was observed.

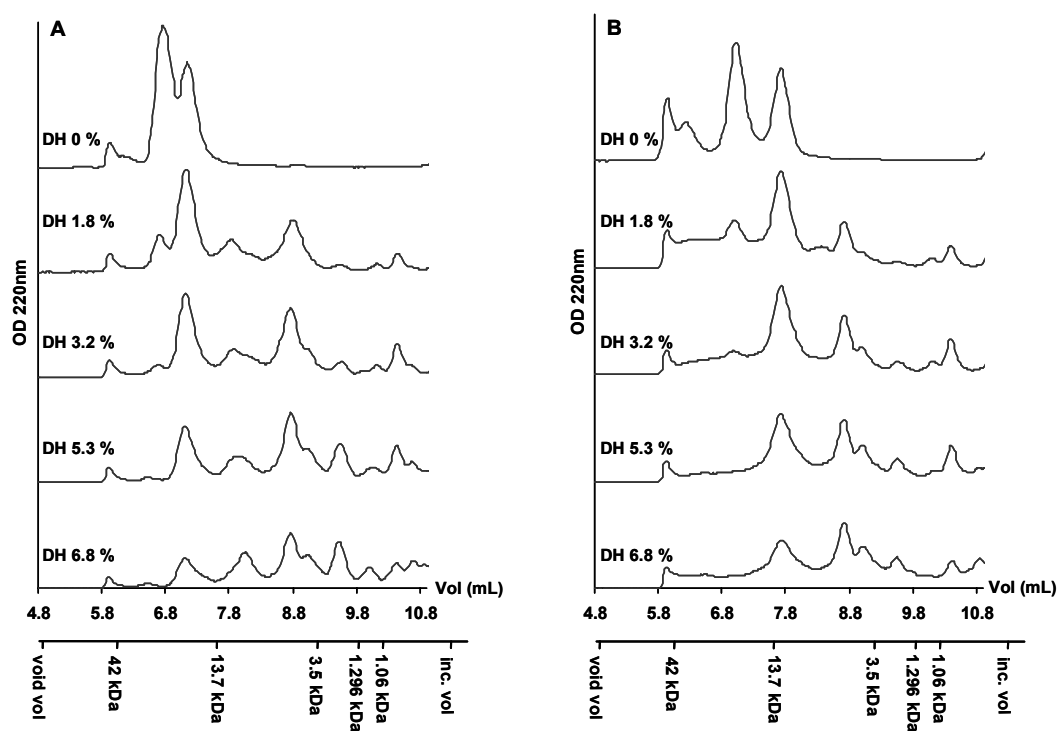


Figure 2.1. Size exclusion chromatograms of the WPI hydrolysates made with *Bacillus licheniformis* protease (DH 0, 1.8, 3.2, 5.3 and 6.8 %) under reducing (A) and non-reducing conditions (B). Ig = immunoglobulins; BSA = bovine serum albumin; β -lg = β -lactoglobulin; α -la = α -lactalbumin; inc. vol = included volume.

Digestion of the substrate became slower when approaching DH 6.8 %, indicating that this DH was close to the end point of hydrolysis. In the size exclusion pattern, under reducing conditions, eight peptide fractions can be distinguished, in the DH 6.8 % hydrolysate, eluting at 8.0, 8.8, 9.0, 9.5, 10.0, 10.5, 10.7 and 10.8 mL, respectively. The number average mass of the peptides present in the DH 6.8 % hydrolysate was estimated to be 2068 Da (about 18 amino acid residues), which is in reasonable agreement with the theoretical average mass of the peptides of 1530 Da (13.5 amino acid residues) at this DH. Assuming that BLP cleaves after Glu residues and considering that the WPI is composed of β -lg, α -la and BSA only, the maximum theoretical attainable DH is 9.7 %. Steric hindrances (presence of cystine residue in C-terminal position of Glu residue) and electrostatic hindrances (neighbouring of other Glu or Asp residues) could influence the selectivity of the enzyme for the substrate. Taking into account these hindrances, a maximum DH of 7.0 % was to be expected.

Figure 2.1B shows the size exclusion patterns of the hydrolysates under non-reducing conditions. From the comparison of the DH 6.8 % chromatograms, under reducing (**Figure 2.1A**) and non-reducing conditions (**Figure 2.1B**), it might be deduced that the peptide fractions eluting at 8.8, 9.0, 10.5 and 10.8 mL under both reducing and non-reducing conditions originated from peptides devoid of disulfide bridges. The peptide fractions eluting at 8.0 and 10.0 mL were present in the pattern under reducing conditions and were, therefore, assumed to originate from disulfide bridged peptides, as they were not present in the pattern obtained under non-reducing conditions. A significant decrease in the area of the peaks eluting at 9.5 and 10.7 mL when going from reducing to non-reducing conditions indicated that these peaks contained both disulfide-bridged peptides and non-disulfide bridged peptides co-eluting under non-reducing conditions.

2.3.2. Aggregate formation in the hydrolysates: effects of DH, temperature and ionic strength

The proportion of aggregation within the hydrolysates, at pH 7.0, as a function of DH (3.2, 5.3 and 6.8 %), temperature (20, 40 and 60°C) and ionic strength (0.020, 0.075 and 0.200 M) is shown in **Table 2.1**.

Table 2.1. Proportion of aggregation in WPI hydrolysates as a function of the degree of hydrolysis (at pH 7.0) at temperatures of 20°C, 40°C and 60°C, and at ionic strengths of 0.020 M, 0.075 M and 0.200 M. Values are means for duplicates \pm standard deviation.

Temperature (°)	Ionic strength (M)	Aggregation (%) ^a		
		DH 3.2%	DH 5.3%	DH 6.8%
20	0.020	7.87 \pm 5.11	37.7 \pm 3.07	49.1 \pm 2.98
	0.075	13.0 \pm 7.18	36.17 \pm 4.80	46.7 \pm 4.94
	0.200	17.67 \pm 0.83	33.47 \pm 5.35	45.0 \pm 4.50
40	0.020	12.23 \pm 6.04	38.9 \pm 4.54	46.8 \pm 5.04
	0.075	28.0 \pm 5.48	40.2 \pm 5.44	45.7 \pm 5.20
	0.200	28.83 \pm 2.79	39.53 \pm 2.94	46.85 \pm 5.03
60	0.020	19.6 \pm 7.7	42.83 \pm 2.65	45.75 \pm 6.39
	0.075	31.63 \pm 3.81	43.5 \pm 3.09	44.75 \pm 5.78
	0.200	30.97 \pm 5.13	43.57 \pm 3.74	46.85 \pm 4.43

^aNo aggregation was obtained at DH 0 and 1.8%.

The proportion of aggregated material at pH 7.0 increased with increasing the DH. It reached approximately 45 % of the material present, at DH 6.8 %. The proportion of aggregation also varied with ionic strength and temperature. Aggregation was higher at higher temperature and at higher ionic strength. However, the influence of both decreased with increasing DH and was even absent at DH 6.8 %.

In order to investigate which peptide fractions were responsible for aggregation, the composition of the pellets was determined with size exclusion chromatography, under both reducing and non-reducing conditions. **Figure 2.2** shows the chromatograms of aggregates obtained at DH 3.2, 5.3 and 6.8 %, respectively, at an ionic strength of 0.075 M and at temperatures of 40 and 60°C, together with the parental material. The chromatograms of the aggregates obtained at 20°C were also recorded, but not shown, as they completely resembled the chromatograms of the aggregates obtained at 40°C. The chromatograms of the aggregates were normalized to the same total area in order to emphasize compositional differences between the aggregates formed at various conditions.

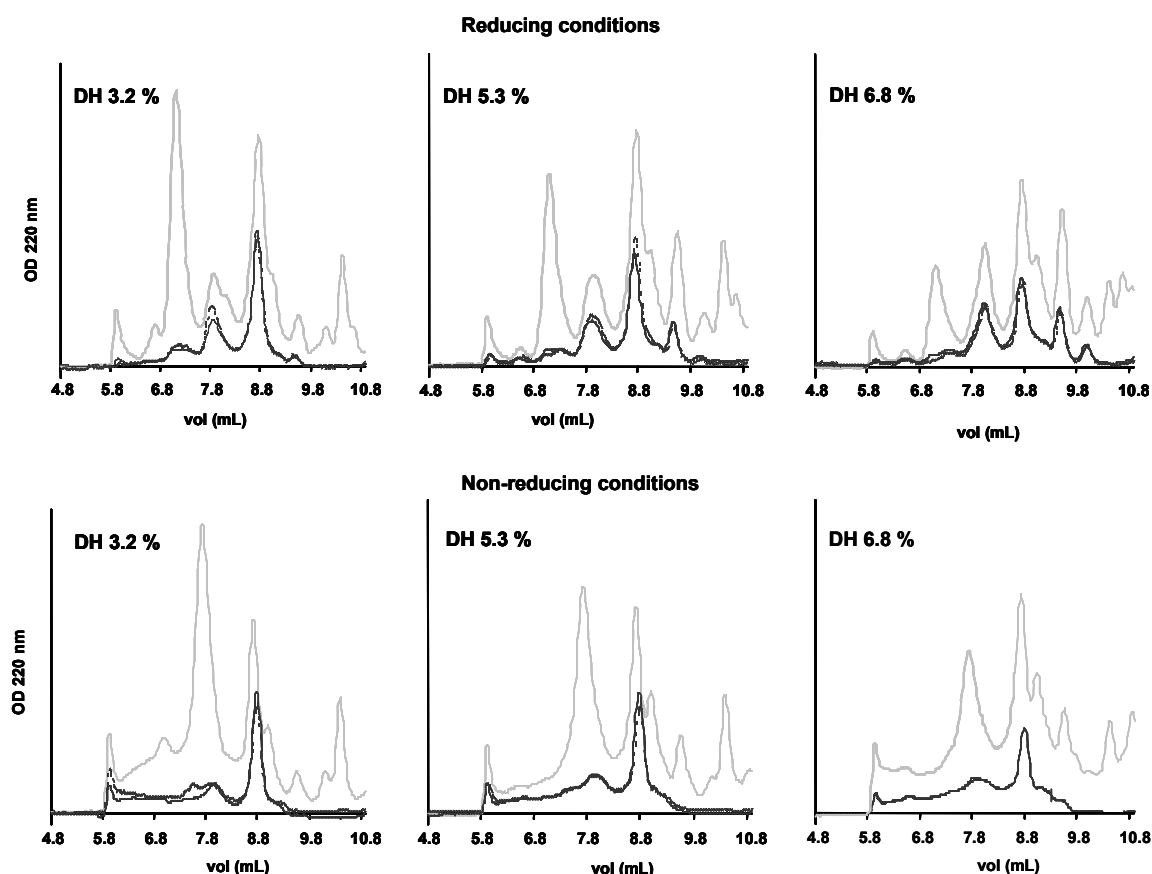


Figure. 2.2. Size exclusion chromatograms of enzyme induced aggregates obtained from hydrolysates with DH 3.2, 5.3 and 6.8 %, at pH 7.0, at an ionic strength of 0.075 M, at 40°C (black line) and 60°C (dashed line), under reducing and non-reducing conditions. The gray line in the figures represents the complete hydrolysate.

Under reducing conditions, at DH 3.2 %, peptide fractions eluting at around 7.8 mL and around 8.7 mL were the main fractions involved in aggregation. With increasing the DH, more and more smaller peptides were incorporated in the aggregates (9.5 and 10.0 mL). Ionic strength (results not shown) and temperature hardly influenced the peptide size composition of the aggregates at DH 5.3 and 6.8 %. In the DH 3.2 % hydrolysate, the size of the peptides involved in aggregation seemed to increase with temperature.

Under non-reducing conditions, the aggregating materials formed at the three DH values were largely similar but contained a peptide fraction eluting at 8.8 mL (not originating from disulfide bridged peptides) and material eluting from 5.9 to 8.1 mL, corresponding to disulfide bridged peptides. Temperature did not influence the relative composition of the peptide fractions involved in aggregation of the hydrolysates with DH 5.3 and 6.8 %. In the hydrolysate with DH 3.2 %, increasing the temperature to 60°C caused a small enrichment of the aggregates with the peptide fraction eluting at 7.5 mL.

2.3.3. Mixing of hydrolysates with intact whey protein isolate: effects of DH, temperature and ionic strength

The enzyme-induced aggregates that were formed at pH 7.0 could be solubilized at pH 2.0. Because intact WPI does not aggregate at pH 7.0, the ability of hydrolysates to aggregate intact whey proteins at pH 7.0 can be investigated by mixing both at pH <7.0, and

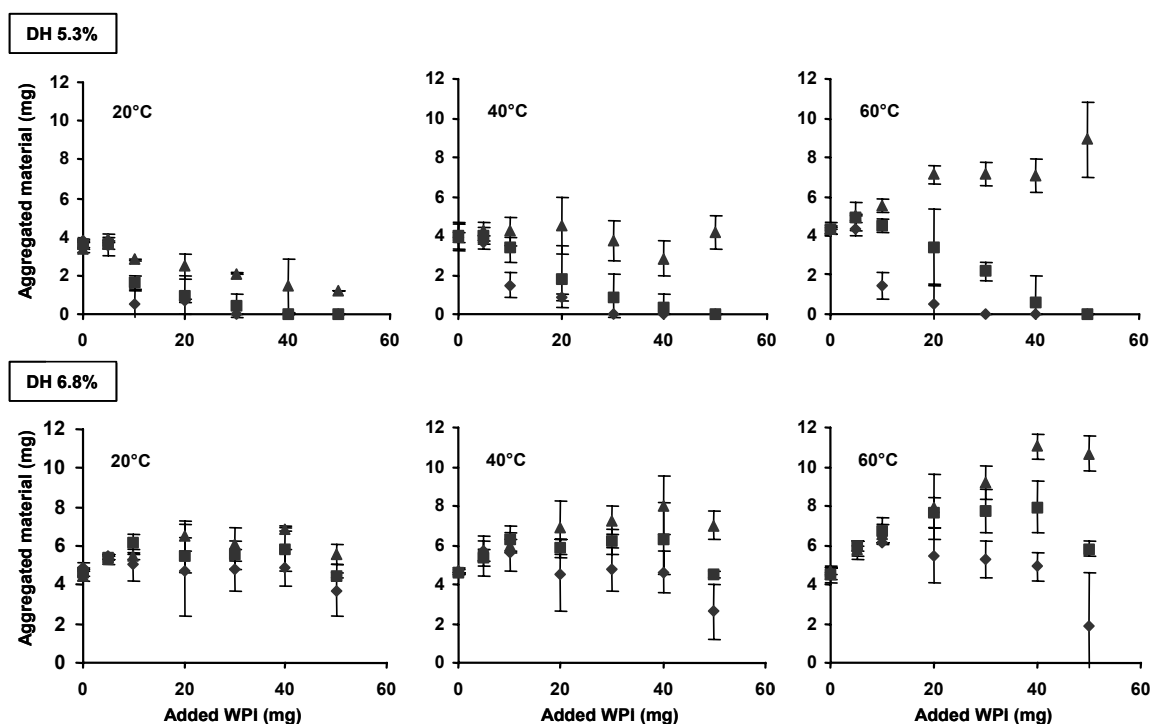


Figure 2.3. Amount of aggregated material (pH 7.0) as a function of the amount of added intact WPI to hydrolysates of DH 5.3 and 6.8 %, at 20°C, 40°C and 60°C. Ionic strengths were: 0.020 M (◆), 0.075 M (■) and 0.200 M (▲). Values are means for duplicates with standard deviation indicated by vertical bars.

subsequently determining the amount of aggregated material at pH 7.0, at various conditions of temperature and ionic strength. The aim of this experiment was to determine if and how much additional WPI the hydrolysates could aggregate. For this experiment, the total amount of aggregated material was expressed as a function of the amount of intact WPI added, as is shown in **Figure 2.3**.

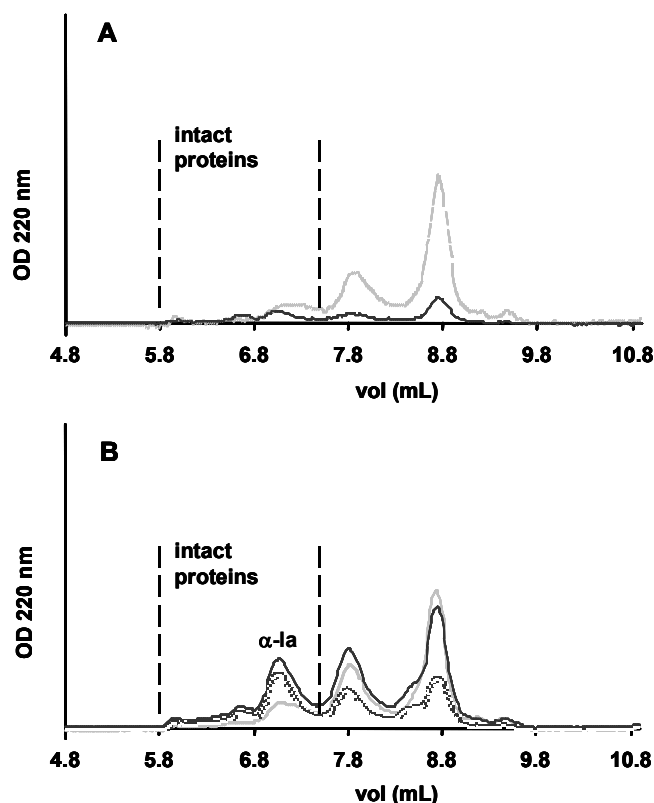


Figure 2.4. Size exclusion chromatograms, under reducing conditions, of aggregates obtained at pH 7.0 from mixtures of a hydrolysate of DH 3.2 % with intact WPI at 40°C (A) and 60°C (B), at an ionic strength of 0.200 M (no added WPI: gray line; 20 mg of WPI added: black line; 40 mg of WPI added: dotted line).

Additional aggregation was considered to have occurred when more aggregation was observed than in the hydrolysate itself. No precipitation occurred in the mixture of control WPI and added intact WPI. Upon adding intact WPI to the hydrolysates of DH 3.2% (results not shown) and DH 5.3 % (**Figures 2.4A, B and C**), a decrease in the amount of aggregated material was observed for all conditions tested (except at 60°C and an ionic strength of 0.200 M). This decrease became larger with increasing amounts of added WPI until it eventually became absent. Thus, aggregation seemed to be inhibited by the addition of intact WPI. When WPI was added to the hydrolysate of DH 6.8 % (**Figure 2.3**), an increase in the amount of aggregated material was observed, until a maximum was reached when 40 mg of WPI was added. Above 40 mg a decrease in the amount of aggregation was observed. In general, the amount of additionally aggregated material increased with increasing DH. In

addition, increasing the temperature or the ionic strength favored aggregation at all DH's, being maximal at an ionic strength of 0.200 M and at 60°C.

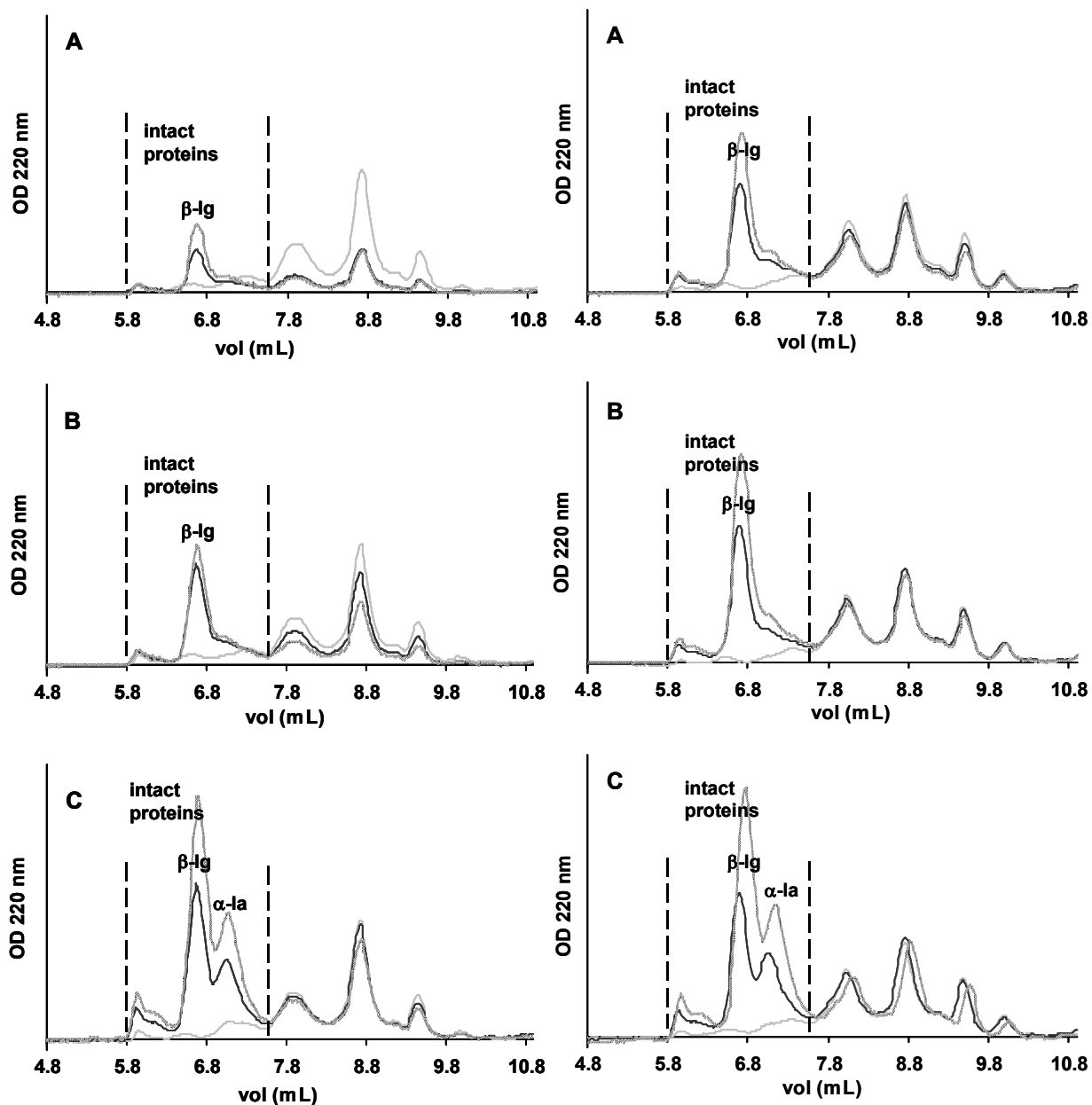


Figure 2.5. Size exclusion chromatograms, under reducing conditions, of aggregates obtained at pH 7.0 from mixtures of a hydrolysate of DH 5.3 % with intact WPI at 20°C (A), 40°C (B) and 60°C (C), at an ionic strength of 0.200 M (no added WPI: gray line; 20 mg of WPI added: black line; 40 mg of WPI added: dotted line).

Figure 2.6. Size exclusion chromatograms, under reducing conditions, of aggregates obtained at pH 7.0 from mixtures of a hydrolysate of DH 6.8 % with intact WPI at 20°C (A), 40°C (B) and 60°C (C), at an ionic strength of 0.200 M (no added WPI: gray line; 20 mg of WPI added: black line; 40 mg of WPI added: dotted line).

In order to determine how the composition of the aggregates varied during these mixing experiments, *i.e.* how much intact WPI was present in the aggregates, the composition of the washed pellets was determined with size exclusion chromatography, under reducing conditions. **Figures 2.4, 2.5 and 2.6** show the chromatograms obtained for the DH 3.2, 5.3 and 6.8 % hydrolysates, respectively. Upon mixing of the hydrolysate of DH 3.2 %, at 20°C, too little material was aggregated in order to be analyzed.

WPI became part of the aggregates upon mixing (peaks from 5.8 to 7.1 mL) and its quantity increased when the DH increased. Also, smaller peptides were involved in aggregation when the DH increased. During mixing of the DH 3.2 % hydrolysate, larger peptides became involved in aggregation when the temperature was increased.

At low ionic strength (data not shown), the amount of peptides present in the aggregates decreased upon mixing. This was observed for all DH's, but was not observed at high ionic strength (0.200 M). Upon mixing the hydrolysates with intact WPI, increasing the ionic strength increased the amount of aggregated WPI, at all temperatures. β -Lg (peak eluting at 6.8 mL) was present in the aggregates at all temperatures and its quantity increased when the temperature increased, while α -la (peak eluting at 7.1 mL) was present in pronounced quantities in the aggregates only above 40°C.

2.4 Discussion

2.4.1 Peptide-peptide interactions

The hydrolysis of WPI by BLP has been shown to lead to the formation of a soft gel with a particulate structure (Otte et al., 1996). The formation of enzyme-induced aggregates thus determines the gelation properties of WPI after hydrolysis (Otte et al., 1996). In the present work, the formation of these aggregates was studied in more detail. Hydrolysis of WPI with BLP was inhibited by acidification in order to reach various degrees of hydrolysis. Subsequently, the amount of aggregating material and its peptide composition, at various temperatures and ionic strengths, was investigated.

In this study, an increase in the amount of aggregating material with increasing DH was observed, at pH 7.0. This is in agreement with the observation by Otte and co-workers (Otte et al., 1996) of a shift in the turbidity maximum from pH 5.6 to pH 7.0, during hydrolysis, due to peptide aggregation.

It was shown that four peptide fractions, eluting at about 7.8, 8.8, 9.5 and 10.0 mL, with molecular masses of 9.0-7.5, 4.0, 2.1 and 1.4 kDa, were responsible for aggregation in the hydrolysates of WPI made with BLP. The size of the aggregating peptides became smaller with increasing DH, while the extent of aggregation became higher (**Figures 2.2 and 2.3**). Small peptides thus seem very important for aggregation. It was also shown that three of

the four aggregating peptide fractions existed in the aggregates as disulfide-linked peptides. It is not known whether the disulfide bridges were originally present or resulted from reshuffled S-S bridges (Caessens et al., 1999). Since the peptide aggregates could be dissolved at $\text{pH} \leq 2$ and $\text{pH} \geq 8$, it seems plausible that mainly non-covalent interactions between the peptides are responsible for aggregation. Otte and co-workers (Otte et al., 1997) also found that aggregates created during hydrolysis of β -lg consisted of peptides held together by non-covalent interactions.

The aggregation behavior within the hydrolysates of various DH's differed as a function of temperature and ionic strength (**Table 2.1**). Indeed, the effect of temperature and ionic strength was absent at high DH, where the attractive forces had become so dominant that further enhancement of attractive forces or a decrease of repulsive forces did not have any effect. The explanation for this change in behavior can be found in the differences in the concentration of aggregating peptides and/or in the peptide composition of the hydrolysates. For example, the peptide fraction of 9.0 kDa at DH 3.2 % was assumed to be cleaved to 7.5 kDa at DH 6.8 %, as observed in **Figures 2.1A and 2.2**. It can thus be hypothesized that this 9.0 kDa aggregating fraction contained a precursor of the 7.5 kDa aggregating fraction, but with a hydrophilic extremity. This can be deduced from the fact that aggregation at low DH was much more sensitive to temperature and ionic strength (**Table 2.1**). In addition, changes in aggregation behavior within the hydrolysates of various DH's can be related to structural changes in the peptides as well since with increasing DH there should be less secondary structures remaining and thus nothing to unfold when the temperature was increased.

Aggregation is the result of the balance between electrostatic interactions, repulsion as well as attraction, hydrophobic interactions and covalent interactions, in which amino acid composition as well as their sequence in the peptides, steric factors and environmental factors plays an important role. Our results show that aggregation within the hydrolysates seemed to be favored mainly by increased hydrophobic interactions and to a lesser extent by reduced electrostatic repulsion, as already suggested by Ju and co-workers (Ju et al., 1995) and Otte and co-workers (Otte et al., 1997).

2.4.2. Protein-peptide interactions

In the present study, it was shown that the enzyme-induced aggregates contain only peptides (**Figure 2.2**), which was also observed by Otte and co workers (Otte et al., 1997 and Otte et al., 2000a). Because in WPI hydrolysates the simultaneous presence of intact β -lg and aggregating peptides was not possible, the interaction between these peptides and whey proteins was studied by mixing the hydrolysates with WPI.

The capacity of the hydrolysates to aggregate protein was characterized both in terms of amount as in term of composition of the aggregates obtained. In hydrolysates with a low DH, the peptide aggregates dissociated upon mixing (**Figure 2.3**). Under these conditions,

there were presumably not enough aggregating peptides to form protein-peptide aggregates. Upon increasing the DH, the presence of WPI in the aggregates became apparent, although the amount of aggregated material decreased with further addition of intact WPI (**Figures 2.3 and 2.5**). It can thus be assumed that protein-peptide interactions did indeed occur but the concentration of aggregating peptides was apparently too low to form stable protein-peptide aggregates. For hydrolysates with a high DH, additional aggregation was indeed observed and the amount and the nature of the aggregating peptides were apparently suited to hold the otherwise not aggregating proteins together in aggregates (**Figures 2.3 and 2.6**). The small aggregating peptide fractions of 2.1 and 1.4 kDa, created at high DH, seemed, therefore, to be more suited for protein-peptide interactions than the bigger peptides detectable at low DH. A change in the composition of the aggregates (not shown) towards less peptides and more intact protein occurred upon further WPI addition. The peptides involved in these conditions are, however, potentially the most interesting, because, although being fewer in number upon WPI addition, they are able to aggregate more protein.

As shown in **Figures 2.5A and 2.6A**, β -lg was present in the aggregates already at a temperature of 20°C. β -Lg is a globular protein that contains two disulfide bridges and a free, buried cysteine residue. Since the denaturation temperature of β -lg, at pH 7.0, is 70-73°C (Hoffmann et al., 1997), it can be assumed that the peptides are able to interact with the surface of the native β -lg. The increasing amount of β -lg in the aggregates with increasing temperature could be explained by the increased hydrophobic interactions between peptides and β -lg. The presence of more β -lg at 60°C could also be explained by the appearance of extra hydrophobic interaction sites on the partly unfolded β -lg. β -lg-peptide interactions were also favored at high ionic strength, indicating that reducing the distance over which electrostatic repulsion is effective allows more aggregation to occur. Pure α -la, which contains four disulfide bonds but no free thiol groups and has a denaturation temperature of 64°C at pH 7.0 (Boye and Alli, 2000), does not aggregate upon heating (Calvo et al., 1993). As shown in **Figure 2.2**, the aggregates isolated from the hydrolysates, at all DH's, at a temperature of 60°C and for an ionic strength of 0.075 M, did not contain remaining intact α -la. Under the same conditions (not shown), α -la and β -lg both participated in the aggregates when intact protein was added to the hydrolysates with DH higher than 3.2 % upon mixing. Therefore, the presence of β -lg together with α -la in the system, combined with high temperature, allows the incorporation of α -la in the aggregates. Indeed, the presence of β -lg, containing a free thiol group, may lead to the formation of intermolecular disulphide bonds and to co-aggregation with α -la (Elfagm and Wheelock, 1978; Matsudomi et al., 1992; Calvo et al., 1993). At 60°C, peptides may interact with β -lg linked with α -la via disulphide bonds. Therefore, especially at high temperature (60°C), reshuffling of S-S bridges between β -lg, α -la and peptides will lead to the formation of new potentially aggregating fragments. However, size-exclusion chromatography under non-reducing conditions (results not shown) indicates that non-covalent forces stabilize the core of the aggregates. A small amount of

intact α -la was incorporated in the aggregates upon mixing of the DH 3.2 % hydrolysate with intact protein, at an ionic strength of 0.200 M, although β -lg was not co-aggregated (**Figure 2.4**). Based on this, it might be hypothesized that high ionic strength allowed peptides to interact directly with the partly unfolded α -la, at 60°C, even though increasing ionic strength stabilizes the α -la (Boye et al., 1997).

Aggregation of protein by peptides thus seemed to be dominated by both hydrophobic interactions and by reduced electrostatic repulsion.

2.4.3. Peptide-peptide versus protein-peptide interactions: similarities and differences

In the mixing experiment, peptide-peptide interactions competed with protein-peptide interactions. Interestingly, the peptides fractions involved in the peptide-peptide interactions were the same as those involved in the protein-peptide interactions. The peptide fractions important for aggregation consisted of small (disulfide linked) peptides.

In hydrolysates with a high DH, peptide-peptide interactions were so strong that they were not sensitive to temperature and ionic strength variations, whereas protein-peptide interactions were sensitive to both temperature and ionic strength variations. This indicates that the balance between hydrophobic and electrostatic interactions is clearly different in the two situations. Presumably, hydrophobic interactions completely dominate peptide-peptide interactions, while the strength of protein-peptide interactions depends more on the balance between hydrophobic attraction and electrostatic repulsions.

2.5 Conclusions

The optimal properties of aggregating peptides, formed when WPI was hydrolyzed with BLP, were reached near the end point of hydrolysis. The hydrolysis products consist of small, hydrophobic, peptides, which were the most effective for peptide-peptide and protein-peptide aggregation. Modulation of the interactions with temperature and ionic strength variations was possible for protein-peptide interactions, but hardly for peptide-peptide interactions.

The method chosen to analyze the composition of the aggregates allowed making a distinction between intact proteins and the aggregating peptide fractions. In order to explore the interactions in the protein-peptide system in more detail, analysis techniques that give a much higher resolution, consisting of reversed phase chromatography or capillary electrophoresis coupled with statistical data analysis are needed.

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CHAPTER 3

Protein-peptide interactions in mixtures of whey peptides and whey proteins

Abstract

The objective of this work was to identify the dominant peptides that have the capability to aggregate intact whey proteins and to understand the underlying protein-peptide interactions leading to aggregation in mixtures of whey protein hydrolysate, made with *Bacillus licheniformis* protease, and whey protein isolate. First, the effect of several conditions on the amount and composition of aggregates formed was investigated using response surface methodology. Next, the peptides present in the aggregates were separated from the intact protein and identified with liquid chromatography-mass spectrometry. There was an optimal amount of added intact WPI that could interact with aggregating peptides, yielding a maximal amount of aggregated material in which the peptide/protein molar ratio was of around 6. Under all conditions applied the same peptides were observed in the protein-peptide aggregates formed. The dominant peptides were β -lg AB [f1-45], β -lg AB [f90-108] and α -la [f50-113]. It was hypothesized that peptides could form a kind of glue network that can include β -lg via hydrophobic interactions at the hydrophobic binding sites at the surface of the protein.

Key words: *Bacillus licheniformis* protease; aggregation; proteins isolate hydrolysate

3.1 Introduction

Specific interactions between protein domains and peptides play an important role in a wide range of biological processes, e.g. peptide interaction with a protein membrane receptor for propagation of information through a signaling system, inhibition of an enzyme with a peptide, formation of molecular complexes, etc. Non-specific protein-peptide interactions are observed in food systems. They mainly apply to protein hydrolysates that often consist of mixtures of intact protein and peptides. Some authors have reported that peptides could bind to β -lactoglobulin (Noiseux et al., 2002; Barbeau et al., 1996), the major whey protein, and that such an interaction could increase the resistance of β -lactoglobulin to thermodenaturation (Barbeau et al., 1996). α -Lactalbumin, the second main whey protein, is known to interact with peptides containing clusters of basic amino acid residues in close proximity with hydrophobic amino acid residues (Gurgel et al., 2001) such as melittin, a 26-residue cytolytic peptide from bee venom (Permyakov et al., 1991). The binding of α -lactalbumin to the synthetic peptide WHWRKR (Gurgel et al., 2001) was even used to develop purification strategy of the protein.

We have shown recently (Chapter 2) that hydrolysates of a whey protein isolate made by a seryl protease from *Bacillus licheniformis* (BLP) could aggregate intact whey proteins. The additional amount of aggregated material, containing peptides and intact protein, increased with increasing degrees of hydrolysis. Peptides involved in the aggregates were found to have apparent molecular weights ranging from 1400 to 7500 (under reducing conditions). It was hypothesized that protein-peptide interactions depended on a balance between hydrophobic attraction and electrostatic repulsion. Since partial hydrolysis of whey protein isolate with BLP induces formation of aggregates that eventually form a gel (Otte et al., 1996), information on protein-peptides interactions could help understanding the gelation mechanism in protein hydrolysates and modulate the properties of protein-peptide mixtures.

The objective of the present work was to bring insight into protein-peptide interactions in mixtures of whey peptides and intact proteins. For that purpose, first temperature, ionic strength and amount of added intact protein were varied, according to an experimental design, in order to define optimal conditions at which a hydrolysate aggregates intact WPI upon a mixing experiment. The effects of the different conditions on WPI/peptide molar ratios, on the amount of aggregated material, the amount of peptides and of intact aggregated protein in the aggregates were investigated using response surface methodology in order to further detail composition and protein-peptide interactions in the aggregates. Next, peptides dominantly present in protein-peptide aggregates were chromatographically separated from intact protein. From peptides identification, hypotheses on protein-peptide interactions were postulated.

3.2 Materials and methods

3.2.1 Materials

A commercial whey protein isolate (WPI) powder (trade name Bipro, Davisco Foods International Inc., Le Sueur, MN, USA) was used for the experiments. According to the manufacturer, it consisted of 74.0 % (w/w) β -lactoglobulin (β -lg), 12.5 % (w/w) α -lactalbumin (α -la), 5.5 % (w/w) bovine serum albumin, and 5.5 % (w/w) immunoglobulins. The protein content of the powder was 93.4 % (w/w) and it contained 0.12 % (w/w) calcium. The enzyme used was a seryl proteinase from *Bacillus licheniformis* (BLP, product name NS-46007, batch PPA 6219; E.C. 3.4.21.19), specific for Glu-X bonds and to a lesser extent for Asp-X bonds (Breddam and Meldal, 1992). The enzyme was kindly provided by Novozymes (Novozymes A/S, Bagsvaerd, Denmark).

All reagents were of analytical grade and purchased from Sigma (Sigma Chemical CO, St Louis, MO, USA) or Merck (Darmstadt, Germany).

3.2.2 Hydrolysis of WPI

WPI was dispersed in Millipore water at a concentration of 120 mg g⁻¹ and stirred overnight at 4°C. The suspension was centrifuged (30 min, 19000 x g, 20°C) and the supernatant was subsequently filtered (Rezist 30GF92 and PVDF 0.45 μ m, Schleicher and Schuell GmbH, Dassel, Germany). After measuring the nitrogen content, using the Dumas method, the supernatant was diluted to 50 mg (protein) g⁻¹ with Millipore water, preheated at 40°C for 15 min and the pH was adjusted to pH 8.0, 5 min before addition of the enzyme solution. The enzyme/substrate ratio (v/v) used was 1/100 to reach a degree of hydrolysis (DH) of 6.8 %. During hydrolysis, the reaction mixture, incubated at 40°C, was maintained at pH 8.0 by addition of a 0.4 M NaOH solution in a pH-STAT (719 S Titrimo, Metrohm Ion Analysis, Metrohm Ltd., Herisau, Switzerland). To permanently inactivate (as verified with size exclusion chromatography) the enzymatic activity of BLP, the pH was adjusted to pH 2.0 with a 6 M HCl solution. The nitrogen content of the hydrolysate was measured using the Dumas method. The hydrolysate was stored at 4°C prior to further analysis and use.

3.2.3 Mixing of hydrolysate with intact whey protein isolate

An experimental design was applied to studying the effects of mixing the hydrolysate with intact WPI. Response surface methodology linked the mixing variables, via a polynome, to the amount of aggregated material, the amount of intact WPI and the amount of peptides in the aggregates as well as the WPI/peptide molar ratios in the aggregates (four responses). Three variables (k=3) were varied: the amount of added intact WPI, the ionic strength and the temperature of incubation. The variables were optimized using a central composite rotatable

design (CCRD), in which the variables vary around a central point. The design contains five levels for each variable, coded as $-\alpha$, -1 , 0 , $+1$, $+\alpha$ (**Table 3.1**). Three types of experiments were performed: center experiments, having all variables set at level 0 (repeated twice to determine the reproducibility); cube experiments, which are combinations of -1 and $+1$ levels (2^k experiments for a full factorial design); and star experiments, with one variable at an extreme value ($-\alpha$ or $+\alpha$) and the other variables at 0 level ($2 \times k$ experiments) (Myers and Montgomery, 1995).

Table 3.1. Central composite design: variables and levels.

Variable	Level of the variables				
	$-1.68 (-\alpha)$	-1	0	$+1$	$+1.68 (+\alpha)$
A (Temperature of incubation, °C)	20	28.1	40	51.9	60
B (Ionic strength, mM)	25	55.4	100	144.6	175
C (Amount of added intact WPI, mg)	0	8.1	20	31.9	40

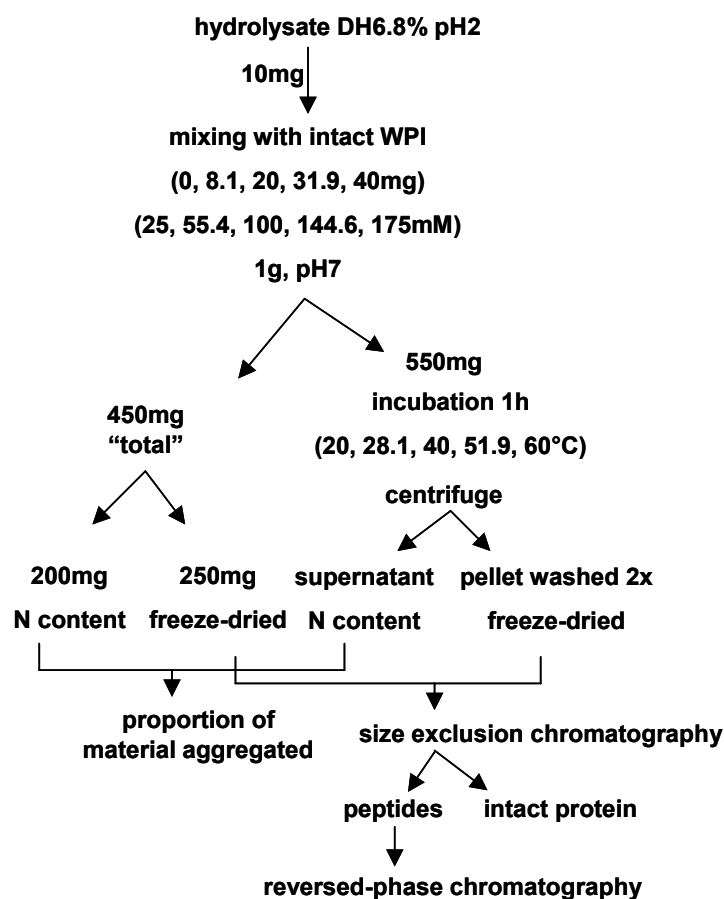


Figure 3.1. Outline of the experiments performed.

Figure 3.1 gives an outline of the experiments performed. WPI hydrolysate, containing 10 mg of proteinaceous material, was mixed with samples containing 0, 8.1, 20, 31.9, and 40 mg of intact WPI solution (pH 7.0). The pH was adjusted to pH 7.0 with 0.25-

2 M NaOH solutions and the ionic strengths to 25, 55.4, 100, 144.6, and 175 mM using a 2 M NaCl solution. The total mass of each sample was set to 1 g with Millipore water, yielding a final hydrolysate concentration of 10 mg g⁻¹ and WPI concentrations of 0, 8.1, 20, 31.9, and 40 mg g⁻¹. Each sample was divided into two parts. One part (0.55 g) was incubated for 1 h at 20, 28.1, 40, 51.9 or 60°C and centrifuged (15 min, 19000 x g, 20°C) while the other part, was left non-centrifuged (further denoted “total”). In order to determine the extent of aggregation, the nitrogen content (N) in the supernatant and in the non-centrifuged part (N₀) was determined using the Dumas method. The proportion of aggregation was defined as $(1 - N/N_0) \times 100 \%$ and gave the amount of aggregated material. The pellets, containing the aggregates, were washed twice with 25, 55.4, 100, 144.6, and 175 mM NaCl solutions at 20, 28.1, 40, 51.9 or 60°C and freeze-dried. As well, 0.25 g of the non-centrifuged part was freeze-dried.

3.2.4 Size exclusion chromatography

Size exclusion experiments were performed with an Äkta Purifier System (GE Healthcare Bio-Sciences, Uppsala, Sweden), with Unicorn software. Samples were separated with an analytical Shodex Protein KW-802.5 column (300 x 8 mm; particle size: 7 µm; pore size: 500 Å; Showa Denko K. K., Kanagawa, Japan). The column was equilibrated with 6 M urea containing 30 % (v/v) acetonitrile and 0.1 % (v/v) trifluoroacetic acid (TFA). The flow rate was 0.5 mL min⁻¹ and the column temperature 20°C. A volume of 100 µL sample was injected onto the column. Detection was performed at 220 and 280 nm.

Sample preparation was as follows. The freeze-dried pellets and “total” samples were dissolved in 550 µL and 250 µL 8 M guanidinium hydrochloride, respectively. Next, a volume of 100 µL of dissolved pellet or “total” was mixed with 600 µL of 0.05 M Tris-HCl buffer containing 0.05 M dithiothreitol (DTT) and 8 M guanidinium hydrochloride (pH 8.0). After 2 h of incubation at room temperature, 300 µL of acetonitrile and 1 µL of TFA were added.

From the pellet samples, containing the aggregated material, the peptides, eluting between 7.8 and 11.2 mL, were collected. A volume of 100 µL of 0.120 mg mL⁻¹ dynorphin A (1-7) (Bachem, Bachem AG, Bubendorf, Switzerland) was added per 1.4 mL of collected fraction and further served as internal standard. Next, the acetonitrile was evaporated with an ALPHA-RVC CMC-1 rotating vacuum concentrator (CHRIST, Osterode am Harz, Germany). A volume of 3.5 µL TFA was added per mL of fraction before further analysis with reversed-phase chromatography.

The areas (at 280 nm) of the peaks corresponding to intact protein present in the aggregates and in the “total” were calculated using the program Peak Fit (SPSS Inc., Chicago, IL, USA). This calculation gave the proportion of intact WPI in the aggregates, and, therefore, the amount of intact WPI in the aggregates. From this value and together with the total amount of aggregated material, the amount of peptides in the aggregates was calculated.

The column was calibrated using a low molecular weight gel filtration calibration kit (GE Healthcare Bio-Sciences). The kit contained blue dextran (2000 kDa), aldolase (42 kDa), ribonuclease A (13.7 kDa). In addition, aprotinin (6.5 kDa), insulin chain B (3.5 kDa), angiotensin I (1.296 kDa), bradykinin (1.06 kDa) (Sigma, product numbers A-6012, I-6383, A-9650 and B-3259, respectively) and leupeptin (0.426 kDa, Roche, F. Hoffmann-La Roche Ltd, Basel, Switzerland, product number 1017101) were used for calibration purposes.

3.2.5 Liquid chromatography- mass spectrometry

Samples were separated on an analytical reversed-phase Vydac C8 column (208MS52; 2.1 x 250 mm; bead diameter: 5 μ m; porosity: 300 nm; Dionex, Sunnyvale, CA, USA) by HPLC (Thermo Separation Products Inc., San Jose, CA, USA), with ThermoQuest software. The flow rate was 0.2 mL min⁻¹, column temperature 20°C, eluent A 0.07 % (v/v) TFA in 5 % (v/v) acetonitrile, and eluent B 0.05 % (v/v) TFA in acetonitrile. A volume of 75 μ L sample was injected onto the column. After 10 min isocratic elution with eluent A, further elution was obtained with a linear gradient from 0 to 55 % eluent B in 100 min, then from 55 to 100 % eluent B in 2 min and 100 % B for 20 min. Detection was performed at 220 nm. Reversed-phase chromatograms were baselined and the area under the peaks was normalized to reach the same area of the internal standard peak for each sample. Because small variations of retention times occur during separation, the program COWTool was used to align the peaks by correlation optimized warping (Nielsen et al., 1998; Nielsen et al., 1999). Mass spectra were recorded with an electrospray ionization mass spectrometer (LCQ ion-trap, Finnigan MAT 95, San Jose, CA, USA), operating in the positive mode using a spray voltage of 2 kV and a capillary temperature of 200°C. The capillary voltage was set at 45 kV and the tube lens voltage at 35 kV. Mass spectra were collected in a full mass scan, followed by a zoom scan and a MS/MS scan of the most intense ion in a window of 1.5-2 m/z and a 30-35 % relative collision energy. The apparatus and data were controlled by Xcalibur software. The accuracy of the mass determination was \pm 0.3 Da. The theoretical masses of peptides were calculated using the program Protein Prospector MS Digest v 4.0.5 by P.R. Baker and K.R. Clauser (<http://prospector.ucsf.edu>). In addition, mass spectra were analyzed by Bioworks Browser software (Thermo Electron Corporation, Waltham, MA, USA), with a FASTA database containing the β -lg A, β -lg B and α -la B sequences.

3.2.6 Nitrogen content determination

Nitrogen contents were measured using the combustion or Dumas method (AOAC, 1995) with a NA 2100 Protein nitrogen analyzer (CE Instruments, Milan, Italy). A 6.38 x N conversion factor was used to convert nitrogen content to protein content.

3.2.7 Statistical analysis

Statistical analysis was performed with The Unscrambler® programme (Camo, Camo ASA, Oslo, Norway). Five responses were considered: four responses that are the amount of aggregated material, the amount of intact WPI and the amount of peptides in the aggregates as well as the WPI/peptide molar ratios in the aggregates and the fifth response is the reverse phase chromatograms.

The population standard deviation, which is the deviation from the means over all samples, and the standard deviation over repeated center samples were calculated for the amount of aggregated material, the amount of intact WPI and the amount of peptides in the aggregates as well as the WPI/peptide molar ratios.

For response surface analysis, the software was used to fit second-order models and generate response surface plots. Quadratic response surface analysis was based on multiple linear regressions taking into account the main, the quadratic and the interaction effects, according to equation 1:

$$Y = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{i < j=2}^3 b_{ij} X_i X_j + e \quad [1]$$

where Y is the estimated response; b_0 is the value of the fitted response at the center point of the design; b_i is linear regression term; b_{ii} is the quadratic regression term; b_{ij} is the interaction regression term; and X_i and X_j are the variables (Montgomery, 1991). The significance of the b-coefficients calculated by regression analysis was tested with the Student t-test with a level of statistical significance defined as $p \leq 0.05$. Analysis of variance (ANOVA) was performed on the models. The R^2 value, the residual error, the pure error (calculated from the repeated measurements) and the lack of fit (lof) were calculated. The lack of fit indicates whether the calculated response surface represents the true surface. The sum of squares (SS) of the lack of fit is calculated as $SS_{\text{residual}} - SS_{\text{pure error}}$. The significance of the lack of fit was tested with the Student t-test with a level of statistical significance defined as $p \leq 0.05$ (Ostle and Malone, 1988; Myers and Montgomery, 1995).

3.3 Results

The objective of this work was to identify peptides that aggregate whey proteins and to understand protein-peptide interactions leading to aggregation. First, statistical analysis was used to determine the influence of several parameters on aggregates composition and to be able to select representative aggregates to study the peptide composition in further detail.

3.3.1 Statistical analysis of data

The enzyme-induced aggregates that were formed upon hydrolysis of WPI to DH 6.8% at pH 7.0 could be solubilized at pH 2.0. Because intact WPI does not aggregate at pH 7.0, the ability of hydrolysates to aggregate intact whey proteins at pH 7.0 can be investigated by mixing both at pH <7.0, and, upon increasing the pH, subsequently determining the amount of aggregated material at pH 7.0, at various conditions of temperature and ionic strength, using experimental design. The aim of this experiment was to determine how the composition and amount of the aggregates varied upon temperature, ionic strength and addition of WPI. Results or responses (amount of aggregated material, amount of intact WPI and of peptides in the aggregates as well as WPI/peptide molar ratios) are given in **Table 3.2**.

Table 3.2. Central composite design: arrangements and responses. With (A) temperature, (B) ionic strength and (C) amount of added WPI.

Run	Coded variable levels	Responses			
	A, B, C	Amount of aggregated material (mg)	Amount of WPI in the aggregates (mg)	Amount of peptides in the aggregates (mg)	WPI/peptide molar ratios in the aggregates
1	0, 0, -1.68	4.57	0.00	4.57	0.000
2	-1, -1, -1	5.38	1.11	4.27	0.048
3	-1, +1, -1	5.95	1.17	4.78	0.045
4	+1, -1, -1	5.89	1.29	5.60	0.042
5	+1, +1, -1	6.20	1.31	4.90	0.049
6	0, 0, 0 (1)	6.12	2.14	3.98	0.099
7	0, 0, 0 (2)	6.06	2.17	3.89	0.103
8	-1.68, 0, 0	5.15	1.91	3.24	0.108
9	0, -1.68, 0	4.56	1.63	2.92	0.103
10	0, +1.68, 0	7.23	2.51	4.72	0.098
11	+1.68, 0, 0	7.98	3.84	4.14	0.170
12	-1, -1, +1	4.82	2.04	2.78	0.135
13	-1, +1, +1	5.68	2.61	3.07	0.156
14	+1, -1, +1	5.03	2.74	2.29	0.220
15	+1, +1, +1	7.19	3.83	3.36	0.210
16	0, 0, +1.68	5.40	2.30	2.40	0.176

The total amount of aggregated material varied from 4.56 to 7.98 mg. In addition, the amount of intact WPI in the aggregates of all the samples varied between 0 and 3.84 mg, which is in the range of the amount of aggregating peptides in the system (2.29-5.6 mg).

The WPI/peptide molar ratios in the aggregates of all the samples varied between 0 and 0.220, which is equivalent to a peptide to WPI molar ratio between 4.5 and infinity. The average calculated peptide molecular weight in the aggregates was 3566, as calculated from the size exclusion chromatograms, under reducing conditions. The average WPI weight taken was 19400, based on the protein relative composition. We chose to express WPI/peptide

molar ratio and not peptide/WPI molar ratio (that would be more expressive) since a peptide to WPI molar ratio of infinity (run 1) cannot be entered in the program.

Table 3.3 gives the range of the variation, the average values and the population standard deviation over the 16 samples for the four responses. The values for the center samples are specified separately since these samples are the only replicates. This provides an indication about the reproducibility of the mixing experiment. The population standard deviation for the four responses is considerably higher than the standard deviation over the repeated center samples. Therefore, the variables influence both the amount of aggregated material, the amount of intact WPI and peptides in the aggregates as well as the WPI/peptide molar ratios, which makes regression analysis interesting.

Table 3.3. Average, standard deviation and range of all points compared with the central points for all the responses in the central composite design.

Response		All samples	Centre samples
Amount of aggregated material (mg)	Range	4.56-7.98	6.12-6.06
	Average	5.83	6.09
	Standard deviation	0.98	0.04
Amount of WPI in the aggregates (mg)	Range	0-3.84	2.14-2.17
	Average	2.08	2.16
	Standard deviation	1.02	0.02
Amount of peptides in the aggregates (mg)	Range	2.29-5.6	3.89-3.98
	Average	3.81	3.94
	Standard deviation	0.98	0.06
WPI/peptide molar ratios in the aggregates	Range	0-0.220	0.099-0.103
	Average	0.110	0.101
	Standard deviation	0.064	0.003

3.3.2 Response surface modeling

Regression coefficients and quadratic response surfaces provide information about the affects of individual variables and of their interactive effects on a response. In the experimental design, three variables were varied, which implies that for every response a model could be built from 10 b-coefficients, *i.e.* the coefficients for 3 main effects, 3 quadratic effects, 3 interactions and 1 intercept. Variables needed to reach a maximum amount of aggregated material, a maximum amount of intact protein in the aggregates, a minimum amount of peptides in the aggregated and a maximum WPI/peptide molar ratio in the aggregates could be estimated. Here we describe in detail the response surface modeling for one response: the amount of aggregated material.

The regression coefficients and the overall performance of two response surface models are shown in **Table 3.4**. The initial regression analysis performed with all regression parameters (“complete model”) revealed that several terms were not significant (p -value > 0.2). In order to simplify the model, non-significant terms were eliminated step by step from the regression model, starting with quadratic terms and interaction terms, ensuring that the R^2 of the model does not change significantly (backward elimination). The quadratic term of ionic strength was eliminated, as well as the interaction terms temperature-ionic strength and temperature-added intact protein. This procedure resulted in a model with 7 regression terms (**Table 3.4**, “simplified model”). The R^2 of this “simplified model” is 0.846, which is close to that of the “complete model” ($R^2=0.867$). The lack of fit of the model indicates whether the calculated response surface represents the true shape of the surface. The lack of fit is not significant in the complete model (p -value is 0.052) and in the simplified model (p -value is 0.062).

Table 3.4. Regression coefficients and their p -values for the regression model for prediction of the amount of aggregated material as a function of temperature (A), ionic strength (B) and amount of added WPI (C).

	Complete model		Simplified model	
	b-coefficient	p-value	b-coefficient	p-value
Intercept	6.093	0.000	5.973	0.000
A	4.455e-02	0.014	4.455e-02	0.003
B	1.378e-02	0.007	1.378e-02	0.001
C	4.28e-03	0.751	4.282e-03	0.714
AB	0.118	0.540		
AC	0.109	0.571		
BC	0.244	0.230	0.244	0.163
AA	0.146	0.421	0.176	0.226
BB	-6.934e-02	0.697		
CC	-0.362	0.076	-0.333	0.036
Other statistics				
R²	0.867		0.846	
Sum of squares		df^a		df
Total error	1.926	6	2.230	11
Pure error	1.800e-03	1	1.800e-03	1
Lack of fit	1.924	5	2.228	10
p-value lack of fit	0.052		0.062	

^a degree of freedom

The regression coefficients and the response surfaces were used to study the effects of the variables on the amount of aggregated material. The influences of temperature and ionic strength are higher than that of the amount of added intact protein, as indicated by the higher

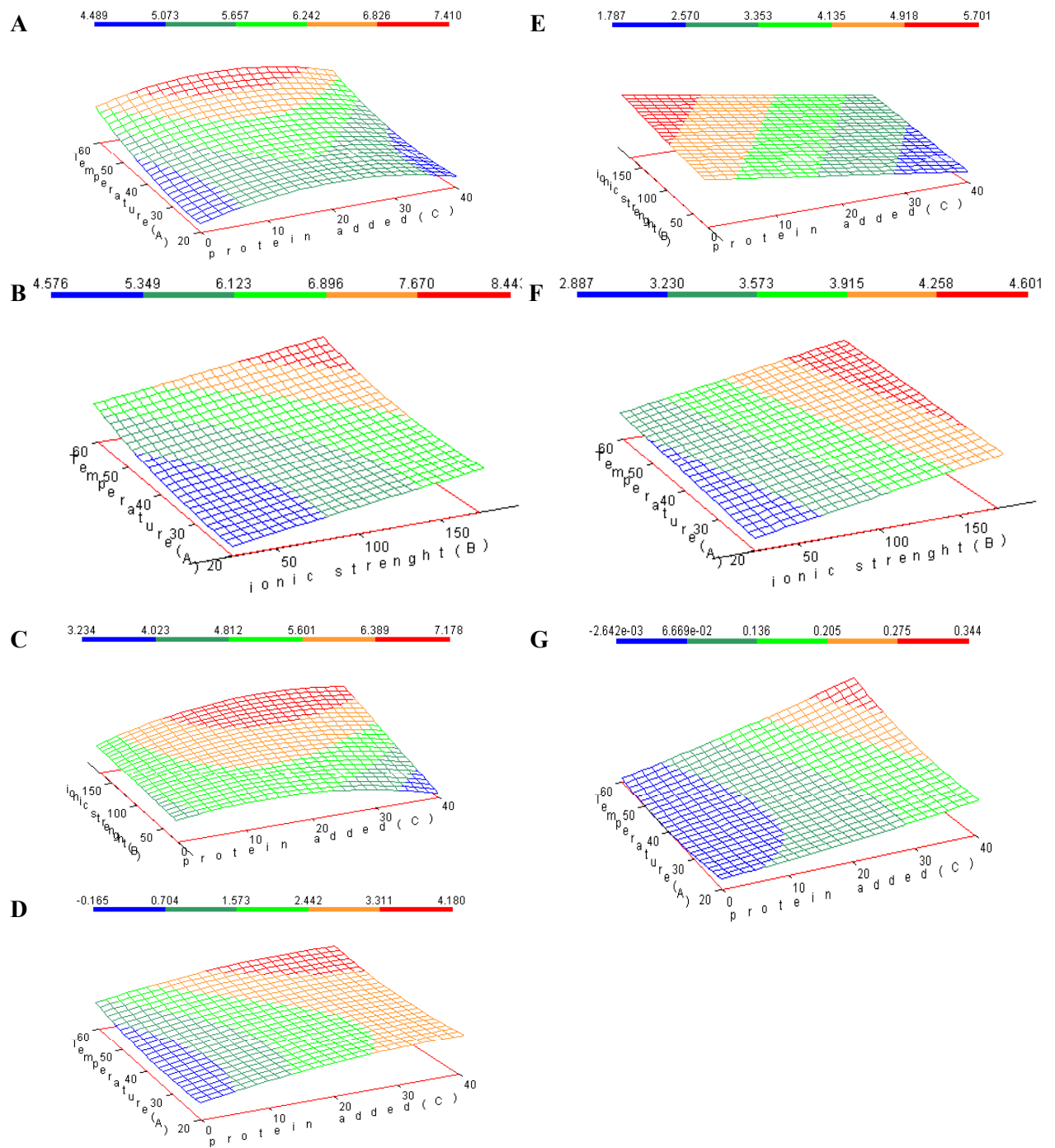


Figure 3.2. Response surfaces of: -the effects of temperature and amount of protein added (A), temperature and ionic strength (B), and ionic strength and amount of protein added (C) on the amount of aggregated material in the aggregates; -the effects of temperature and amount of protein added (D) on the amount of WPI in the aggregates; -the effects of ionic strength and amount of protein added (E) and temperature and ionic strength (F) on the amount of peptides in the aggregates; -the effects of temperature and amount of protein added (G) on the WPI/peptide ratio in the aggregates. (In every figure, the third variable is at its center value).

absolute regression coefficients (main terms). However, the effect of the amount of added intact protein as quadratic term is significant. The effects of temperature, ionic strength and amount of added intact WPI are illustrated in the response surface plots (**Figures 3.2. A, B and C**). An optimum amount of aggregated material is found when 20 mg of WPI is added to the hydrolysate, whatever the temperature, at an ionic strength of 100 mM (**Figure 3.2. A**). A maximum amount of aggregated material was obtained at combinations of high ionic strength with high temperature (**Figure 3.2. B**) and high amount of WPI added with high ionic strength (**Figure 3.2. C**).

With respect to the amount of intact WPI in the aggregates, it can be stated that the effects of temperature and amount of added intact protein are higher than that of ionic strength, as indicated by the higher absolute regression coefficients (**data not shown**). In the response surface plot (**Figure 3.2. D**) a maximum amount of intact protein in the aggregates is observed at high temperature (around 60°C) and for high amount of WPI added.

The effects of temperature and amount of added intact protein on the amount of peptides in the aggregates are higher than that of ionic strength, as indicated by the higher absolute regression coefficients (**data not shown**). In the response surface plots (**Figures 3.2. E and F**), a maximum amount of peptides in the aggregates was obtained at combinations of low amount of WPI added with high ionic strength and high ionic strength with high temperature.

Concerning the WPI/peptide molar ratios in the aggregates, it can be seen that the effects of temperature and amount of added intact protein are higher than that of ionic strength, as indicated by the higher absolute regression coefficients (**data not shown**). In the response surface plot (**Figure 3.3. G**) a maximum WPI/peptide molar ratios in the aggregates is observed at high temperature (around 60°C) and for high amount of WPI added.

3.3.3 Identification of the main peptides involved in protein-peptide interactions

In this study, the 16 pellets containing aggregated material (peptides and intact protein) were analyzed with size exclusion chromatography under reducing conditions (**Figure 3.3**) in order to separate intact protein from peptides. The peptides were collected and afterwards analyzed with reverse phase chromatography (**Figure 3.4**).

Interestingly, the 16 reversed-phase chromatograms obtained were similar with respect to the number of peaks and to the relative proportions of these. Three major peaks were found that correspond well with three major peaks obtained in size exclusion chromatography. Correlations between sample characteristics were studied by partial least squares regression. No stable model could be built from the analysis of the chromatograms. The calibration was satisfying, but could not be validated and only 60% of the information was explained in principal component analysis (instead of 80% at least). Therefore, it was concluded that the variables (temperature, ionic strength and amount of added intact WPI) have no effect on the peptide composition in the aggregates.

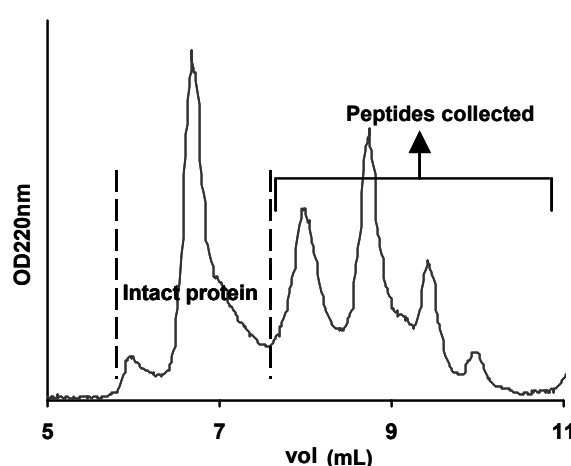


Figure 3.3. Size exclusion chromatogram, under reducing conditions, of aggregates obtained from the mixture of a hydrolysate with intact WPI (run 6 in this case)

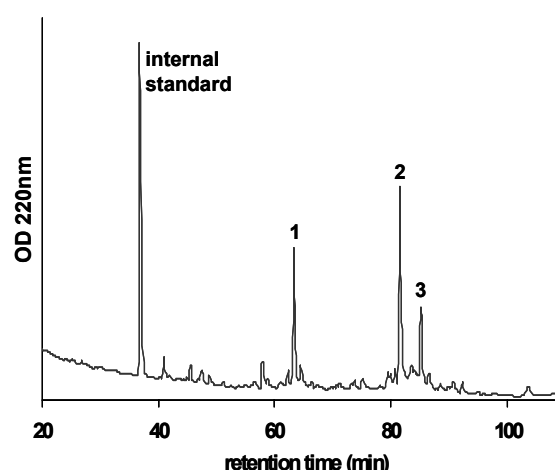


Figure 3.4. Reverse phase chromatogram, under reducing conditions, of peptides isolated from intact protein with size exclusion chromatography (run 6 in this case).

The peptides present in the three major peaks (**Figure 3.4.**) were identified with mass spectrometry (**Table 3.5**), from both full mass scans and MS/MS scans. Unless ionization suppression from these peptides occurred in the electrospray, hiding other peptides, each peak contained one peptide. Peak 1 (β -Ig AB [f90-108]) and peak 3 (α -Ia [f50-113]) contain peptides originating from disulfide-bridged fragments. Peak 2 contained the peptide β -Ig AB [f1-45], which corresponds to the N-terminal extremity of β -Ig.

Table 3.5. Mass spectrometry results for the peptide peaks 1, 2 and 3 separated by reverse phase chromatography (see **Figure 3.4**).

Peak	Measured mass (Da)	Possible fragment	Theoretical mass (Da)
1	2335.2	β -Ig AB [f90-108]	2336.8
2	4895.6	β -Ig AB [f1-45]	4898.7
3	7403.5	α -Ia [f50-113]	7408.5

3.4 Discussion

It has been shown that hydrolysis of WPI with BLP leads to the formation of peptide aggregates (Otte et al., 1997). We have recently shown that these WPI hydrolysates have the capacity to aggregate intact WPI (Chapter 2). In order to further investigate aggregates composition and interactions in the protein-peptide system, we used response surface methodology to analyze the effects of mixing conditions on aggregates composition. In

addition, the peptides present in the aggregates were separated from the intact protein and identified with liquid chromatography-mass spectrometry.

3.4.1. Composition of the aggregates

The results obtained with respect to the effects of temperature, ionic strength and the amount of added WPI are schematically summarized in **Figure 3.5**. Increasing both temperature and ionic strength increased both the amounts of intact protein and peptides in the aggregates, and, therefore, the total amount of aggregated material. There were protein-peptide interactions leading to formation of stable protein-peptide aggregate since WPI alone did not aggregate under these conditions. Since elevated temperature favors hydrophobic interactions and high ionic strength reduces the distance over which electrostatic repulsion is effective, protein-peptide interactions depend on a balance between hydrophobic attractions and electrostatic repulsions, as already assumed in a former study (Chapter 2).

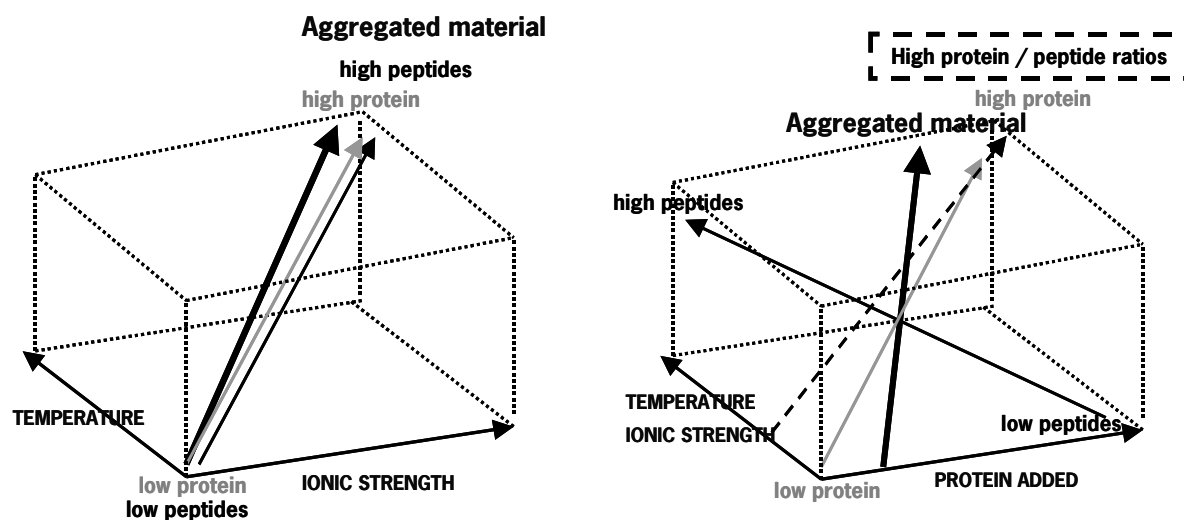


Figure 3.5. Schematic representation of the variations of composition in the aggregates as a function of temperature, ionic strength and amount of intact WPI added.

In addition, a change in the composition of the aggregates towards less peptides and more intact protein occurred upon WPI addition (**Figure 3.5**). There was an optimal amount of added intact WPI (20 mg) that could interact with aggregating peptides (at 60°C, 175 mM NaCl), yielding the maximum amount of aggregated material (**Figure 3.2 A**) in which the WPI/peptide molar ratio was around 0.166 (**Figure 3.2. G**; equivalent to a peptide/protein molar ratio of around 6). The main protein present in the aggregates was β -lg, as already identified in a former study (Chapter 2). Therefore, in these conditions, 6 moles of aggregating peptides were interacting with 1 mole of β -lg. Upon further WPI addition, the WPI/peptide ratio in the aggregates increased: protein-peptide interactions were assumed to give soluble adducts since the amount of aggregated material decreased.

In addition, it was shown that variables (temperature, ionic strength and the amount of added WPI) have no effect on peptide composition since the same peptides were present in the aggregates in all the conditions tested (**Figure 3.4**) at the same relative proportions. However, the variables have an effect on the total amount of peptides present in the aggregates (**Figure 3.2. E and F**).

3.4.2. Main peptides involved in protein-peptide interactions

Two of the identified peptides originated from β -lg. β -Lg is a globular protein with a molar mass of 18.3 kDa. It is constituted by 162 amino acid residues, has a pI of 5.2 and contains two stabilizing disulfide bridges and a thiol function on Cys 121. While the disulfide bond between residues 66-160 is on the outer surface, the disulfide bond between residues 106-119 and the free thiol group 121 are buried in the interior of the native molecule. The monomer of β -lg has a calyx fold consisting of an 8-stranded antiparallel β -barrel, typical of the lipocalin protein superfamily; this structure confers a hydrophobic pocket to the β -lg.

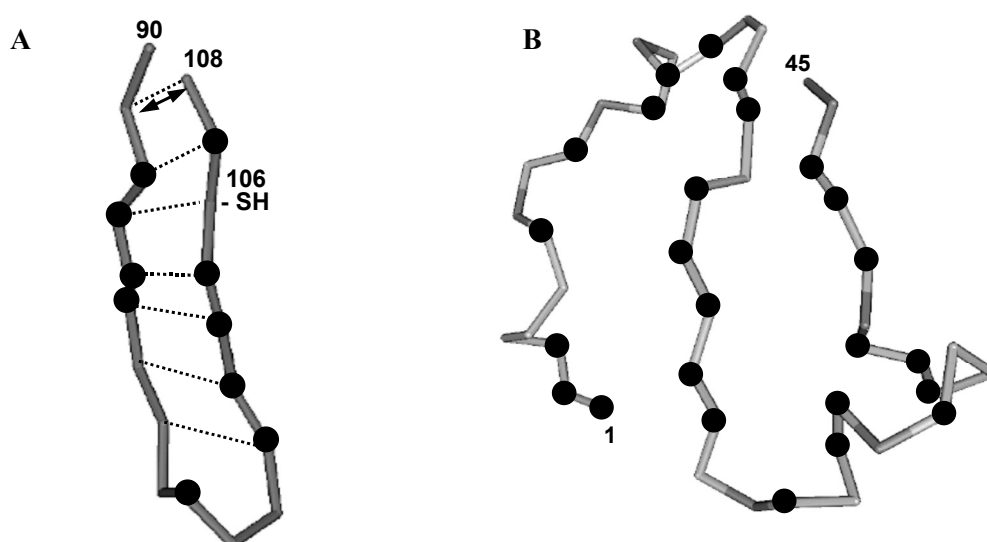


Figure 3.6. Molecular view of (A) the peptide β -lg A [f90-108] and (B) β -lg A [f1-45] in a crystal of β -lg A based on Adams and co-workers (Adams et al, to be published; PDB: 2AKQ). Bonds between atoms of the backbone are represented as thick tubes; hydrophobic residues are represented with black balls; dotted lines represent putative H-bonds stabilizing the putative β -sheet structure; the arrow represents a putative stabilizing salt bridge. From Cn3D version 4.1, produced by National Center for Biotechnology Information.

The structure of the fragment β -lg AB [f90-108] in the β -lg protein is known as 2 β -strands belonging to the same β -barrel, stabilized with hydrogen bonds and with a salt bridge and having hydrophobic residues pointing to both sides of the structure (**Figure 3.6 A**). This peptide β -lg AB [f90-108] has some hydrophobic nature since 53% of the amino acid residues are hydrophobic. According to the sequence of β -lg and to the specificity of the

enzyme, the peptide β -lg AB [f90-108] would be linked via a disulfide bridge, between residues Cys 106-Cys 119, with the minimum sequence β -lg AB [f114-127]. However, despite careful analysis, the latter peptide was not found in the aggregates. The fragment β -lg AB [f90-108] could also possibly be linked to other fragments via reshuffling of the disulfide bond Cys 106-Cys 119. For example, Caessens and co-workers (Caessens et al., 1999) proved reshuffling of disulfide bonds during hydrolysis of β -lg with plasmin. In this study, peptides from the N-terminal part of the molecule were linked to peptides of the middle part of the molecule, in many different combinations with a newly formed intermolecular disulfide bond between Cys 66 and Cys 106, 119 or 121. However, it could not be proven whether the disulfide bond between Cys 106 and Cys 119 remained intact or that this bond also took part in the reshuffling (Caessens, 1999).

The structure of the fragment β -lg AB [f1-45] in the β -lg protein is known as 3 β -strands, belonging to the same β -barrel (**Figure 3.6 B**). Since this peptide is resistant to enzymatic cleavage of the three aspartic acid residues that it contains, it is assumed to adopt a different and/or more compact structure than in the β -lg molecule where the β -strands are “loose” and, therefore, to hide the cleavable peptide bonds. This is supported by the fact that 56% of the amino acid residues in the peptide are of hydrophobic nature.

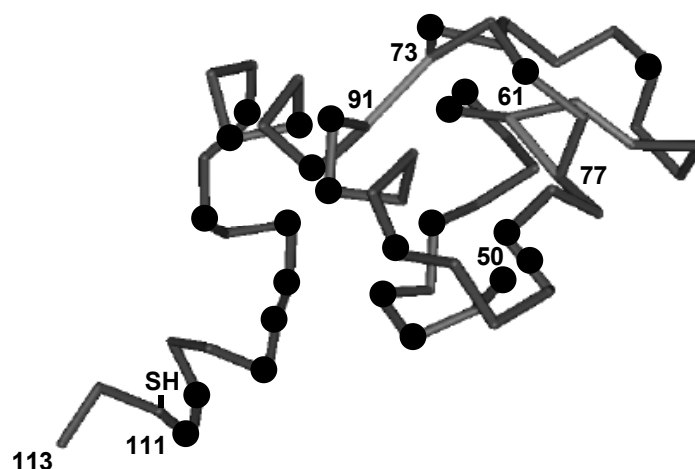


Figure 3.7. Molecular view of the peptide α -la [f50-113] in α -la (PDB: 1F6S). Bonds between atoms of the backbone are represented as thick tubes; hydrophobic residues are represented with black balls. Disulfide bonds are between residues 66-77 and between residues 73-91. From Cn3D version 4.1, produced by National Center for Biotechnology Information.

The third identified peptide originated from α -la. α -La is a globular protein with a molar mass of 14.4 kDa. It is a metalloprotein constituted by 123 amino acid residues, and with a pI of 4.8. α -La is comprised of two subdomains (α and β). The α -subdomain consists of helices in the amino- and carboxyl-terminal regions of the polypeptide chain (residues 1 to 34 and 86 to 123). The β -subdomain consists of the remaining of the protein (residues 35 to

85), that are a three-stranded antiparallel β -sheet, a small 3_{10} helix and some loops and coils (Demarest et al., 1999).

The peptide α -la [f50-113] (**Figure 3.7.**) belongs to both α - and β -subdomains of α -la. According to the sequence of α -la and to the specificity of the enzyme, the peptide α -la [f50-113] could be linked via a disulfide bridge with the minimum sequence α -la [26-37]. However, the latter peptide was not found in the aggregates. As the peptide α -la [f50-113] is rather large (7.408 kDa; around 50% of mass of the intact α -la), we cannot draw assumptions on its structure.

3.4.3. Peptides- β -lactoglobulin interactions

Peptides β -lg AB [f1-45], α -la [f50-113] and intact β -lg have a negative net charge at pH 7.0 (around -2.0 , -2.9 and -9.0 respectively). The peptide β -lg AB [f90-108] is neutral at pH 7.0. In addition, the peptides β -lg AB [f1-45] and β -lg AB [f90-108] contain a high proportion of hydrophobic amino acid residues possibly arranged in clusters (**Figure 3.6**). This corresponds well with the results indicating protein-peptide interactions via hydrophobic interactions and reduced electrostatic repulsion.

It has been reported that β -lg has the ability to bind a variety of small hydrophobic molecules (retinol, fatty acids, aromatic molecules, toluene, etc). Three binding sites have been reported (Sawyer et al., 1998): one in the hydrophobic calyx formed by the β -barrel (for retinol and hydrophobic ligands), another one in an external hydrophobic pocket between the α -helix and the β -barrel (for fatty acids), and the third on the outer surface close to the parallel stack of Trp 19/Arg 124 (for aromatic ligands). However, there was no evidence for the two latter binding sites. In the case of the interaction of β -lg with peptides, it was already speculated that hydrophobic peptides (with masses of 0.554 and 1.383 kDa) could interact with the hydrophobic core of β -lg (Noiseux et al., 2002) and promote a more compact structure to the protein (Barbeau et al., 1996). In addition, charged peptides could also bind to β -lg. In this situation, negatively charged peptides (with masses of 0.703 and 1.245 kDa) were speculated to interact electrostatically with positively charged regions at the surface of the protein (Noiseux et al., 2002). However, in the present study, peptides involved in protein-peptide interactions are larger than the ligands described in literature and reported to interact with β -lg. Indeed, the peptides β -lg AB [f1-45] (~ 4.9 kDa) and α -la [f50-113] (~ 7.4 kDa), account for around 27 and 40%, respectively, of the mass of β -lg and are probably too large to enter the hydrophobic calyx of β -lg for example. However, it cannot be excluded that the peptide β -lg AB [f90-108] (2.3 kDa), especially if it is folded in a β -sheet and if it is disulfide linked with a small peptide, could possibly enter the hydrophobic calyx of β -lg.

It is hypothesized that the peptides form a network, based on hydrophobic interactions, which can include protein. Peptides could form a kind of glue network that can include β -lg, at an optimal peptide to protein molar ratio of 6, via hydrophobic interactions

with the hydrophobic binding sites at the surface of the protein, as described above and reduced electrostatic repulsions. As a selection of aggregating peptides did not occur upon addition of WPI, it is assumed that the peptides interact with the protein with the same affinity. When there was an excess of protein in the system, less aggregation was observed, meaning that the glue network of peptides was disrupted into soluble protein-peptide adducts. That proves again that non-covalent interactions hold the peptide network. However, it cannot be excluded that the free SH group of the peptides could form covalent protein-peptide interactions.

3.5 Conclusions

The present results, based on response surface methodology, showed that there is an optimal amount of added intact WPI that could interact with aggregating peptides, yielding a maximum amount of aggregated material in which the peptide to protein molar ratio was of around 6. Protein-peptide interactions were mainly of hydrophobic nature. The aggregates consisted of a network of peptides, mainly β -lg AB [f1-45], β -lg AB [f90-108] and α -la [f50-113], and in which β -lg was included.

Acknowledgements

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CHAPTER 4

Hydrolysis of whey protein isolate with *Bacillus licheniformis* protease.

1. Fractionation and identification of aggregating peptides

Abstract

The objective of this work was to identify the dominant aggregating peptides from a whey protein hydrolysate (degree of hydrolysis of 6.8%) obtained with *Bacillus licheniformis* protease. The aggregating peptides were fractionated with preparative reversed-phase chromatography and identified with liquid chromatography-mass spectrometry.

The results showed that the dominant aggregating peptide, at pH 7.0, was β -lg AB [f1-45]. In addition, the peptides β -lg AB [f90-108]-S-S- α -la [f50-113], α -la [f12-49]-S-S- α -la [f50-113], β -lg AB [f90-108]-S-S- β -lg AB [f90-108], β -lg A [f90-157] and β -lg AB [f135-157/158] were also identified as main aggregating peptides. The results further showed that aggregation, via hydrophobic interactions, prevented further digestion (at pH 8.0), thereby explaining the large size of the aggregating peptides. It is hypothesized that *Bacillus licheniformis* protease breaks down hydrophilic segments in the substrate and, therefore, preserves hydrophobic segments that aggregate once exposed to the solvent.

Key words: *Bacillus licheniformis* protease; fractionation; peptide identification; aggregation; whey protein isolate

4.1. Introduction

Hydrolysis is usually used to improve the solubility of proteinaceous material. For example, whey protein hydrolysates are usually more soluble and more heat stable than intact protein. For example, trypsin (specific for Lys and Arg residues) hydrolysis inhibits the heat-induced gelation of whey proteins (Ju et al., 1995). As well, hydrolysis of soy proteins usually enhances their solubility (Inouye et al., 2002). For example, trypsin and Alcalase 0.6L (a non-specific commercial protease preparation) generated soy hydrolysates that show good solubility (around 50%) at pH 4.5, a pH at which intact soy proteins are insoluble (Kim et al., 1990).

However, both limited and extensive hydrolysis of globular proteins can also lead to aggregation and gelation. A lot of research has been performed on the hydrolysis of whey proteins with *Bacillus licheniformis* protease (BLP; glutamyl endopeptidase) since Otte and co-workers (Otte et al., 1996) showed that limited hydrolysis, at a degree of hydrolysis (DH) of approximately 2%, led to formation of peptide aggregates, which formed a gel. As the aggregates could be solubilized in either sodium dodecyl sulfate, urea or at extreme pH values, mainly electrostatic and hydrophobic interactions were concluded to be the major interacting forces (Otte et al., 1997). The aggregates formed upon hydrolysis of β -lactoglobulin (β -lg), the main protein in bovine whey, consisted of 6 to 7 major peptides (2-6 kDa; Otte et al., 2000a). According to the work of the same authors, the fragment β -lg [f135-158] was be initiator of aggregation, since it was present at high concentrations in the aggregates, as determined with MALDI-TOF mass spectrometry.

In addition, Doucet and co-workers (Doucet et al., 2001) observed that aggregation and gelation of whey protein isolate occurred during extensive hydrolysis (DH>18%) with Alcalase 2.4L ®, at a high protein concentration (20% w/v) and at pH 6.0. Alcalase 2.4L ® is a *Bacillus licheniformis* protease preparation, with Subtilisin Carlsberg (preference for large uncharged residues) as main proteolytic component. As reported later (Doucet et al., 2003a), glutamyl endopeptidase activity is also present in Alcalase 2.4L ®. Gelation occurred by aggregation of peptides with an average chain length of 4.3 residues, mainly via hydrophobic interactions (Doucet et al., 2003b). Spellman and co-workers (Spellman et al., 2005) further showed, after isolating subtilisin and glutamyl endopeptidase activities from Alcalase 2.4L ®, that the glutamyl endopeptidase activity is responsible for the peptide aggregation in whey protein hydrolysates obtained with Alcalase 2.4L ®.

In complement to these findings, we reported in a former study (Chapter 2) that, within whey protein isolate hydrolysates produced with BLP, the extent of aggregation increased with increasing the degree of hydrolysis, until the experimental end point of hydrolysis (DH 6.8%). The proportion of the peptides that aggregated was around 45% in the

hydrolysate with a DH 6.8%, and was not affected by variations in temperature or ionic strength.

The objective of the present work is to identify aggregating peptides in a whey protein isolate hydrolysate produced by BLP at DH 6.8% and to understand the peptide aggregation. To this end, aggregating peptides were isolated from a hydrolysate with a DH 6.8% and fractionated with preparative reversed-phase chromatography. Upon subsequent peptide identification, hypotheses on mechanism of enzyme-induced aggregation of whey proteins were postulated.

4.2. Materials and methods

4.2.1. Materials

A commercial whey protein isolate (WPI) powder (trade name Bipro, Davisco Foods International Inc., Le Sueur, MN, USA) was used for the experiments. According to the manufacturer, it consisted of 74.0 % (w/w) β -lactoglobulin (β -lg), 12.5 % (w/w) α -lactalbumin (α -la), 5.5 % (w/w) bovine serum albumin, and 5.5 % (w/w) immunoglobulins. The protein content of the powder was 93.4 % (w/w) and it contained 0.12 % (w/w) calcium. The enzyme used was a seryl proteinase from *Bacillus licheniformis* (BLP, product name NS-46007, batch PPA 6219; E.C. 3.4.21.19), specific for Glu-X bonds and to a lesser extent for Asp-X bonds (Breddam and Meldal, 1992). The enzyme was kindly provided by Novozymes (Novozymes A/S, Bagsvaerd, Denmark).

All reagents were of analytical grade and purchased from Sigma (Sigma Chemical CO, St Louis, MO, USA) or Merck (Darmstadt, Germany).

4.2.2. Hydrolyses

WPI was dispersed in Millipore water at a concentration of 120 mg g⁻¹ and stirred overnight at 4°C. The suspension was centrifuged for 30 min (19000 x g, 20°C) and the supernatant was subsequently filtered (Rezist 30GF92 and PVDF 0.45 μ m, Schleicher and Schuell GmbH, Dassel, Germany). After measuring the nitrogen content, using the Dumas method, the supernatant was diluted to 50 mg g⁻¹ with Millipore water, preheated at 40°C for 15 min and the pH was adjusted to pH 8.0, 5 min before addition of the enzyme solution. The enzyme/substrate ratio used was 1/100 (v/v) to reach a DH value of 6.8 %. During hydrolysis, the reaction mixture, incubated at 40°C, was maintained at pH 8.0 by addition of a 0.4 M NaOH solution in a pH-STAT (719 S Titrino, Metrohm Ion Analysis, Metrohm Ltd., Herisau, Switzerland). To permanently inactivate the enzymatic reaction of BLP (as verified with size exclusion chromatography), the pH of the hydrolysate, diluted to 20 mg mL⁻¹, was

adjusted to pH 2.0 with a 6.5 M trifluoroacetic acid (TFA) solution and kept for one hour at least.

A part of the hydrolysate was freeze-dried while the other part was used to isolate peptides aggregating at pH 7.0. For that purpose, the pH of the hydrolysate was brought from pH 2.0 to pH 7.0 with 2.5 M NH_4HCO_3 . After 1 h at room temperature, the hydrolysate was centrifuged (19000 x g, 20 min, 20°C) and the pellet was washed twice with water and freeze-dried. The supernatant was freeze-dried and desalted with preparative reversed-phase chromatography. Part of the pellet was fractionated into 4 fractions (P1, P2, P3 and P4) using preparative reversed-phase chromatography.

WPI and the fraction P3 were also hydrolyzed in presence of 4 M urea. A WPI solution, at a concentration of 20 mg g⁻¹, in 75 mM Tris-HCl buffer (pH 8.0) containing 4 M urea, 40°C, was hydrolyzed with BLP. The enzyme/substrate ratio used was 1/100 (v/v). The hydrolysis was stopped after 1 h by transfer to ice. The fraction P3 was suspended at 3.5 mg g⁻¹ in 75mM Tris-HCl buffer, with and without 4 M urea, 40°C, pH 8.0, and was hydrolysed with BLP. The enzyme/substrate ratio used was 1/250 (v/v). The hydrolysis was stopped after 3 h by transfer to ice. Samples were analyzed with reversed phase chromatography.

4.2.3. Preparative reversed-phase chromatography

Fractionation of the aggregating peptides (pellet) was performed with a preparative HPLC system (Waters, Milford, MA, USA) controlled by MassLynx Version 4.0 software using an XTerra® Prep MS C₁₈ OBD™ column (50x100 mm; bead diameter: 5 µm, Waters), at 20°C. The flow rate was 82.7 mL min⁻¹, column temperature 20°C, eluent A 0.07 % (v/v) TFA in 5 % (v/v) aqueous acetonitrile, and eluent B 0.05 % (v/v) TFA in acetonitrile. Sample preparation was as follows: the freeze-dried pellet was dissolved in 8 M guanidinium hydrochloride containing 5 % (v/v) acetonitrile and 0.07 % (v/v) TFA, at a concentration of 20 mg g⁻¹. A volume of 10 mL sample was injected onto the column. After 5 min isocratic elution with eluent A, further elution was obtained with a linear gradient from 0 to 31 % eluent B in 9.8 min, then from 31 to 40 % eluent B in 17.1 min, from 40 to 100 % B in 6.7 min, and 100 % B for 3.7 min. Detection was performed at 220 nm. Four fractions (P1, P2, P3 and P4) were collected as further shown in **Figure 4.3**.

Desalting of the supernatant, containing the non-aggregating peptides, was performed with the same equipment, column and eluents. Sample preparation was as follows: the freeze-dried supernatant was dissolved in 8 M guanidinium hydrochloride containing 5 % (v/v) aqueous acetonitrile and 0.07 % (v/v) TFA, at a concentration of 40 mg g⁻¹. A volume of 10 mL sample was injected onto the column. After 5 min isocratic elution with eluent A, peptides were collected upon elution with a linear gradient from 0 to 100 % eluent B in 5 min. Detection was performed at 220 nm.

Acetonitrile was evaporated from all the eluates with a rotative evaporator and samples were subsequently freeze-dried.

4.2.4. Nitrogen content determination

Nitrogen contents were measured using the combustion or Dumas method (AOAC, 1995) with a NA 2100 Protein nitrogen analyzer (CE Instruments, Milan, Italy). A 6.38 x N conversion factor was used to convert nitrogen content to protein content.

4.2.5. Analytical size exclusion chromatography

Experiments were performed with an Äkta Purifier System (GE Healthcare Bio-Sciences, Uppsala, Sweden) with Unicorn software. Samples were separated with an analytical Shodex Protein KW-802.5 column (300 x 8 mm; particle size: 7 µm; pore size: 500 Å; Showa Denko K. K., Kanagawa, Japan). The column was equilibrated with 6 M urea containing 30 % (v/v) aqueous acetonitrile and 0.1 % (v/v) TFA. The flow rate was 0.5 mL min⁻¹ and the column temperature 20°C. Sample preparation was as follows. The freeze-dried samples (hydrolysate, supernatant and pellet) were dissolved in 950 µL of 0.05 M Tris-HCl buffer containing 0.05 M dithiothreitol (DTT) and 8 M guanidinium hydrochloride (pH 8.0). After 2 h of incubation at room temperature, 50 µL of acetonitrile and 0.5 µL of TFA were added. The final peptide concentration was 1 mg mL⁻¹. Samples were also prepared under non-reducing conditions with the same sample preparation as above, without using Tris-HCl buffer and DTT. A volume of 20 µL sample was injected onto the column. Detection was performed at 220 nm. The chromatograms were normalized so that the area under the peaks in the supernatant and in the pellet samples represented 50% of the total area under the peaks in the hydrolysate sample because the proportion of aggregating material in the hydrolysate was ~50% (Chapter 2).

The column was calibrated using a low molecular weight gel filtration calibration kit (GE Healthcare Bio-Sciences). The kit contained blue dextran (2000 kDa), aldolase (42 kDa), ribonuclease A (13.7 kDa). In addition, aprotinin (6.5 kDa), insulin chain B (3.5 kDa), angiotensin I (1.296 kDa), bradykinin (1.06 kDa) (Sigma, product numbers A-6012, I-6383, A-9650 and B-3259, respectively) and leupeptin (0.426 kDa, F. Hoffmann-La Roche Ltd, Basel, Switzerland, product number 1017101) were used for calibration purposes.

4.2.6. Analytical reversed-phase chromatography

Samples obtained from WPI hydrolysis made at a WPI concentration of 50 mg g⁻¹ were separated on an analytical Vydac C8 column (208MS52; 250 x 2.1 mm; bead diameter: 5 µm; porosity: 300 nm; Dionex, Sunnyvale, CA, USA) by HPLC (Thermo Separation Products Inc., San Jose, CA, USA) with ChromQuest software. The flow rate was 0.2 mL min⁻¹, column temperature 20°C, eluent A 0.07 % (v/v) TFA in 5 % (v/v) acetonitrile,

and eluent B 0.05 % (v/v) TFA in acetonitrile. Sample preparation was as follows. The freeze-dried samples (hydrolysate, supernatant, pellet and peptide fractions) were dissolved in 950 μL of 0.05 M Tris-HCl buffer (pH 8.0) containing 0.05 M DTT and 8 M guanidinium hydrochloride. After 2 h of incubation at room temperature, 50 μL of acetonitrile and 0.5 μL of TFA were added. The final peptide concentration was 0.5 mg mL^{-1} . Samples were also prepared under non-reducing conditions with the same sample preparation as above, without using Tris-HCl buffer and DTT. The chromatograms were normalized so that the area under the peaks in the supernatant and in the pellet samples represented 50% of the total area under the peaks in the hydrolysate sample.

WPI and P3 hydrolysates obtained in presence or absence of urea during hydrolysis were analyzed as follows. A volume of 25 μL WPI hydrolysate (20 mg g^{-1}) and of 71 μL P3 hydrolysate (3.5 mg g^{-1}) were mixed with 450 μL and 404 μL , respectively, of 0.05 M Tris-HCl buffer (pH 8.0) containing 0.05 M dithiothreitol (DTT) and 8 M guanidinium hydrochloride. After 2 h of incubation at room temperature, 25 μL of acetonitrile and 0.25 μL of TFA were added. The final WPI hydrolysate and P3 hydrolysate concentrations were 1 and 0.5 mg mL^{-1} , respectively.

For all samples, a volume of 30 μL sample was injected onto the column. After 10 min isocratic elution with eluent A, further elution was obtained with a linear gradient from 0 to 55 % eluent B in 100 min, then from 55 to 100 % eluent B in 2 min and 100 % B for 20 min. Detection was performed at 220 nm.

4.2.7. Mass Spectrometry analysis

4.2.7.1. Electrospray mass spectrometry

Mass spectra were recorded with an electrospray ionisation mass spectrometer (LCQ ion-trap, Finnigan MAT 95, San Jose, CA, USA) connected to the reversed phase chromatography unit. It was operating in the positive mode using a spray voltage of 2 kV and a capillary temperature of 200°C. The capillary voltage was set at 45 kV and the tube lens voltage at 35 kV. Mass spectra were collected in a full mass scan, followed by a zoom scan and a MS/MS scan of the most intense ion in a window of 1.5-2 m/z and a 30-35 % relative collision energy. The apparatus and data were controlled by Xcalibur software. The accuracy of the mass determination is ± 0.3 Da. The theoretical masses of peptides were calculated using the program Protein Prospector MS Digest v 4.0.5 by P.R. Baker and K.R. Clauser (<http://prospector.ucsf.edu>). In addition, mass spectra were analyzed by Bioworks Browser software (Thermo Electron Corporation, Waltham, MA, USA), with a FASTA database containing the β -lg A, β -lg B and α -lg B sequences.

4.2.7.2. MALDI TOF mass spectrometry

The equipment used was an UltraFlex TOF (Brucker Daltonik GmbH, Bremen, Germany) in the positive mode. A volume of 1 μL of sample solution was mixed with 9 μL of matrix solution. The matrix solutions consisted of dimethoxy-4-hydroxycinnamic acid (SA, 10 mg) or α -cyano-4-hydroxycinnamic acid (α -CN, 10 mg) dissolved in 500 μL acetonitrile, 100 μL 3% (v/v) TFA and 400 μL water. The SA matrix was used to detect masses from 5 kDa to 100 kDa (with linear detector), and the α -CN matrix to detect masses < 5 kDa (with reflective detector). A volume of 1 μL of sample/matrix mixture was loaded onto a ground steel plate and left to dry at room temperature during 15 min. All samples were applied, in duplicate, with each of the two matrices. For the SA matrix, external calibration was performed with bovine insulin (5734.56 Da), ubiquitin (8565.89 Da), cytochrome C from horse heart (12361.09 Da and 6181.05 Da for single and double protonated molecules, respectively), and horse myoglobin (16952.55 Da and 8476.77 Da for single and double protonated molecules, respectively). For the α -CN matrix, the external calibration was performed with bradykinin (1-7; 757.85 Da), angiotensin II (1047.19 Da), angiotensin I (1297.48 Da), substance P (1348.64 Da), bombesin (1620.86 Da), rennin substrate (1760.02 Da), ACTH (clip 1-17; 2094.43 Da), ACTH (clip 18-39; 2466.68 Da), and somatostatin (3149.57 Da).

4.3. Results

4.3.1. Fractionation of aggregating peptides

The hydrolysate was first fractionated into aggregating (pellet) and non-aggregating peptides (supernatant) by centrifugation under non-reducing conditions at pH 7.0. The complete hydrolysate, the aggregating and the non-aggregating peptides were analyzed with reversed phase chromatography and size exclusion chromatography, under both reducing and non-reducing conditions (**Figures 4.1 and 4.2**).

Complete digestion of β -lg and α -la, the two main proteins in WPI, would create 20 or 38 peptides under reducing conditions, depending if the BLP would cleave after only Glu residues or after Glu and Asp residues, respectively (excluding the free Glu and Asp residues that would not be detected). As the hydrolysate contained at least 28 peptide peaks, according to reversed phase chromatography under reducing conditions (and at least 23 peptide peaks under non-reducing conditions), it can already be assumed that BLP cleaved after both Glu and Asp residues, but missed cleavage of some of these.

Under non-reducing conditions, the aggregating peptides (pellet) eluted later in the reversed phase chromatogram (**Figure 4.1**) compared to the non-aggregating peptides (supernatant). The pellet was, therefore, enriched in large and/or hydrophobic peptides. In

addition, the pellet was enriched in a peptide eluting at 82 min, under both reducing and non-reducing conditions and contained mainly three other peptide peaks (eluting at 58, 66 and 81 min) under non-reducing conditions.

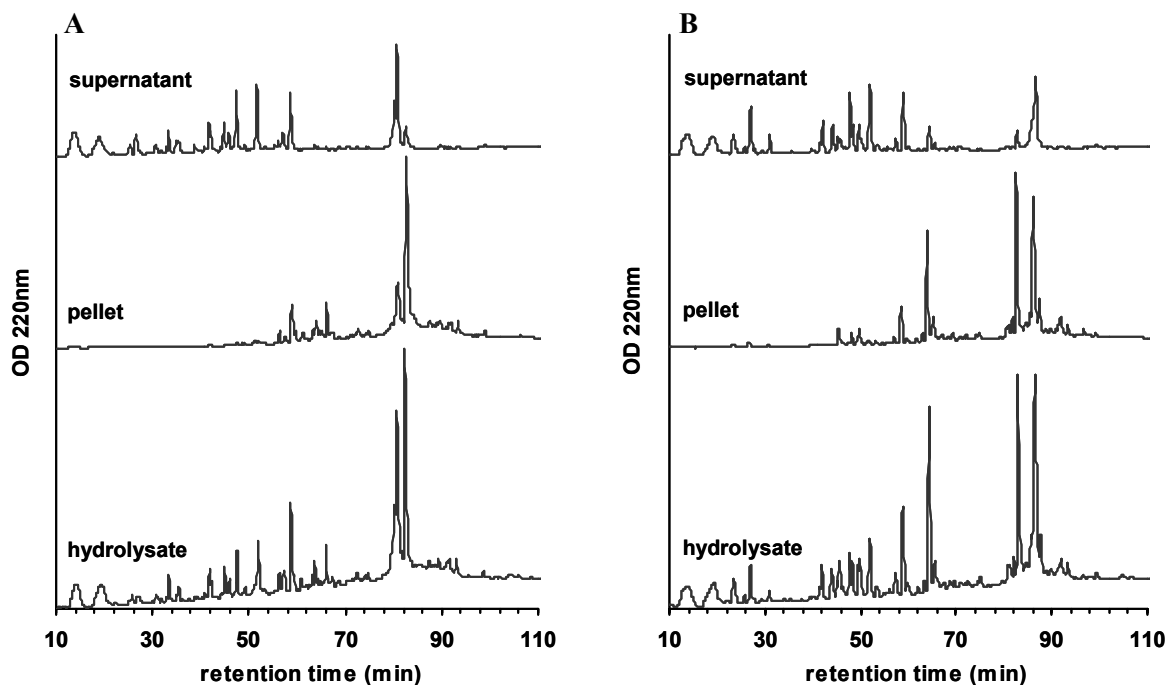


Figure 4.1. Reversed phase chromatograms, under non-reducing (A) and reducing (B) conditions, of the complete hydrolysate (DH 6.8%), and of the pellet and supernatant isolated from the complete hydrolysate by centrifugation.

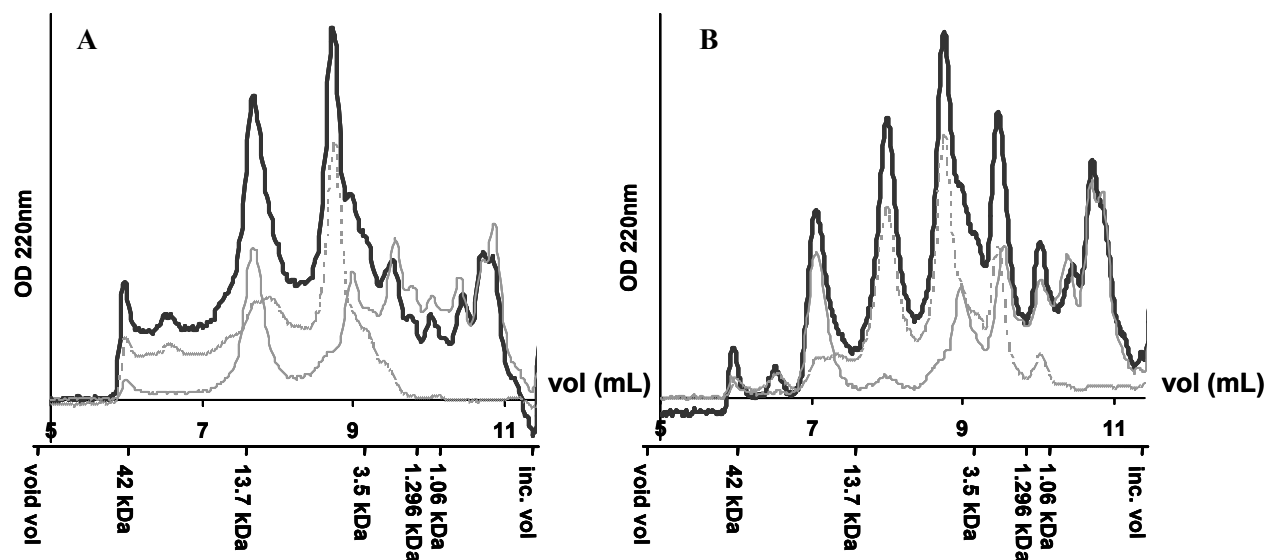


Figure 4.2. Size-exclusion chromatograms, under non-reducing (A) and reducing (B) conditions, of the complete hydrolysate (DH 6.8%, solid line), and of the pellet (dashed gray line) and supernatant (gray line) isolated from the complete hydrolysate by centrifugation.

According to the size exclusion patterns shown in **Figure 4.2**, remaining intact α -la, which eluted at 7.0 mL (Figure 2.1; from Chapter 2) under reducing conditions, was present in the supernatant after fractionation of the hydrolysate into supernatant and pellet. The hydrolysate contained mainly 8 peptide fractions, with an average mass around 2 kDa, under reducing conditions (Chapter 2). Under both reducing and non-reducing conditions, it was noticed that the pellet was enriched in larger peptides (eluting early) than the supernatant. The aggregating peptides consisted of 4 peptide fractions, under reducing conditions.

Preparative reversed phase chromatography (**Figure 4.3**) was used to further fractionate the aggregating peptides into four fractions (P1, P2, P3 and P4) as a function of the size and/or hydrophobicity of the peptides, under non-reducing conditions. The peaks in P1, P2, P3 and P4 made up about 35%, 18%, 22% and 25% of the total peak area in the pellet chromatogram, respectively.

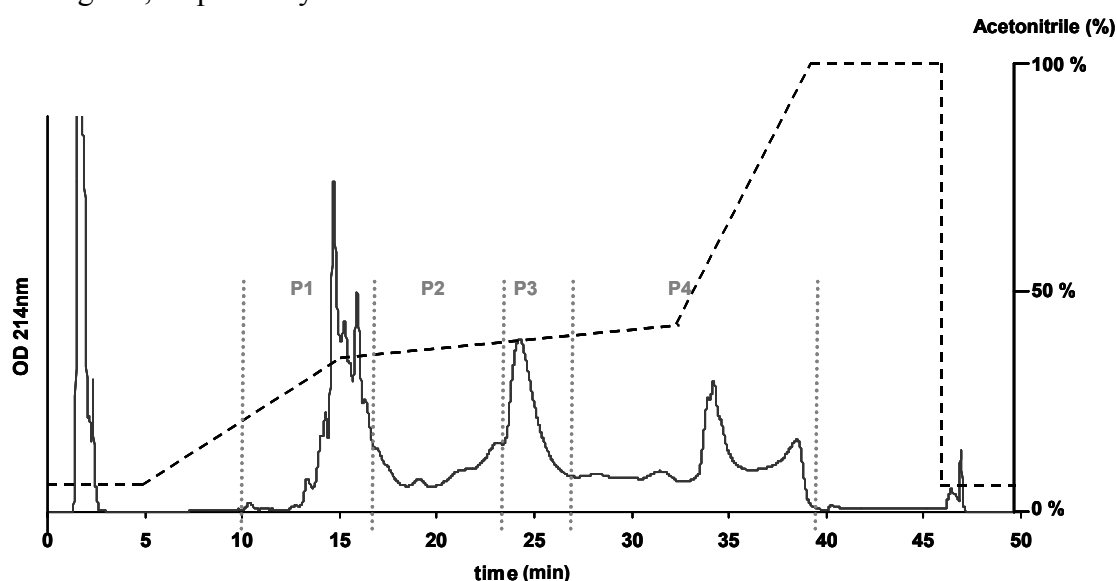


Figure 4.3. Preparative reversed phase chromatogram, under non-reducing conditions, of the pellet isolated from the complete hydrolysate by centrifugation. Four fractions were denoted as P1, P2, P3 and P4.

The four fractions were subsequently analyzed with analytical reversed phase chromatography (**Figure 4.4**) under both reducing and non-reducing conditions. Due to the different column materials, column geometries and elution gradients, the reversed phase chromatograms of preparative and analytical separations were different.

As expected, the peptides of the fractions P1 to P4 eluted at different retention time ranges (**Figures 4.4 and 4.5**). When going from non-reducing to reducing conditions, peptide peaks with high intensity appeared in the pellet, at 64 min (no 4) and at 86 min (letters j to n) and were assumed to originate from disulfide bridged fragments. The peptide peak at 64 min (no 4), in fractions P1, P2 and P4, under reducing conditions, was assumed to be disulfide linked with different peptides (assuming disulfide bond reshuffling) or to a peptide of

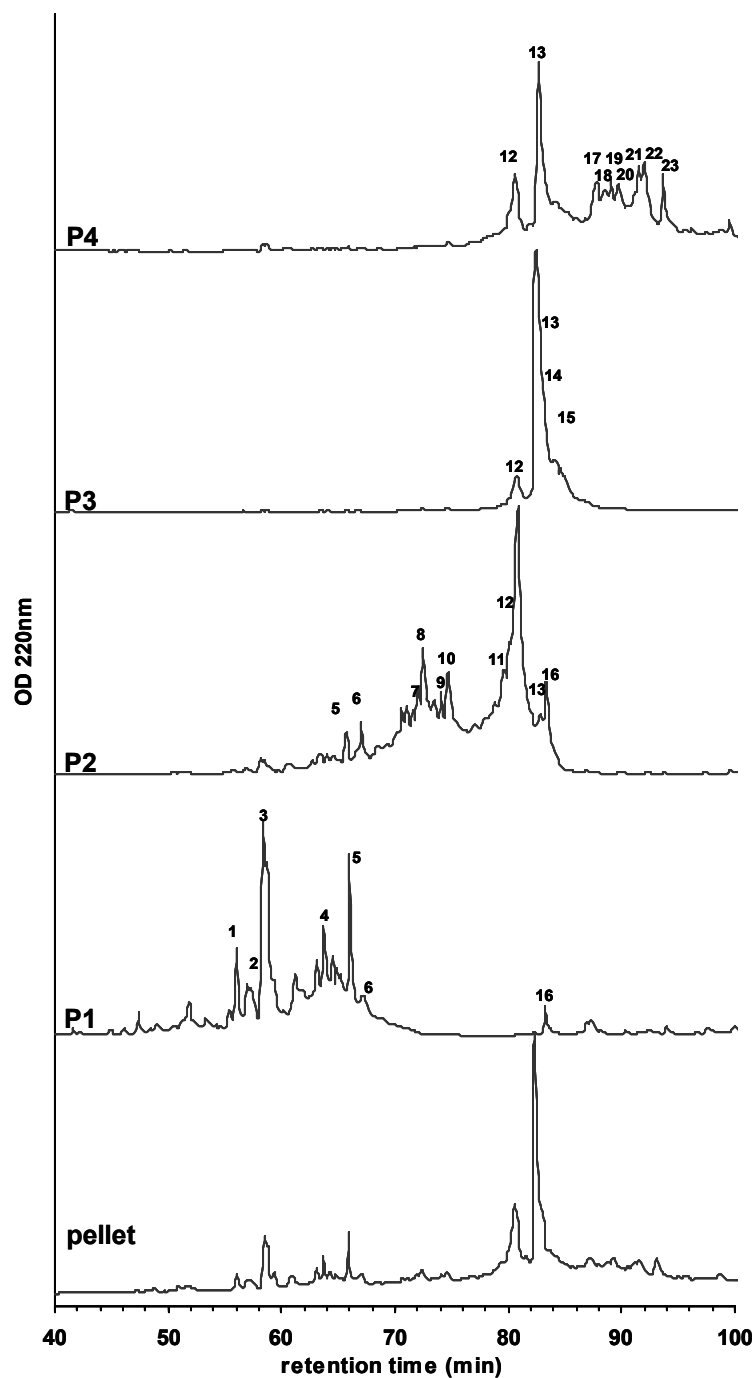


Figure 4.4. Reversed phase chromatograms, under non-reducing conditions, of the pellet and the four fractions isolated from it. Code for the peaks refers to **Tables 4.1**.

variable length (the enzyme can miss cleavage of the substrate at some positions) since fractions P1, P2 and P4 did not have one common peak under non-reducing conditions. The peptide peak eluting at 84 min (no 13), was the major component of fraction P3, and was also present in fraction P4. The peptide fraction P3 seemed rather pure and did not originate from disulfide bridged fragments since the peptide peak had the same retention time under reducing and non-reducing conditions.

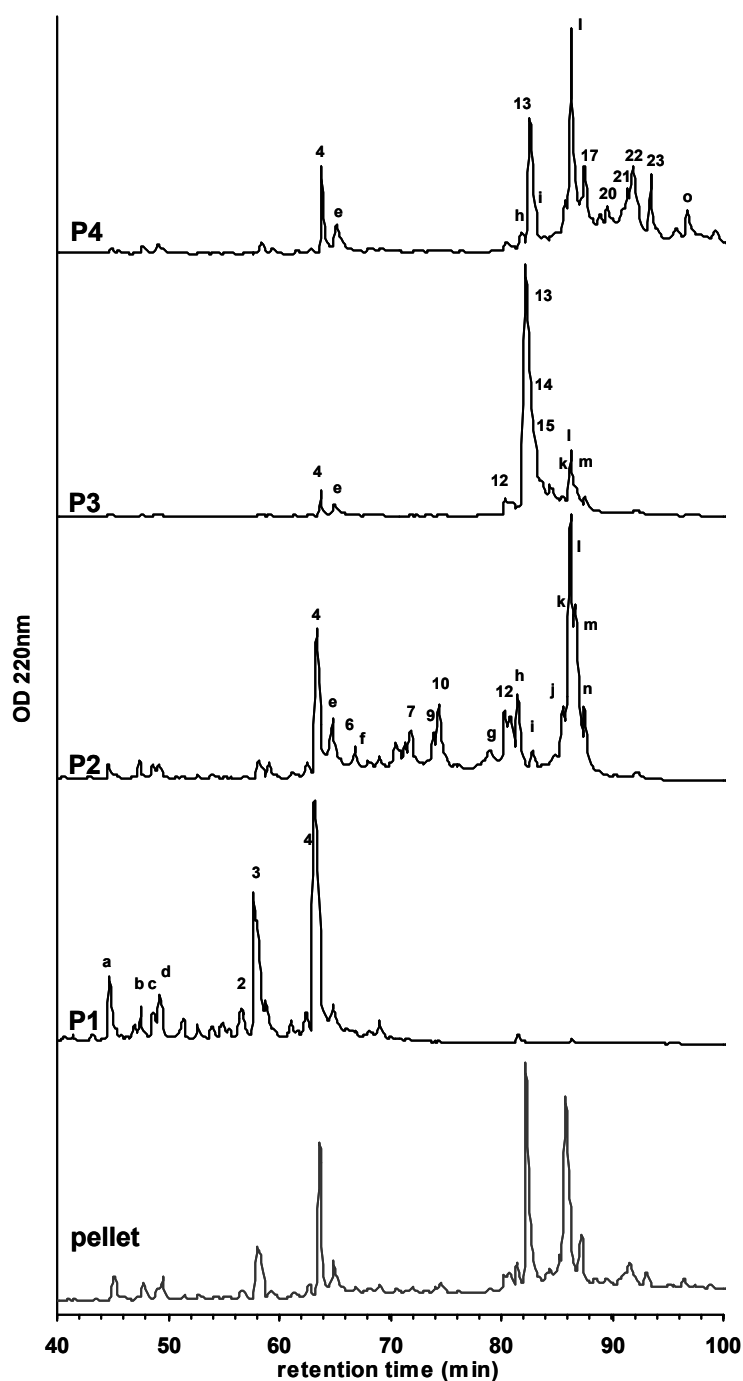


Figure 4.5. Reversed phase chromatograms, under reducing conditions, of the pellet and the four fractions isolated from it. Code for the peaks refers to **Tables 4.1 and 4.2**.

4.3.2. Identification of aggregating peptides

Table 4.1 lists the peptides present at both reducing and non-reducing conditions (peptide peaks denoted by numbers in **Figures 4.4 and 4.5**), and **Table 4.2** lists the peptides only present at reducing conditions (peptide peaks denoted by letters in **Figure 4.5**). The identification was based on the masses determined with both MALDI TOF MS (non-reducing conditions), LC-MS (reducing and non-reducing conditions), the primary structures of β -lg

A, β -lg B and α -la B, and the specificity of the enzyme. Identification of the peptides present at reducing conditions helped identifying peptides present at the non-reduced conditions.

In total, 23 peaks were considered under non-reducing conditions, containing 26 peptides identified deriving from β -lg, two peptides identified deriving from α -la, and as well 9 disulfide linked peptides. Additionally, 15 peaks were considered under reducing conditions, containing 9 peptides identified deriving from β -lg and 11 peptides identified deriving from α -la. Under non-reducing conditions, the masses of the non-disulfide linked peptides ranged from ~1.4 to 14.2 kDa and those of the disulfide linked peptides ranged from ~3.3 to 11.6 kDa.

It was remarkable that the fragment β -lg AB [f90-108] (peptide no 4 under reducing conditions) was present in the aggregates either non-disulfide linked or linked via a disulfide

Table 4.1. MS results for the peptides present under non-reducing conditions only (bold) and under both non-reducing and reducing conditions (not bold) in the fractions separated by RP-HPLC (Figures 4.4 and 4.5).

RP-HPLC peak	RT (min)	Measured masses (Da)	Possible fragment	Theoretical mass (Da)
1	56	3335.8	β-lg AB [f66-74]-S-S-β-lg AB [f90-108]	3336.8
2	57.4	2435.2	β -lg AB [f138-158]	2436.3
		2307.3	β -lg AB [f138-157]	2307.3
3	58.3	2826.3	β -lg AB [f135-158]	2826.5
		2699.2	β -lg AB [f135-157]	2697.5
4	63.7	2336.8	β -lg AB [f90-108]	2335.2
5	66	4668.0	β-lg AB [f90-108]-S-S-β-lg AB [f90-108]	4668.4
6	67	2173.7	β -lg AB [fX-45] ¹	
7	72.1	3608.5	# ²	
8	72.4	5580.0	β-lg AB [f90-108]-S-S-# (3245.8 Da)	5580.9
9	74.1	1395.5	β -lg AB [f52-62]	1343.7
10	74.7	5637.2	β -lg A [f86-134]	5633.7
		3695.9	β -lg AB [f12-45]	3695.9
11	79.9	4797.3	#	
		8404.3	α-la [f1-49]-S-S-α-la [f12-37]	8408.0
		#	#	
		#	#	
12	80.7	3553.8	β -lg AB [f1-33]	3584.9
		3750.2	β -lg A [f99-130] or β -lg AB [f131-162]* ³	3750.8
		4276.5	#	
		5790.1	β -lg AB [f1-53] 2ox	5765.0
		9740.1	β-lg AB [f90-108]-S-S-α-la [f50-113]	9736.7
		11576.5	α -la [f12/15-113/116]	11574.5
		11582.4	α-la [f12-49]-S-S-α-la [f50-113]	11591.5
		14177.1	α -la [f1-123]	14177.8

Table 4.1. (continuation)

RP-HPLC peak	RT (min)	Measured masses (Da)	Possible fragment	Theoretical mass (Da)
13	82.5	4895.6	β -lg AB [f1-45]	4895.6
14	82.6	5561.4	β -lg AB [f1-51]	5565.7
		4767.8	β -lg AB [f1-44]	4766.5
15	83.3	5880.1	β -lg AB [f1-53] 2ox	5765.0
		4956.6	β -lg B [f46-98]	4959.3
		6234.4	β -lg B [f109-162]	6135.0
		#	#	
16	83.5	#	#	
17	87.9	7706.2	β -lg A [f90-157]	7868.9
18	88.7	#	#	?
19	89.1	6568.3	β -lg A [f75-129]	6301.2
20	89.7	7577.8	β -lg B [f1-65]	7185.7
		8420.3	#	
		6137.1	β -lg A [f12-65]	6044.1
		4083.2	#	
		#	#	
21	91.6	3448.2	β -lg A [f34-62]	3351.8
22	92	5808.6	β -lg AB [f1-55]	5975.1
		3594.2	β -lg B [f45-74]	3422.8
23	94.7	4467.9	β -lg AB [f1-X]	

¹ peptide may result from non specific cleavage

² no identification based on mass (no fragmentation pattern available)

³ peptide present or mass present, but not identified

bond to different peptides: β -lg AB [f66-74] and α -la [f50-113]. It was also present as a covalently linked dimer. The peptide β -lg AB [f90-108] contains a cysteine at position 106 that is involved in a disulfide bridge with the cysteine at position 119 in the parental β -lg. Therefore, the disulfide bridge 106-119, although buried in the interior of the native protein, could take part in disulfide bridge reshuffling with peptides from β -lg (as was already observed by Otte and co-workers - Otte et al., 2000) and peptides from α -la.

According to **Figures 4.4 and 4.5** and to **Tables 4.1 and 4.2**, the fraction P1 consisted mainly of the peptides β -lg AB [f135-157/158] and the peptide β -lg AB [f90-108]-S-S- β -lg AB [f90-108]. The fraction P2 consisted mainly of β -lg AB [f90-108]-S-S- α -la [f50-113]. It also contained large (disulfide linked and non-disulfide linked) peptides (8.4-14.2 kDa) of truncated α -la that are α -la [f12-49]-S-S- α -la [f50-113], α -la [f1-123] and α -la [f12/15-113/116]. The fraction P3 contained mainly the peptide β -lg AB [f1-45]. The fraction P4 contained β -lg AB [f1-45] and other peptides with higher retention times (peaks 20 to 23) having a partial common sequence with the fragment β -lg AB [f1-45] (see section 4.3.3); it

Table 4.2. MS results for the peptides only present in reducing conditions under the fractions separated by RP-HPLC (**Figure 4.5**).

RP-HPLC peak	RT (min)	Measured masses (Da)	Possible fragment	Theoretical mass (Da)
a	44.7	1447.7	β -lg B [f115-127]	1447.7
		1416.6	α -la [f26-37]	1416.6
b	47.5	2717.2	α -la [f26-49]	2717.2
c	48.8	2400.1	α -la [f26-46]	2400.1
d	49.3	1476.0	β -lg A [f115-127]	1475.7
e	64.8	4171.0	α -la [f12-49]	4174.0
		3857.4	α -la [f12-46]	3856.8
		2873.4	α -la [f12-37]	2873.4
f	66.8	5534.9	α -la [f1-49]	5536.6
g	79	5142.0	β -lg B [f113-157] ox	5139.6
		5016.8	β -lg B [f115-157]	4897.5
h	81.5	3245.8	#	
i	82.8	4053.1	α -la [f79-113]	4053.1
		3549.2	α -la [f83-113]	3549.8
j	85.5	2962.4	β -lg AB [f90-114]	2962.4
k	85.6	7421.4	α -la [f50-113] ox	7419.5
l	86.2	7403.5	α -la [f50-113]	7403.5
m	86.7	7092.0	β -lg B [f54-114] ox	7083.7
		#	#	
		#	#	
n	87.5	5880.2	β -lg B [f34-85] or β -lg B [f45-96]*	5877.2/5872.2
		6924.6	#	
		5052.6/8420	β -lg A [f115-158]*	5054.6
		#	#	
o	96.8	7215.6	β -lg A [f66-129]	7285.8

Legend: see **Table 4.1**.

also contained the peptides β -lg A [f90-157], α -la [f12-49]-S-S- α -la [f50-113] and β -lg AB [f90-108]-S-S- α -la [f50-113].

4.3.3. Peptide β -lg AB [f1-45]

The peptide β -lg AB [f1-45] was the main aggregating peptide in a WPI hydrolysate made with BLP at high DH, since it made up about 22% of the total peak area of the aggregating peptides present in the chromatogram of the total pellet (**Figure 4.3**). Beside β -lg AB [f1-45], the aggregating peptides comprised also peptides that had partial common sequence with it (**Tables 4.1 and 4.2**) like β -lg AB [f12-45] and β -lg AB [f1-33], that eluted before β -lg AB [f1-45] and like β -lg AB [f1-51], β -lg AB [f1-44], β -lg AB [f1-65], β -lg AB

[f12-65], β -lg AB [f34-62] and β -lg AB [f1-55] that eluted after β -lg AB [f1-45] in the reversed phase chromatogram. For this reason, it was allowed to study and interpret the hydrophobicity profile solely on the amino acid sequence of the peptide β -lg AB [f1-45] (**Figure 4.6**). It could be seen that within the peptide β -lg AB [f1-45], the segment β -lg AB [f20-34] is indeed relatively hydrophobic and, therefore, could strongly contribute to peptide aggregation.

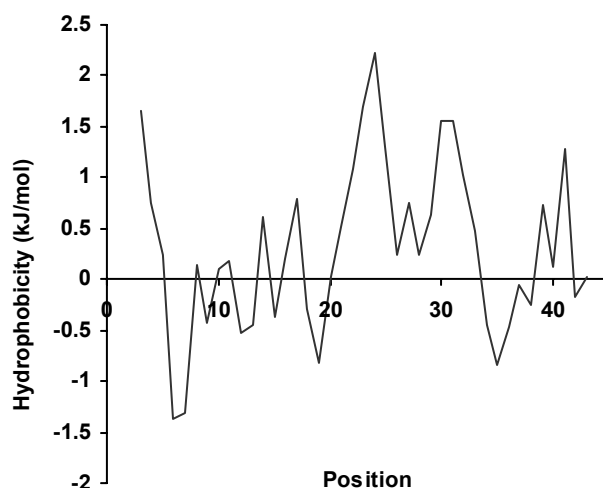


Figure 4.6. Schematic representation of hydrophobicity in the peptide β -lg AB [f1-45], according to Kyte and Doolittle (1982) with a window of 5 residues.

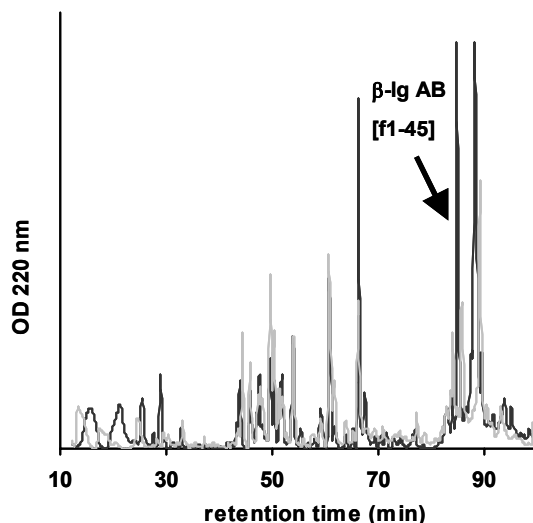


Figure 4.7. Reversed phase chromatograms, under reducing conditions, of hydrolysates of WPI made with BLP, in absence (black) and presence of 4 M urea (gray).

The fragment β -lg AB [f1-45] resisted digestion by BLP at pH 8.0 although the enzyme can both theoretically as well as at the conditions applied cleave at positions Asp 11, Asp 33 and Glu 44 as peptides such as β -lg AB [f1-33], β -lg AB [f12-45] and β -lg AB [f1-44] were observed. It was, therefore, assumed that as soon as the fragment β -lg AB [f1-45] was created, it aggregated, thereby preventing further hydrolysis. In order to verify this

assumption, hydrolysis of both WPI and the fraction P3 in the presence of urea was performed. The hydrolysates were subsequently analyzed with reversed phase chromatography. It was first positively verified that the BLP was active in presence of 4 M urea as WPI could be digested into many peptides (**Figure 4.7**). As shown in the reversed phase chromatograms in **Figure 4.7**, the fragment β -lg AB [f1-45] could be digested by BLP in presence of 4 M urea as there was a considerable decrease in the height of the peak containing this peptide. It was cleaved at least at position Asp 33, since the peptide β -lg AB [f34-45] was produced upon hydrolysis of the fraction P3 (**not shown**). From that last result, it was confirmed that peptide aggregation occurred during hydrolysis at pH 8.0, despite not detected after centrifugation of the hydrolysate at this pH value. As well, it was confirmed that aggregation of the peptide β -lg AB [f1-45] prevented its further digestion.

4.4. Discussion

4.4.1 Characteristics of the main aggregating peptides

Within the whey protein isolate hydrolysates produced with BLP, the extent of aggregation at neutral pH is the highest when the experimental end point of hydrolysis (DH 6.8%) is reached (Chapter 2). The proportion of peptides that aggregate is around 45% at this point. The proportion of aggregation is independent of temperature or ionic strength variations. Therefore, it was concluded that hydrophobic interactions dominate peptide aggregation and that the peptides do not possess substantial remaining secondary structure (Chapter 2).

Peptides originating from only β -lg substrate or from only α -la substrate can aggregate since enzyme-induced aggregation and gelation of β -lg (Otte et al., 1997) and α -la (Ipsen et al., 2001b) was reported. According to the results, all the peptides listed in the **Table 4.1** can aggregate, but it is clear from a quantitative point of view that the main aggregating peptide is β -lg AB [f1-45]. Next to this, the peptides β -lg AB [f90-108]-S-S- α -la [f50-113], α -la [f12-49]-S-S- α -la [f50-113], β -lg AB [f90-108]-S-S- β -lg AB [f90-108], β -lg A [f90-157] and β -lg AB [f135-157/158] are also present in considerable amounts. The main aggregating peptides are, therefore, either single fragments or native/non-native disulfide bridged fragments, the latter mainly involving β -lg AB [f90-108] (**Figure 4.8**).

The masses of the aggregating peptides largely deviate from the estimated average masses of the peptides in the hydrolysate (2 kDa; Chapter 2) since they are 2.8, 4.9 and 7.9 kDa (β -lg AB [f135-158], β -lg AB [f1-45] and β -lg A [f90-157], respectively) for the single fragments and 4.7, 9.7 and 11.6 kDa (β -lg AB [f90-108]-S-S- β -lg AB [f90-108], β -lg AB [f90-108]-S-S- α -la [f50-113] and α -la [f12-49]-S-S- α -la [f50-113], respectively) for the disulfide bridged fragments. Therefore, peptide distribution between pellet and supernatant is

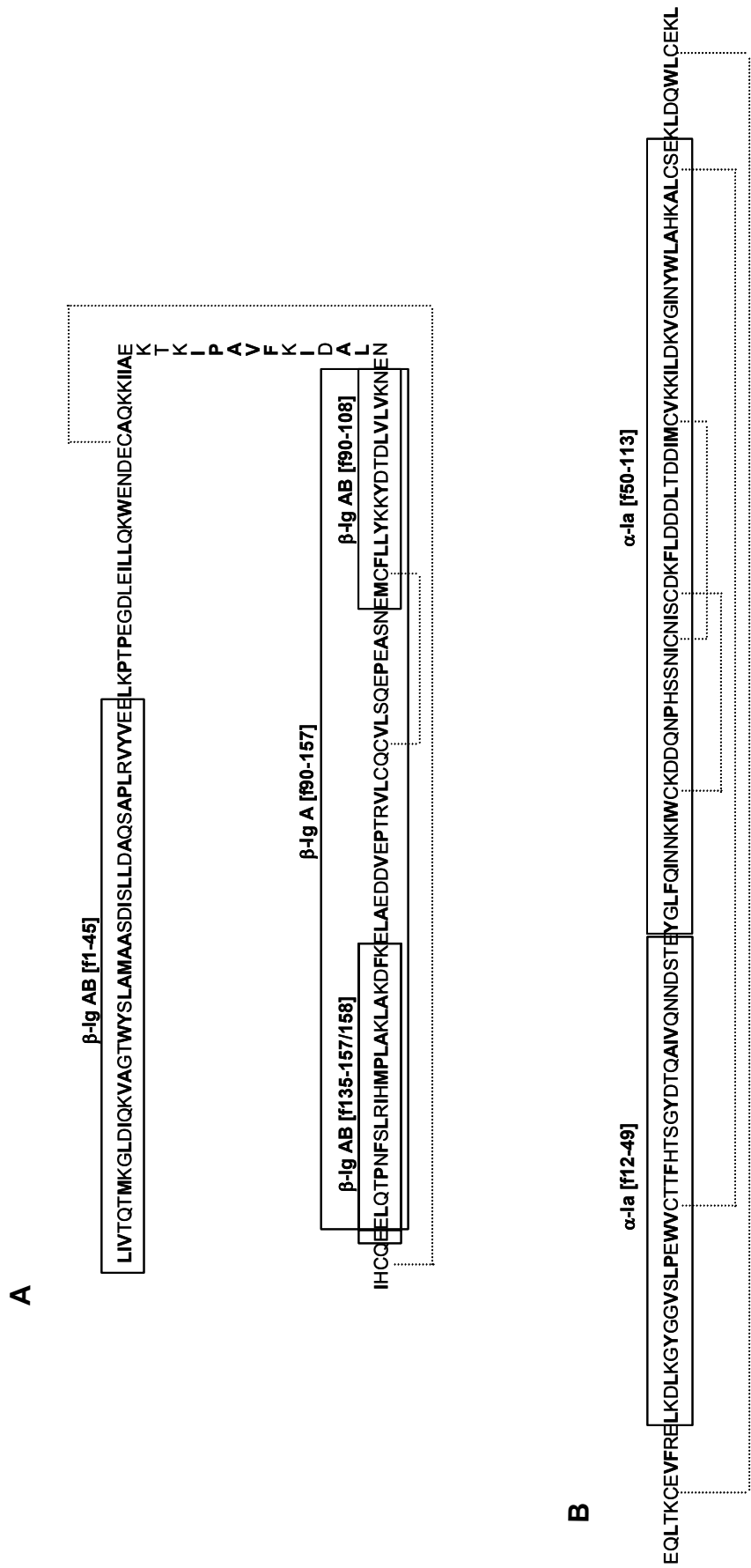


Figure 4.8. Amino acid sequences of bovine β-Ig A (A) and bovine α-Ia B (B). The dotted lines indicate the native disulfide bonds. Hydrophobic residues are in bold. The sequences of the main aggregating peptides are framed.

Table 4.3. Characteristics of the main aggregating peptides in a hydrolysate of WPI made with BLP.

Peptide	pI ^a	MW (Da)	Hf _{av} (kcal res ⁻¹) ^b	Maximum size of hydrophobic segment (aa) ^c	Net charge at	
					pH 2.0	pH 7.0
β -lg A	5.18 ^d	18367.3	1.23	14	+18.1	-9.2
β -lg AB [f1-45]	4.14	4895.6	1.19	14	+3.6	-2.0
β -lg AB [f90-108]	6.97	2335.2	1.46	5	+3.7	0.0
β -lg A [f90-157]	4.52	7868.9	1.21	9	+9.6	-4.0
β -lg AB [f135-157/158]	9.86	2826.5	1.31	5	+5.7	+1.1
α -la B	4.30 ^d	14186.0	1.15	6	+16.4	-6.7
α -la [f12-49]	4.14	4174.0	0.95	6	+2.7	-2.9
α -la [f50-113]	4.64	7403.5	1.19	6	+9.5	-2.9

^a Isoelectric point was determined from ExPASy Proteomics tools. ^b Average hydrophobicity was calculated according to the method of Bigelow (1967). ^c according to Kyte and Doolittle (1982) with a window of 5 residues (aa means amino acid). ^d from Walstra et al., 2006.

such that the largest peptides of the hydrolysate are the aggregating ones and the smallest peptides are the non-aggregating ones.

Experiments demonstrated that hydrophobic interactions dominate peptide aggregation (Chapter 2). The peptide β -lg AB [f90-108], and its covalent dimer, possesses the highest average hydrophobicity (1.46 kcal res⁻¹) of the main aggregating peptides, which, together with a net charge of 0 at neutral pH (**Table 4.3**), might contribute to aggregation. For comparison, the average hydrophobicity of β -lg is 1.23 kcal res⁻¹ and that of α -la is 1.15 kcal res⁻¹. It is assumed that the fragment β -lg AB [f90-108] is an aggregating peptide that could act as an “anchor”, since it is present in the aggregates linked via a disulfide bond to various peptides. The peptide β -lg AB [f1-45] has a lower average hydrophobicity than intact β -lg (**Table 4.3**), but contains hydrophobic amino acid residues belonging to an assumed exposed hydrophobic segment at the region β -lg AB [f20-34] (**Figure 4.6**) that could contribute to aggregation. According to reversed phase chromatography, the fragment α -la [f50-113], was more hydrophobic than its parental non-reduced peptides (α -la [f12-49]-S-S- α -la [f50-113] and β -lg AB [f90-108]-S-S- α -la [f50-113]), since the latter eluted earlier under non-reducing conditions. Although no hydrophobic segment was observed in the sequence of α -la [f50-113] (**Table 4.3**), which has a lower average hydrophobicity than intact α -la, surface hydrophobicity could appear from folding of the peptide and offer exposed interaction sites for hydrophobic interactions.

Otte and co-workers (Otte et al., 2000a) reported that the peptide β -lg [f135-158] is present at high concentrations in the enzyme-induced aggregates and responsible for initiation of aggregation in β -lg hydrolysates made with BLP. This peptide is indeed present in the aggregates (peak 3 in **Figures 4.4 and 4.5**), but it is not the dominating peptide when comparing its abundance with the other peptide peaks, assuming an equal weight based

response factor at 220 nm of the different peptides. In addition, the peptide β -lg AB [f135-157/158] is present in both the pellet and the supernatant, as shown in **Figure 4.1**, while the peptide β -lg AB [f1-45] is not. This indicates that the peptide β -lg [f135-157/158] is more soluble than the peptide β -lg AB [f1-45]. The average hydrophobicity of the peptide β -lg [f135-157/158] is higher than that of intact β -lg and also it is the only peptide from the main aggregating ones that is slightly positively charged at neutral pH (**Table 4.3**), allowing electrostatic interactions with the other negatively charged aggregating peptides (Otte et al., 2000a).

The presence of the peptide β -lg AB [f90-108]-S-S- α -la [f50-113] in the aggregates, having a fragment from β -lg and a fragment from α -la, is an indication of SH/SS exchanges during hydrolysis at pH 8.0. At this pH, the sulfhydryl groups are more reactive than at pH 7.0 since thiol groups have a pKa of 9.0-9.5. As enzyme-induced aggregation of WPI was reported upon hydrolysis at pH 7.0 (Otte et al., 1996), with assumedly less SH/SS exchanges than at pH 8.0, disulfide linked peptides are presumably not required for aggregation. However, as each of the fragments could aggregate without the other one, it is not known whether peptide aggregation proceeds faster when disulfide linked peptides are involved.

4.4.2 Mechanism of enzyme-induced aggregation of whey protein isolate

In the present situation, BLP or glutamyl endopeptidase, by its specificity, cleaves hydrophilic segments in a polypeptide and, therefore, preserves hydrophobic segments. According to our results, BLP first cleaves the substrate after Glu residues since it is more specific for Glu than for Asp residues (Breddam and Meldal, 1992). Because of the uneven partition of the Glu residues in β -lg and α -la, large fragments such as β -lg AB [f1-45] and α -la [f50-113] and also β -lg AB [f90-108] are produced. Next, these peptides containing Asp residues are hardly further digested because, as the peptides produced aggregate via hydrophobic interactions, cleavage sites involving Asp residues become less accessible. This explains why aggregating peptides are rather large peptides at high DH.

It is assumed that aggregation of whey protein hydrolysates could occur with two different mechanisms since in a hydrolysate made with glutamyl endopeptidase activity (BLP), aggregation occurs at relatively limited hydrolysis (DH ~2 to 7%, pH 7.0) and involves rather large peptides (2-10 kDa), while in a hydrolysate made with both glutamyl endopeptidase and subtilisin activities (Alcalase 2.4L ®), aggregation occurs after extensive hydrolysis (DH > 18%, pH 6.0) and involves small peptides (<2 kDa; Doucet et al., 2003b). In the case aggregation occurs at limited hydrolysis, it possibly requires an enzyme with narrow specificity for polar residues and a substrate containing hydrophobic segments. In the case aggregation occurred after extensive hydrolysis, it possibly required several enzymes with specificity for both polar and non polar residues to cleave most hydrophilic and hydrophobic segments so that resulting oligopeptides, having a net charge of 0 at pH 6.0 (Doucet et al., 2003a), at high concentration, physically aggregated. This hypothesis is supported by the fact

that Spellmann and co-workers (Spellmann et al., 2005) showed that a hydrolysate of whey proteins made with subtilisin activity only does not aggregate but does when glutamyl endopeptidase activity is added. This further indicates that adding glutamyl endopeptidase breaks down the hydrophilic segments and allows aggregation.

4.5. Conclusions

The present result show that the main aggregating peptide in a WPI hydrolysate made with BLP, at high DH, is β -lg AB [f1-45]. It is hypothesized that BLP breaks down hydrophilic segments, containing Glu residues, and, therefore, preserves hydrophobic segments that can participate in aggregation. Comparative aggregating properties of the isolated peptide fractions will be investigated in a future study.

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Hydrolysis of whey protein isolate with *Bacillus licheniformis* protease.

2. Aggregating capacities of peptide fractions

Abstract

In the previous study peptides aggregating at pH 7.0 derived from a whey protein hydrolysate made with *Bacillus licheniformis* protease (BLP) were fractionated and identified. The objective of this work was to investigate the solubility of the fractionated aggregating peptides, as a function of concentration, and their aggregating capacities towards added intact proteins. The amount of aggregated material and the composition of the aggregates obtained were measured by nitrogen content and size exclusion chromatography, respectively.

The results showed that the aggregating peptides consist of insoluble peptides, mainly β -lg AB [f1-45], β -lg A [f90-157] and peptides having a partial common sequence with the fragment β -lg AB [f1-45]. Hydrophobic interactions drove peptide aggregation. These peptides co-aggregated, assumedly via hydrophobic interactions, other relatively more soluble peptides (β -lg AB [f90-108]-S-S- α -la [f50-113] and α -la [f12-49]-S-S- α -la [f50-113]). It was also shown that aggregating peptides could aggregate intact protein non specifically since the same peptides were involved in the aggregation of whey proteins, β -cn and BSA. Both insoluble and partly insoluble peptides were required for the aggregation of intact protein.

Key words: *Bacillus licheniformis* protease; whey protein isolate; peptides; aggregation; solubility

5.1 Introduction

In the first part of this study (Chapter 4) peptides aggregating at pH 7.0 obtained from a whey protein hydrolysate made with *Bacillus licheniformis* protease (BLP) were fractionated and identified. The dominant aggregating peptide was β -lg AB [f1-45]. This peptide has a mass of 4.9 kDa and a negative net charge at neutral pH. In addition, it contains a relatively hydrophobic segment that could strongly contribute to peptide aggregation. Otte and co-workers (Otte et al., 2000a) and Doucet and co-workers (Doucet et al., 2005) earlier reported about aggregating peptides from whey proteins. It was shown (Otte et al., 2000a) that in hydrolysates of β -lactoglobulin (β -lg) made with BLP, once the solution was saturated by certain peptides, the latter appeared in the pellet with increasing amounts. Mainly electrostatic and hydrophobic interactions were involved (Otte et al., 1997) in the aggregation of the 6 to 7 major peptides present in the aggregates (Otte et al., 2000a). Interestingly, in hydrolysates of β -lg made with Subtilisin Carlsberg, the same peptides were present in the supernatant and the precipitate (Doucet et al., 2005) during hydrolysis. Mainly hydrophobic interactions were involved in aggregation of these peptides that had an average chain length of 4.3 residues, an average hydrophobicity of 1.0-1.5 kcal per residue, and a net charge of 0 at pH 6.0 (Doucet et al., 2003a). In addition, peptide-peptide interactions leading to aggregation were already reported in tryptic hydrolysates of β -lg at acidic conditions (Groleau et al., 2003). Mainly hydrophobic interactions were involved in the aggregation of peptides identified as β -lg [f1-8], β -lg [f15-20] and β -lg [f41-60]. In the latter study, peptide-peptide interactions were induced by selectively mixing isolated peptide fractions (Groleau et al., 2003).

We have already shown that the extent of aggregation within hydrolysates of whey proteins made with BLP increased with increasing degrees of hydrolysis (DH) (Chapter 2). These hydrolysates were able to aggregate added whey protein isolate (WPI) and the additional amount of aggregated material increased with increasing DH. Hydrophobic interactions dominated peptide-peptide interactions, while the protein-peptide interactions depended on the balance between hydrophobic attractions and electrostatic repulsions (Chapter 2). After further investigating the protein-peptide interactions (Chapter 3), it was found that there was an optimal amount of added intact WPI that could interact with aggregating peptides, yielding a maximal amount of aggregated material. Under these conditions the peptide/protein molar ratio was around 6. The aggregates consisted of a network of peptides, mainly β -lg AB [f1-45], β -lg AB [f90-108] and α -la [f50-113], in which β -lg was included. In the first part of this study, we have fractionated the aggregating peptides into four fractions according to their size and/or hydrophobicity. In the present study, we aimed at understanding the solubility behavior of fractionated aggregating peptides

and also at understanding their aggregation capacities towards the aggregation of intact protein.

5.2 Materials and methods

5.2.1 Materials

A commercial whey protein isolate (WPI) powder (trade name Bipro, Davisco Foods International Inc., Le Sueur, MN, USA) was used for the experiments. According to the manufacturer, it consisted of 74.0 % (w/w) β -lactoglobulin (β -lg), 12.5 % (w/w) α -lactalbumin (α -la), 5.5 % (w/w) bovine serum albumin, and 5.5 % (w/w) immunoglobulins. The protein content of the powder was 93.4 % (w/w) and it contained 0.12 % (w/w) calcium. Bovine β -casein (β -cn) was purchased from Eurial (Nantes, France) and bovine serum albumin (BSA) was from Sigma (product number A-4503; Sigma Chemical CO, St Louis, MO, USA). The enzyme used was a seryl proteinase from *Bacillus licheniformis* (BLP, product name NS-46007, batch PPA 6219; E.C. 3.4.21.19), specific for Glu-X bonds and to a lesser extent for Asp-X bonds (Breddam and Meldal, 1992). The enzyme was kindly provided by Novozymes (Novozymes A/S, Bagsvaerd, Denmark).

All reagents were of analytical grade and purchased from Sigma or Merck (Darmstadt, Germany).

5.2.2 Hydrolysis of WPI

WPI (50 mg g⁻¹) was hydrolyzed by BLP at pH 8.0 and 40°C to DH 6.8% as described previously (Chapter 4). After hydrolysis, the pH of the hydrolysate, diluted to 20 mg mL⁻¹, was adjusted to pH 2.0 with a 6.5 M trifluoroacetic acid (TFA) solution to permanently inactivate the enzymatic reaction of BLP. Part of the hydrolysate was freeze-dried while the other part was used to isolate peptides aggregating at pH 7.0 (pellet) from non-aggregating peptides (supernatant). Part of the peptides aggregating at pH 7.0 was further fractionated into 4 fractions (P1, P2, P3 and P4) with preparative reversed-phase chromatography as described in Chapter 4. Subsequently, all materials (hydrolysate, supernatant, pellet, and the fractions P1 to P4) were freeze-dried.

5.2.3 Solubility and aggregation experiments

Solubility experiments were performed with the total hydrolysate and peptide fractions in order to express the peptide concentration in the supernatant and the proportion of aggregation as a function of concentration. The freeze-dried peptide fractions were dissolved in 800 μ L of 53 mM sodium phosphate buffer, pH 8.0. Next, the pH was adjusted to pH 7.0 with 0.5 M NaOH or HCl solutions. The final peptide concentrations ranged from 3.0 to

23.22 mg g⁻¹. Each sample was divided into two parts. After incubation for 1 h at 40°C, one part was centrifuged (20 min at 19000 x g, at 20°C) while the other part was not centrifuged. In order to determine the extent of aggregation, the nitrogen contents (N) of the supernatant and of the non-centrifuged part (N₀) were determined using the Dumas method. The proportion of peptides that aggregate was defined as $(1 - N/N_0) \times 100 \%$. Aggregated material was defined as the material removed using the centrifugation and solvent conditions applied. Selected pellets, containing the aggregates, and supernatants were further analyzed with reversed phase chromatography.

Next, the capacities of aggregating intact protein of the various peptide fractions were determined. The protein aggregation capacity was defined as the amount of protein aggregated per mg of peptide. A mass consisting of either 10 mg of total hydrolysate or 5 mg of pellet, supernatant and fractions P1 to P4 was mixed with 1 mL of 53 mM sodium phosphate buffer, pH 7.3, containing 20 mg of intact WPI. The protein aggregation capacities of the pellet fraction and of the peptide fraction P4 were also determined using intact β -cn and BSA, following the same procedure. Next, the pH was adjusted to 7.0 with 0.5 M NaOH or HCl. Each sample was divided into two parts. One part (0.55 g) was incubated for 1 h at 40°C and centrifuged (15 min, 19000 x g, 20°C) while the other part (0.45 g) was left non-centrifuged (further denoted “total”). In order to determine the extent of aggregation, the nitrogen content (N) in the supernatant and in the non-centrifuged part (N₀) was determined using the Dumas method. The pellets, containing the aggregates, were washed twice with 53 mM sodium phosphate buffer pH 7.0 at 40°C and freeze-dried. As well, 0.25 g of the non-centrifuged part was freeze-dried. The “total” and pellets samples were further analyzed with size exclusion chromatography.

5.2.4 Nitrogen content determination

Nitrogen contents were measured using the Dumas method (AOAC, 1995) with a NA 2100 Protein nitrogen analyzer (CE Instruments, Milan, Italy). A 6.38 x N conversion factor was used to convert nitrogen content to protein content for intact WPI and whey protein peptides. For β -cn and BSA, conversion factors used were 6.30 x N and 6.07 x N, respectively.

5.2.5 Analytical size exclusion chromatography

Experiments were performed with an Äkta Purifier System (Amersham, Pharmacia Biotech, Uppsala, Sweden) with Unicorn software. Samples were separated with an analytical Shodex Protein KW-802.5 column (300 x 8 mm; particle size: 7 μ m; pore size: 500 Å; Showa Denko K. K., Kanagawa, Japan). The column was equilibrated with 30 % (v/v) aqueous acetonitrile containing 6 M urea and 0.1 % (v/v) TFA. The flow rate was 0.5 mL min⁻¹ and the column temperature 20°C. Sample preparation was as follows. The freeze-dried pellets

and “totals” from the mixing experiment (peptides mixed with intact protein) were dissolved in 550 μL and 250 μL 8 M guanidinium hydrochloride, respectively. Next, an aliquot (100 μL) of dissolved pellet or total hydrolysate was mixed with 600 μL of 0.05 M Tris-HCl buffer (pH 8.0) containing 0.05 M dithiothreitol (DTT) and 8 M guanidinium hydrochloride. After 2 h of incubation at room temperature, 300 μL of acetonitrile and 1 μL of TFA were added. A volume of 20 μL sample was injected onto the column. Detection was performed at 220 and 280 nm. After estimating the areas (at 280 nm) of the peaks corresponding to intact protein present in the aggregates and in the “total”, using the program Peak Fit (SPSS Inc., Chicago, IL, USA), the proportion of intact protein in the aggregates and, therefore, the amount of intact protein in the aggregates, was calculated.

The fraction containing the aggregating peptides (pellet fraction) was fractionated with size-exclusion chromatography (**Figure 5.1A**) in order to further determine the peptide composition in every peak with reversed phase chromatography. For that purpose, the freeze-dried pellet fraction was dissolved in 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1 M dithiothreitol (DTT) and 8 M guanidinium hydrochloride at peptide concentration of 14.3 mg mL^{-1} . After 2 h of incubation at room temperature, TFA and acetonitrile were added to reach final concentrations of 0.7 % (v/v) and 30 % (v/v), respectively. The final protein concentration was 10 mg mL^{-1} . A volume of 100 μL sample was injected onto the column. Detection was performed at 220 nm. Peptide peaks were collected. Next, the acetonitrile was evaporated with an ALPHA-RVC CMC-1 rotating vacuum concentrator (CHRIST, Osterode am Harz, Germany). Samples were further analyzed with reversed-phase chromatography. The column was calibrated as described earlier (Chapter 2).

5.2.6 Analytical reversed-phase chromatography

Samples (supernatants and pellets) were analyzed on an analytical Vydac C8 column (208MS52; 250 x 2.1 mm; bead diameter: 5 μm ; porosity: 300 nm; Dionex) by HPLC (Thermo Separation Products Inc., San Jose, CA) with ChromQuest software. The flow rate was 0.2 mL min^{-1} , the column temperature was 20°C. Eluent A was 0.07 % (v/v) TFA in 5 % (v/v) acetonitrile and eluent B was 0.05 % (v/v) TFA in acetonitrile. Sample preparation was as follows. The pellets and supernatants were first dissolved in 8 M guanidinium hydrochloride to a concentration of 3 mg mL^{-1} . Next, a volume of 160 μL of dissolved material was mixed with 315 μL of 8 M guanidinium hydrochloride, 25 μL of acetonitrile and 0.25 μL of TFA. The final peptide concentration was 1 mg mL^{-1} . A volume of 30 μL sample was injected onto the column. After 10 min isocratic elution with eluent A, further elution was obtained with a linear gradient from 0 to 55 % eluent B in 100 min, then from 55 to 100 % eluent B in 2 min and 100 % B for 20 min. Detection was performed at 220 nm. The chromatograms were normalized (using the program Peak Fit) in such a way that the sum of the areas under the peaks in the supernatant and those in the pellet samples obtained for the fractions analyzed were the same for all samples. In addition, the relative areas under the

peaks in the supernatant and in the pellet samples were normalized to the proportion of aggregation in the respective samples.

The peptide peaks obtained from the pellet fraction with size-exclusion chromatography (**Figure 5.1A**) were analyzed with reversed-phase chromatography (**Figure 5.1B**). A volume of 50 μL was injected onto the column. Separation was the same as described above. The chromatograms were normalized to make an optimal comparison possible. Identification of the peptides was based on their retention times and mass spectrometry data (Chapter 4).

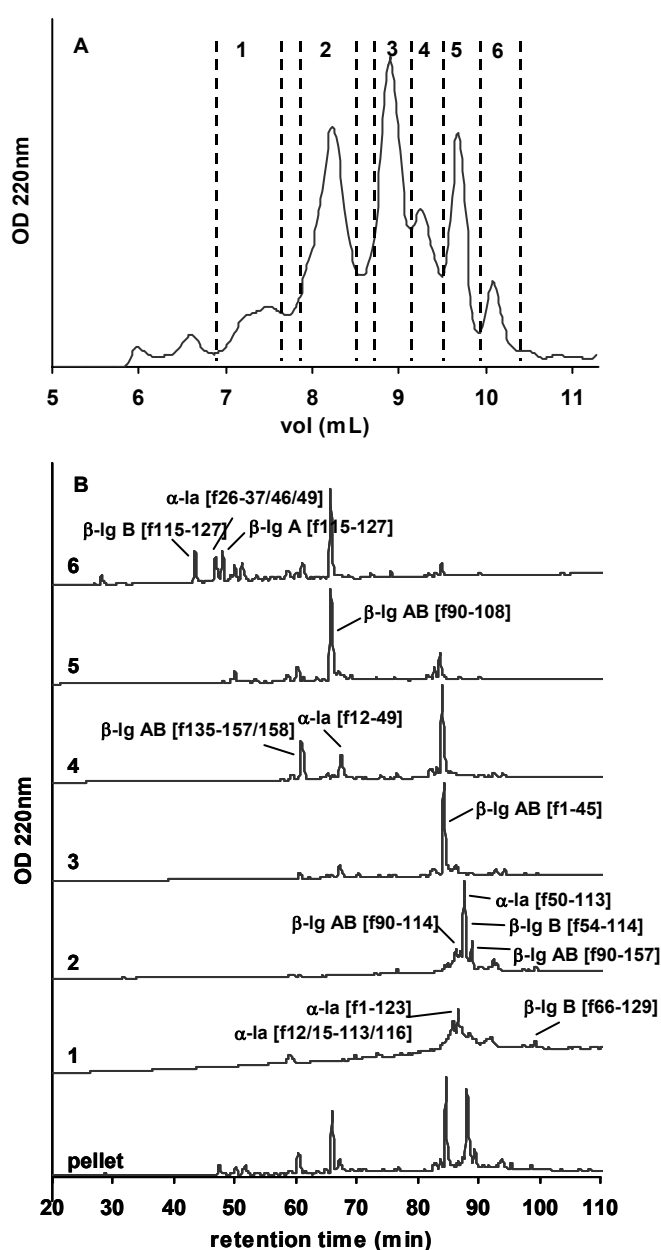


Figure 5.1. A) Size exclusion chromatogram, under reducing conditions, and fractionation of the aggregating peptide fraction; B) reversed-phase chromatograms of the fractionated peptide peaks.

5.3 Results

5.3.1 Solubility of the hydrolysate and of the peptide fractions

Effects of peptide concentration on the solubility of the total hydrolysate

The peptide concentration in the supernatant as a function of hydrolysate concentration was measured at pH 7.0, 40°C, at an ionic strength of 100 mM. The results are given in **Figure 5.2A**. First, to facilitate the comparison, an example of solubility data is given for two fictive peptides, with saturation concentrations of 5 and $> 25 \text{ mg g}^{-1}$. Typically there would be a linear relation (slope of 1.0) between the peptide concentration in the supernatant and its total concentration, until saturation of the solution is reached at 5 and $> 25 \text{ mg g}^{-1}$. Upon increasing peptide concentration, concentration in the supernatant would be constant (plateau) (**Figure 5.1A**). In the hydrolysate, the lowest concentration measured was 3 mg g^{-1} . There was a linear relation, with a slope of 0.46, between peptide concentration in the supernatant and hydrolysate concentration (from 3 to 20 mg g^{-1}). In a hydrolysate, that is a mixture of peptides, solubility depends on the proportion and on the saturation concentration of each peptide present. The slope obtained meant that the hydrolysate contained a mixture of peptides, which around 46% of them have a relative high saturation concentration ($\geq 11 \text{ mg g}^{-1}$; within the experimental range) because the peptide concentration in the supernatant (11 mg g^{-1}) did not reach a plateau at the highest concentration tested (20 mg g^{-1}).

The proportion of aggregation in the hydrolysate with DH 6.8% was also expressed as a function of hydrolysate concentration (**Figure 5.2B**). Again, an example of solubility data is given for the fictive peptides. Typically the proportion of aggregation would start increasing at total peptide concentration of 5 and $> 25 \text{ mg g}^{-1}$. Next, the proportion of aggregation would increase asymptotically from 0 to 100% as total peptide concentration increased, following this equation:

$$\text{proportion of aggregation (\%)} = \frac{T - s}{T} \times 100 \quad (\text{for } T \geq s)$$

where T is the total peptide concentration and s is the saturation concentration of the peptide. In the hydrolysate, the proportion of aggregation started to increase at an estimated hydrolysate concentration of $\sim 2.5 \text{ mg g}^{-1}$. Next, the proportion of aggregation increased asymptotically from 0 to around 50% until a hydrolysate concentration of 15 mg g^{-1} , indicating that the hydrolysate contained peptides with high and peptides with low saturation concentrations (within the experimental range), but no peptides with intermediate saturation concentrations. Indeed, a mixture of peptides with high, intermediate and low saturation concentrations would give a more linear relation between the proportion of aggregation and

the total peptide concentration. The hydrolysate contained around 50% of peptides with low (at hydrolysate concentration of 2.5 mg g^{-1}) and 50% peptides with high saturation concentrations (at hydrolysate concentration $\geq 11 \text{ mg g}^{-1}$).

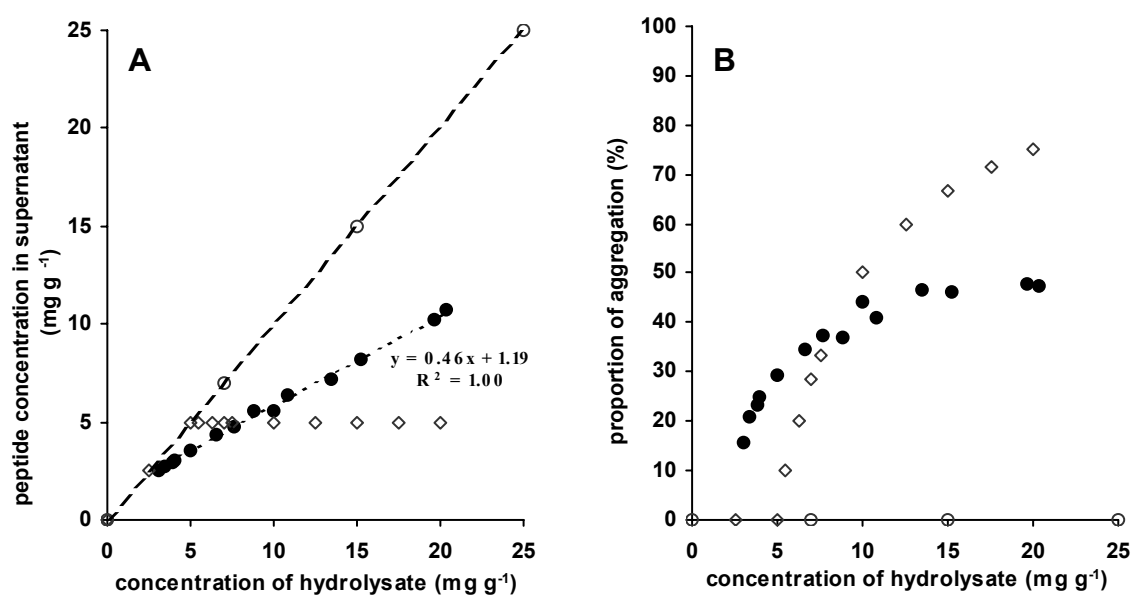


Figure 5.2. Solubility data of the hydrolysate (\bullet) and of fictive peptides that have saturation concentrations of 5 mg g^{-1} (\diamond) and $> 25 \text{ mg g}^{-1}$ (\circ) expressed as: (A) peptide concentration in the supernatant and (B) proportion of aggregation, after centrifugation, as a function of the total hydrolysate concentration.

Effects of peptide concentration on the solubility of different peptide fractions

The hydrolysate was fractionated (Chapter 4) into different fractions in order to compare their solubilities as a function of concentration. **Figure 5.3A** shows that the supernatant fraction was almost completely soluble until at least a concentration of 23 mg g^{-1} (slope of 0.97) and that the pellet fraction was almost completely insoluble. The saturation concentration of all the peptides in the pellet fraction was reached at a pellet fraction concentration of around 2 mg g^{-1} , since the peptide concentration in the supernatant of this fraction reached a plateau at this value.

The solubilities of the four fractions isolated from the pellet fraction were also measured. **Figure 5.3A** shows that fractions P1 and P2 were more soluble than the parental pellet fraction, and fractions P3 and P4 were less soluble than the parental pellet fraction. The fractions P3 and P4 were completely insoluble as no proteinaceous material was detected in their supernatants. The linear relation, with a slope of ~ 0.4 , between peptide concentration in the supernatant of fractions P1 and P2 and total P1 and P2 concentrations (**Figure 5.3A**) indicated that these fractions contained a relatively high proportion of soluble peptides at total peptide concentrations between ~ 4 and $\sim 14 \text{ mg g}^{-1}$. The saturation concentration of all the peptides in the fractions P1 and P2 was not reached at a concentration of 14 mg g^{-1} (being

the highest concentration tested), since the peptide concentration in the supernatant (7 mg g^{-1}) of these fractions did not reach a plateau.

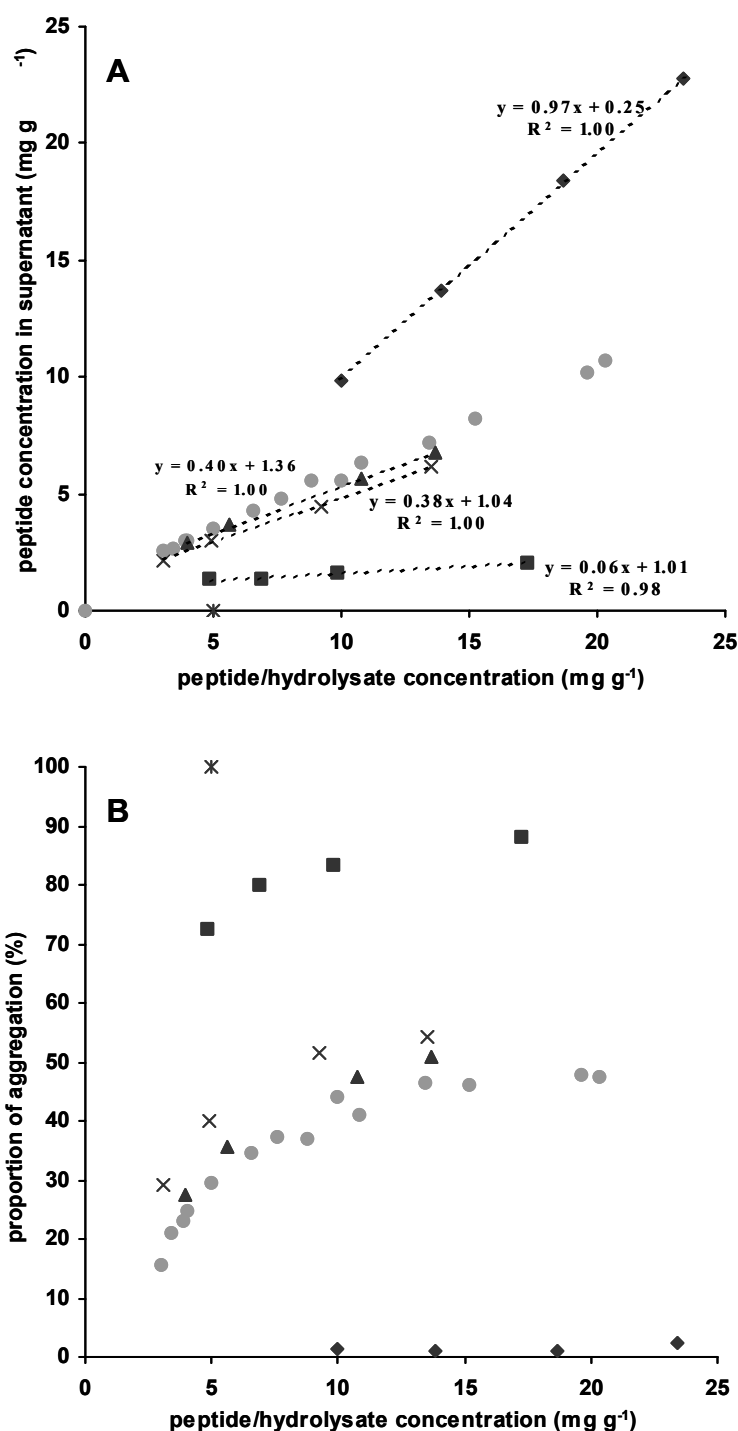


Figure 5.3. Solubility data of the hydrolysate (●) and of the peptide fractions supernatant (◆), pellet (■), P1 (▲), P2 (×), P3 (◆) and P4 (◆) expressed as: (A) peptide concentration in the supernatant and (B) proportion of aggregation, after centrifugation, as function of the total hydrolysate concentration.

Figure 5.3B shows that the proportion of aggregation in the supernatant fraction was negligible, as expected, while the proportion of aggregation in the pellet fraction was double the proportion of aggregation in the complete hydrolysate. The proportion of aggregation increased asymptotically from 0 to around 45% as the pellet fraction concentration increased.

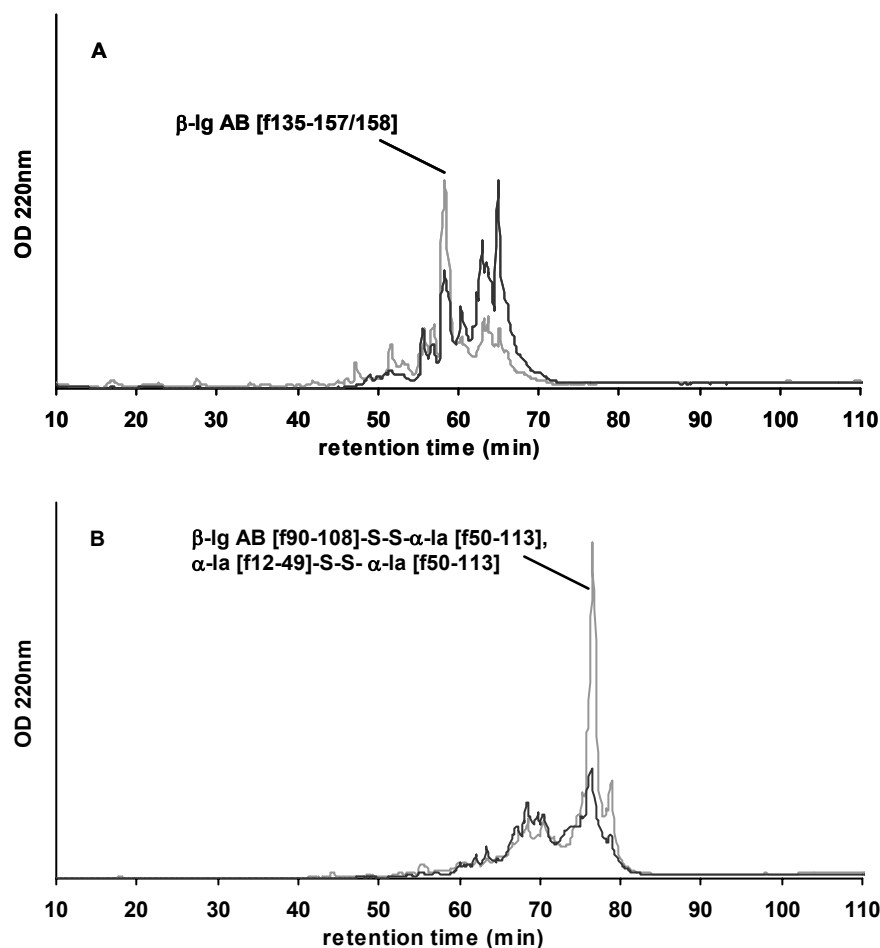


Figure 5.4. Reversed phase chromatograms of the pellets (black line) and supernatants (gray line) isolated, by centrifugation, from the peptide fractions P1 (A) and P2 (B), at a concentration of 9 mg g^{-1} .

The same was shown for the fractions P1 and P2 (**Figure 5.3B**). In addition, the proportion of aggregation in the two fractions tended to reach a plateau at 50%. This indicates that these two fractions are peptide mixtures containing 50% of peptides with relatively low saturation concentration (~ 1 to 2 mg g^{-1}) and 50% of peptides with relatively high saturation concentration ($\geq 7 \text{ mg g}^{-1}$).

The peptide distribution between the soluble and insoluble material in fractions P1 and P2 was further investigated with reversed phase chromatography at a total peptide concentration of 9 mg g^{-1} . The chromatograms are shown in **Figure 5.4**. The peptide β -Ig AB [f135-157/158] was the most soluble peptide of the fraction P1 since among all peptides of this fraction it was significantly more present in the supernatant than in the pellet. For the

same reason, the peptides β -lg AB [f90-108]-S-S- α -la [f50-113] and α -la [f12-49]-S-S- α -la [f50-113] were the most soluble peptides of the fraction P2.

The peptide fractions were further tested for their intact protein aggregation capacities.

5.3.2. Intact protein aggregating capacities of the peptide fractions

It was shown in Chapter 3 that an optimal amount of aggregated material, at pH 7.0, was obtained when a mass of 10 mg hydrolysate (DH 6.8%) was mixed with a mass of 20 mg intact WPI, at 100 mM, whatever the temperature. In the present study, in order to investigate intact protein aggregation capacities of the peptide fractions, we have chosen to mix 10 mg of hydrolysate (DH 6.8%) with 20 mg of intact WPI, at 40°C and 100 mM. In addition, it was decided to mix 5 mg of peptide fractions (supernatant, pellet, and peptide fractions P1 to P4) with 20 mg intact WPI, because the hydrolysate contains ~50% of aggregating peptides (peptides with low solubility).

Table 5.1. Amounts of aggregated intact WPI upon mixing the hydrolysate (10 mg) and the peptide fractions (5 mg) with intact WPI (20 mg).

	WPI aggregation capacity of peptide fractions (mg protein per mg peptides)
Hydrolysate	0.254 +/- 0.005
Supernatant fraction	0.000 +/- 0.000
Pellet fraction	0.429 +/- 0.040
Fraction P1	0.336 +/- 0.055
Fraction P2	0.198 +/- 0.011
Fraction P3	0.247 +/- 0.050
Fraction P4	0.535 +/- 0.076

The amounts of intact WPI in the aggregates per mg of peptide upon mixing the hydrolysate and the peptide fractions with WPI are shown in **Table 5.1**. It could be noticed that, as expected, the supernatant fraction did not aggregate intact WPI. In addition, the pellet fraction did not completely aggregate twice the amount of WPI than the hydrolysate although it contained twice the amount of aggregating peptides. Next, upon comparing the protein aggregation capacities of the pellet fraction with those of the fractions P1 to P4, it was observed that all peptide fractions of the pellet could aggregate intact WPI. The amount of WPI in the aggregates followed the order P2<P3<P1<P4.

Table 5.2. Total amount of peptides precipitated and relative abundance of peptides from the different fractions in peptide and WPI-peptide aggregates, analysed with size-exclusion chromatography.

Fraction	Peptide	without intact WPI		with intact WPI	
		Amount of aggregated peptides (mg)	Relative abundance of peptides ^a	Amount of aggregated peptides (mg)	Relative abundance of peptides ^a
P1		2.00		1.50	
	β-Ig AB [f135-157/158]		+++++		+++
	β-Ig AB [f90-108]		+++++		+++++
	β-Ig AB [f115-127], α-la [f26-37/46/49]		++++		+++++
P2		2.00		1.20	
	α-la [f1-123], α-la [f12/15-113/116]		+++		++
	α-la [f50-113]		++++		+++++
	β-Ig AB [f1-45]		++++		++++
	α-la [f12-49]		+++		++
	β-Ig AB [f90-108]		++++		++++
	β-Ig AB [f115-127], α-la [f26-37/46/49]		+		-
P3		5.00		5.00	
	β-Ig AB [f54-114], α-la [f50-113]		+++++		+++++
	β-Ig AB [f1-45]		+++++		+++++
P4		5.00		5.00	
	β-Ig A [f90-157], β-Ig B [f66-129]		++		++
	α-la [f50-113]		+++++		+++++
	β-Ig AB [f1-45]		++++		++++
	β-Ig AB [f90-114], α-la [f12-49]		++		++
	β-Ig AB [f90-108]		++		++

^a Peptide peak: not detectable [-], detectable [+], ..., ++++++ high intensity.

The protein-peptide aggregates induced by the peptide fractions P1 to P4 were analyzed with size exclusion chromatography, under reducing conditions. Peptide peaks were annotated after identification from peak fractionation (**Figure 5.1**) and LC-MS (Chapter 4). The relative abundance of peptides in the different fractions in the peptide aggregates and in the WPI-peptide aggregates is summarized in **Table 5.2**. It was shown that in the protein-peptide aggregates induced by the peptide fraction P1, the peptide β-Ig AB [f90-108] was mainly involved in aggregation of intact protein. The peptide β-Ig AB [f135-157/158] was present in the peptide aggregates, but less in the protein-peptide aggregates. There were less aggregating peptides of the fraction P2 taking part in protein-peptide aggregation (with mainly the peptides β-Ig AB [f90-108] and α-la [f50-113]) than in peptide-peptide aggregation. Possibly protein-peptide interactions occurred leading to formation of soluble adducts. On the contrary, the aggregating peptides of the fractions P3 (mainly β-Ig AB [f1-45]) and P4 (with α-la [f50-113] as dominant peptide) did not show soluble protein-peptide adducts since there was a similar amount of aggregating peptides in peptide-peptide aggregates and in protein-peptide aggregates.

Table 5.3. Amounts of aggregated intact WPI, β -cn and BSA upon mixing the pellet fraction (5 mg) and the peptide fractions P4 (5 mg) with intact WPI, β -cn and BSA (20 mg).

Protein aggregation capacity of peptide fractions (mg protein per mg peptides)				
	WPI	β -cn	BSA	
Pellet fraction	0.429 +/- 0.040	0.471 +/- 0.004	0.287 +/-	0.093
Fraction P4	0.535 +/- 0.076	1.228 +/- 0.113	0.270 +/-	0.059

Table 5.4. Relative abundance of peptides from the aggregating peptides fraction and from the fraction P4 in peptide aggregates and in protein (WPI, β -cn and BSA)-peptide aggregates, analysed with size-exclusion chromatography.

Fraction	Peptide	without intact WPI			with intact WPI			with intact β -cn			with intact BSA		
		Amount of aggregated peptides (mg)	Relative abundance of peptides	Amount of aggregated peptides (mg)	Relative abundance of peptides	Amount of aggregated peptides (mg)	Relative abundance of peptides	Amount of aggregated peptides (mg)	Relative abundance of peptides	Amount of aggregated peptides (mg)	Relative abundance of peptides	Amount of aggregated peptides (mg)	Relative abundance of peptides
Pellet	α -la [f1-123], α -la [f12/15-113/116], β -lg AB [f90-157], β -lg B [f66-129]	3.60	+++	3.60	+++	3.60	+++	1.90	+++	3.60	+++	3.60	+++
	α -la [f50-113]		++++		++++		++++		++++		++++		++++
	β -lg AB [f1-45]		+++++		+++++		+++++		+++++		+++++		+++++
	β -lg AB [f135-157/158], β -lg AB [f54-114], β -lg AB [f90-114], α -la [f12-49]		+++		+++		+++		+++		+++		+++
	β -lg AB [f90-108]		++++		++++		++++		++++		++++		++++
	β -lg AB [f115-127], α -la [f26-37/46/49]		+		+		+		+		+		+
P4	β -lg A [f90-157], β -lg B [f66-129]	5.00	++	5.00	++	5.00	++	5.00	++	5.00	++	5.00	++
	α -la [f50-113]		+++++		+++++		+++++		+++++		+++++		+++++
	β -lg AB [f1-45]		++++		++++		++++		++++		++++		++++
	β -lg AB [f90-114], α -la [f12-49]		++		++		++		++		++		++
	β -lg AB [f90-108]		++		++		++		++		++		++

Peptide peak: not detectable [-], detectable [+], ..., ++++++ high intensity.

The amount of intact protein in the aggregates upon mixing peptide fractions (pellet fraction and fraction P4) with intact proteins (WPI, β -cn and BSA) is shown in **Table 5.3**. The pellet fraction and the peptide fraction P4 aggregated intact proteins in the same order β -cn>WPI>BSA. The peptide fraction P4 has a high protein aggregation capacity for β -cn. It aggregated 2.6 times more β -cn and 1.2 times more WPI than the pellet fraction, respectively. On the contrary, both peptide fraction P4 and the pellet fraction aggregated the same amount of BSA.

The protein-peptide aggregates induced by the pellet fraction and the peptide fraction P4 were analyzed with size exclusion chromatography, under reducing conditions. The relative abundance of peptides from the aggregating peptides fraction and the fraction P4 in the peptide aggregates and in the protein-peptide aggregates is summarized in **Table 5.4**. It was shown that in pellet fraction- β -cn aggregates, there were less aggregating peptides taking part in protein-peptide aggregation than in peptide-peptide aggregation. Possibly protein-peptide interactions indeed occurred, but led to soluble adducts. This was not the case in peptide fraction P4- β -cn aggregates where all aggregating peptides took part to protein-peptide aggregates. For both fractions P and P4, the same peptides were involved in protein-peptide aggregates with the different proteins.

5.4 Discussion

5.4.1. Peptide solubility

The solubility of a solute is the maximum quantity of solute that can dissolve in a certain quantity of solvent at specified conditions. The authors realize that in the present study, the data presented are not real solubility data since these should be expressed in unit per volume. In the present study, solubility denotes the proportion of a given amount of peptide that goes into solution under specific conditions and is not sedimented by moderate centrifugal forces (Kinsella, 1984). When solution saturation is reached, the excess of peptide aggregates and precipitates. Hydrophobicity is usually the driving force for peptide aggregation. As the hydrolysate is a peptide mixture, we obtain an average solubility for all peptides that it contains since solubility depends on the proportion and on the saturation concentration of each peptide present.

It was shown that the hydrolysate contains two distinct populations of peptides: 50% of them have low saturation concentration ($\leq 2 \text{ mg g}^{-1}$; they were separated as the pellet fraction) and the other 50% have high saturation concentration ($\geq 23 \text{ mg g}^{-1}$; they were separated as the supernatant fraction). Interestingly, the hydrolysate did not seem to contain peptides of intermediate solubility. In addition, we have isolated aggregating peptide fractions from the pellet fraction that have different solubility: the fractions P3 and P4 that

are completely insoluble and the fractions P1 and P2 that are more soluble than the parental pellet fraction.

The peptides in fractions P3 and P4 are the most hydrophobic and/or the largest peptides of the aggregating peptides since they elute late in reversed-phase chromatography (Chapter 4). The fraction P3 contains mainly the peptide β -lg AB [f1-45], that is, therefore, intrinsically insoluble. The fraction P4 contains mainly the peptides β -lg AB [f90-108]-S-S- α -la [f50-113], α -la [f12-49]-S-S- α -la [f50-113], β -lg AB [f1-45], β -lg AB [f90-157] and peptides having a partial common sequence with the fragment β -lg AB [f1-45]. The peptide mixture is insoluble. The solubility of a peptide mixture or of a hydrolysate might not only depend on the solubility of every single peptide, but also on aggregation of different compounds together, as it was shown that it is possible to induce peptide-peptide interactions by mixing peptide fractions (Groleau et al., 2003). Therefore, peptide co-aggregation in fraction P4, via induction of peptide-peptide interactions, could not be excluded.

It was shown that the fractions P1 and P2 contain peptide mixtures of around 50% peptides with low saturation concentration (~ 1 to 2 mg g^{-1} fraction concentration) and, unexpectedly, around 50% peptides with relatively high saturation concentration ($\geq 7 \text{ mg g}^{-1}$ fractions concentration). The peptide β -lg AB [f135-157/158] is the most soluble peptide in the fraction P1 and the peptides β -lg AB [f90-108]-S-S- α -la [f50-113] and α -la [f12-49]-S-S- α -la [f50-113] are the most soluble peptides in the fraction P2. Interestingly, peptide fractions P2 and P4, despite fractionation, have some peptides in common: β -lg AB [f90-108]-S-S- α -la [f50-113] and α -la [f12-49]-S-S- α -la [f50-113]. As these peptides were the most soluble ones in the fraction P2, and as the fraction P4 is completely insoluble, possibly the other peptides of the fraction P4 (β -lg AB [f1-45], β -lg AB [f90-157] and peptides having a partial common sequence with the fragment β -lg AB [f1-45]) induced co-aggregation of β -lg AB [f90-108]-S-S- α -la [f50-113] and α -la [f12-49]-S-S- α -la [f50-113], probably via hydrophobic interactions (Chapter 2).

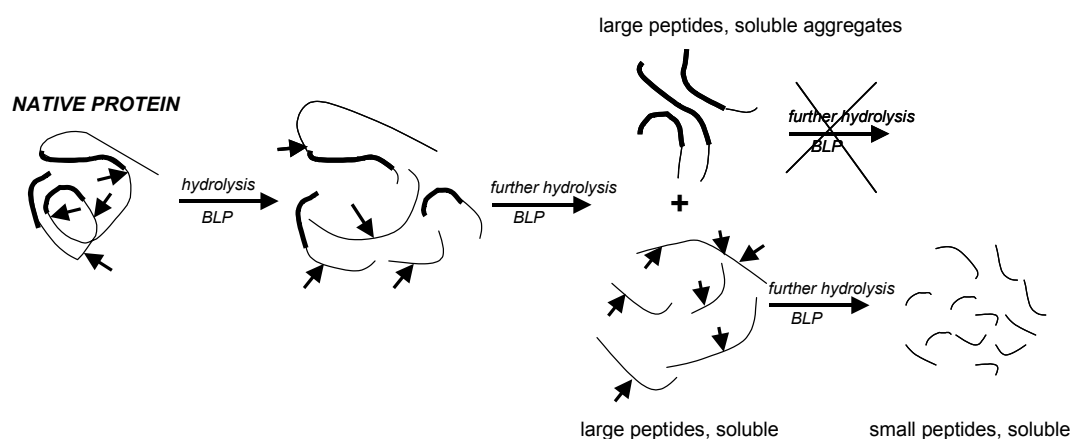


Figure 5.5. Schematic representation of the production of large and small peptides upon hydrolysis, at pH 8.0, of whey proteins by BLP. Arrows indicate enzymatic cleavage, bold lines indicate hydrophobic segments.

It was shown in Chapter 4 that upon hydrolysis of whey proteins by BLP, at pH 8.0, peptide aggregation (at least the peptide β -lg AB [f1-45]) prevented further digestion, explaining that aggregating peptides are relatively large. This is in agreement with the production of large peptides with (apparently) low saturation concentration at pH 7.0 (this Chapter; **Figure 5.4**). The non-aggregating peptides are further digested upon extensive hydrolysis (at pH 8.0) so that small peptides with high saturation concentration at pH 7.0 were produced (**Figure 5.4**).

5.4.2. Protein aggregation

The dominant aggregating peptide of the hydrolysate, β -lg AB [f1-45], which is the main compound of the fraction P3, was shown (**Tables 5.1 and 5.2**) to have poor intact protein aggregating capacities. This indicated that insolubility is not enough for a peptide to aggregate intact protein. It is assumed that the insoluble peptide prefers peptide-peptide interactions over protein-peptide interactions. Fewer protein-peptide aggregates were obtained with the fraction P2 than with the fraction P4. Presumably soluble protein-peptide adducts were obtained with the peptides from the fraction P2. On the contrary, the aggregating peptides of the fraction P4 were the most effective to aggregate intact protein. The fraction P4, that could aggregate more WPI than the fraction containing all the aggregating peptides, consisted of 25% of the peptides of the latter fraction (Chapter 4). It was shown that the fractions P2 and P4 have a quite similar peptide composition (Chapter 4) despite their fractionation with preparative reverse phase chromatography. They both contain the fragment α -la [f50-113], linked via a disulfide bridge to the fragments β -lg AB [f90-108] and α -la [f12-49], that was further shown to be the dominant peptide in the protein-peptide aggregates induced by the peptide fractions P2 and P4. It was also shown above that the latter peptides are partly insoluble. The fraction P4 contained more intrinsically insoluble peptides (β -lg AB [f1-45], β -lg AB [f90-157] and peptides having a partial common sequence with the fragment β -lg AB [f1-45]) than the fraction P2. Based on this, it is hypothesized that the presence of both insoluble and partly insoluble peptides is required to aggregate intact protein.

The aggregating peptides could aggregate different proteins and the same peptides were each time involved in the aggregation. Therefore, no specific interactions took place in protein-peptide aggregates. Remarkably, both the peptides from the pellet fraction and the peptides from the fraction P4 aggregated more β -cn than the whey proteins and BSA. β -cn is an amphipathic protein with the N-terminal part containing many negatively charged groups and the C-terminal part containing many hydrophobic groups. It has a low amount of secondary structure and a molecular mass of ~24 kDa. At temperatures higher than 4°C and above a critical concentration β -cn forms micelles (Payens and van Markwijk, 1963; Schmidt and Payens, 1972). BSA is a rather large globular protein (larger than β -lg and α -la) with a molecular mass of ~69 kDa. In addition, it is highly structured with 17 intramolecular

disulfide bridges. Therefore, the aggregating peptides network containing both insoluble and partly insoluble peptides could include intact proteins, globular or not, with a preference for unfolded amphipathic proteins that offer more possibilities for hydrophobic interactions with hydrophobic peptides.

5.5 Conclusions

The present results indicated that the WPI hydrolysate made with BLP, at high DH, contains small soluble peptides and large peptides with low solubility that resulted from peptide co-aggregation (at pH 7.0). It was shown that low solubility peptides have a high capacity to aggregate with each other, but not with proteins. For the situation studied, it was shown that non-specific protein aggregation required presence of both insoluble and partly insoluble peptides.

Acknowledgements

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CHAPTER 6

General discussion

6.1. Introduction

In contrast to hydrolysis with most proteases (**Table 1.1**), limited hydrolysis of whey proteins with a protease from *Bacillus licheniformis* (BLP) yields peptide aggregates that ultimately form a gel (Otte et al., 1996). Improved knowledge on how to modulate the gelation properties of whey proteins is valuable for extending applications of whey protein hydrolysates, for creating new products and/or for a more economic use of whey protein ingredients. It is also of interest to use this knowledge to control properties of protein/peptide mixtures.

In this study we aimed at understanding the underlying mechanism of peptide-induced aggregation of whey protein hydrolysates made with *Bacillus licheniformis* protease. Based on literature data (Otte et al., 1997) it was hypothesized that remaining intact protein in the whey protein hydrolysate plays a role in the gelation behavior of the hydrolysate. In order to evaluate if aggregating peptides could aggregate intact proteins, it was decided to uncouple hydrolysis from aggregation, isolate aggregating peptides, and to subsequently induce aggregation in presence of added intact proteins (**Figure 1.6**). In this way the proportions of intact protein and peptides are not interrelated as it is in the case with total (non-fractionated) hydrolysates. To this end the research project was divided into three parts:

(I) Hydrolysis of whey proteins with *Bacillus licheniformis* protease. It consisted in finding hydrolysis conditions to reach a large DH range without aggregation and gelation, and inhibit the enzyme without a thermal treatment.

(II) Peptide aggregation. The formation of peptide aggregates was studied with respect to quantification, identification of the type of interactions, identification of the aggregating peptides, and proposing an aggregation mechanism.

(III) Protein-peptide aggregation. The set up used was analogous to the study of peptide aggregation (II).

6.2. Hydrolysis of whey proteins with *Bacillus licheniformis* protease

Whey protein isolate (WPI) was hydrolyzed at pH 8.0. In contrast to lower pH values, at this pH no extensive aggregation was observed. In this way, hydrolysis, aggregation and gelation could be separated, contrary to previous studies using the same protease and substrate in which hydrolyses were performed at pH 7.0-7.5 (Otte et al., 1996; Otte et al., 1997; Otte et al., 2000a; Ipsen et al., 2000). Despite not detected after centrifugation of the hydrolysates at pH 8.0, peptide aggregation at this pH was not completely prevented. It was shown in Chapter 4 that peptide aggregation occurred during hydrolysis at pH 8.0, because the whey protein peptides obtained could be further degraded by BLP in the presence of 4 M urea.

Aggregation of peptides released upon protein hydrolysis has been observed in several publications (Ju et al., 1995; Caessens et al., 1999; Doucet et al., 2001; Ipsen et al., 2001b; Inouye et al., 2002; Kuipers et al., 2005; Fisher et al., 2002). It is remarkable that the currently used hydrolysis theories to predict differences in composition of low DH (DH<10%) hydrolysates (Adler-Nissen, 1986) are still based on the assumption that upon hydrolysis only soluble intermediate products are formed. In the theory of Linderstrøm-Lang (1952), the zipper ($v_0 \gg v_1$) and one-by-one ($v_0 \ll v_1$) reaction models are derived from the hydrolysis of native and denatured globular proteins (**Figure 6.1**; Adler-Nissen, 1986). It is proposed to extend the theory of Linderstrøm-Lang to take into account aggregation of intermediate peptides upon protein hydrolysis (**Figure 6.1**) that are not further degraded.

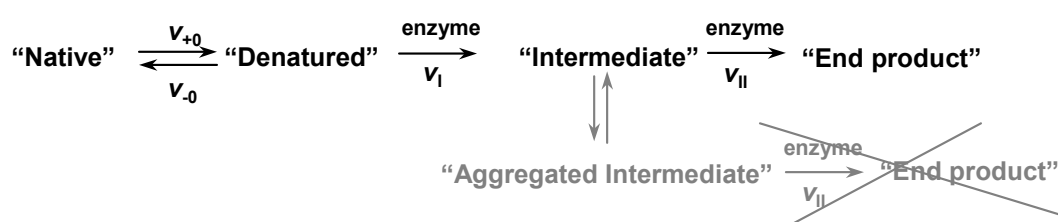


Figure 6.1. Current scheme (in black) of the Linderstrøm-Lang kinetic model (Linderstrøm-Lang, 1952) for the hydrolysis of native and denatured globular proteins (from Adler-Nissen, 1986). The proposed extension of the model is shown in gray. (Reaction rate of denaturation: $v_0 = v_{+0} - v_{-0}$)

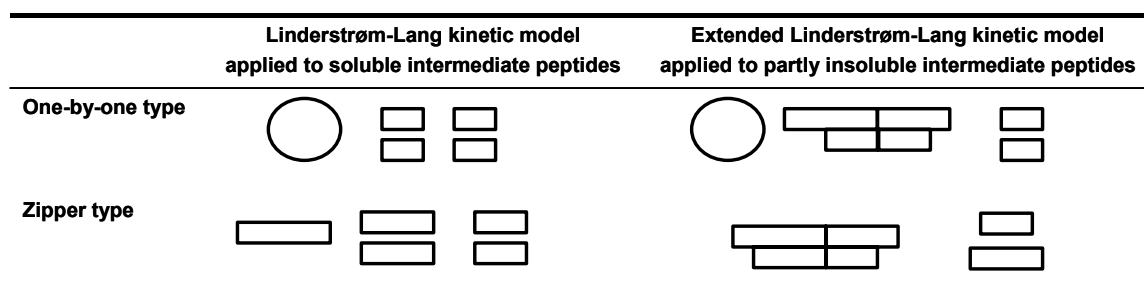


Figure 6.2. Composition of protein hydrolysates as a function of the type of initial degradation (DH < 10%). Circles denote native protein, rectangles denote peptides.

Figure 6.2 shows the predicted compositions of the hydrolysates at low DH values, as a function of the kinetic models. In the case that the peptides released upon protein hydrolysis stay soluble, the hydrolysate, in a one-by-one reaction, will consist of native proteins and small peptides. If the reaction is of the zipper type, the hydrolysate will consist of peptides with a broad distribution of molecular weights. In the case that the peptides released upon protein hydrolysis form aggregates, thereby preventing further hydrolysis, the hydrolysate, in a zipper reaction, would still consist of peptides with a broad distribution of molecular weights. However, if the reaction is an one-by-one type, the hydrolysate would consist of both native protein, small peptides and peptides with a broad distribution of molecular

weights. In the latter situation, the composition of the hydrolysate, at low DH, deviates from the current Linderstrøm-Lang kinetic model.

No matter whether aggregation does or does not take place, protease inhibition was needed in order to handle the hydrolysates without the possibility of further enzymatic degradation during subsequent experiments. Several known inhibitors specific for seryl proteases were tested to inhibit BLP (benzyloxycarbonyl-L-leucine-L-glutamic acid-chloromethyl ketone –Z-LE-CMK; phenylmethanesulphonylfluoride –PMSF-, aminoethyl-benzosulfonyl-fluorohydrochloride –EABSF-, aprotinin, trypsin soybean inhibitor, leupeptin, and an inhibitor cocktail). None of them were able to successfully inactivate BLP. In contrast, BLP could be successfully irreversibly inhibited by acidification of the hydrolysate to pH 2.0. This allowed reaching various DH's without the need of heat inactivation. In addition, the adjustment to pH 2.0 had the beneficial effect that all peptides were soluble at this pH. This allowed adding solubilized peptides to intact protein rather than adding pre-aggregated peptides.

In accordance with literature it was observed, from the identification of aggregating peptides in Chapter 4, that the enzyme cleaved both the Glu-X and the Asp-X bonds of the substrate. Considering that the WPI used is composed of β -lactoglobulin (β -lg; 80.5%), α -lactalbumin (α -la; 13.5%) and bovine serum albumin (BSA; 6%) only, and taking into account steric (e.g. presence of a cystine residue in C-terminal side next to Glu or Asp residues) and electrostatic (e.g. neighboring of other Glu or Asp residues) hindrances, a maximum DH of 10.8 % was to be expected. For the individual whey proteins, the maximum DH's reachable for β -lg, α -la and BSA are 11.1%, 9.8% and 9.6%, respectively. In practice, the maximum DH reached was 6.8%. Prolonged incubation did not yield higher DH's. As hydrolysis stopped at DH 6.8% (practical end of hydrolysis), it is proposed that peptide aggregation prevented further hydrolysis (Chapter 4). Nevertheless, hydrolysates with a large DH range (DH 0 to 6.8%) could be obtained, larger than the ranges already studied (DH 0 to ~2.0% by Otte et al., 1996 and DH 0 to 4.9% by Ju et al., 1995), allowing investigation over a relative large DH range.

6.3. Peptide aggregation and gelation

Aggregated material was arbitrarily defined as the material removed using the centrifugation and solvent conditions applied. In the present study, the extent of aggregation in a whey protein hydrolysate made with BLP at neutral pH was the highest (until ~45%) at the practical endpoint of hydrolysis (**Figure 6.3**).

Since the peptide aggregates could be dissolved at pH 2.0 and pH \geq 8.0, mainly non-covalent interactions between the peptides are considered to be responsible for aggregation. At neutral pH it was found that aggregation at low DH was much more sensitive to temperature and ionic strength than at high DH (**Figure 6.3**). The effects of temperature

indicate that aggregation within the hydrolysates is favored (at high DH) mainly by increased hydrophobic interactions and to a lesser extent by reduced electrostatic repulsion (Chapter 2), as already suggested by others (Ju et al., 1995; Otte et al., 1997).

The enzyme-induced aggregates contain only peptides (Chapter 4), as already found by Otte et al. (1997). The main aggregating peptide (at DH 6.8%) is β -lg AB [f1-45]. Based on peak area, this peptide represents around 22% of all the aggregating peptides. Next to this, the peptides β -lg AB [f90-108]-S-S- α -la [f50-113], α -la [f12-49]-S-S- α -la [f50-113], β -lg AB [f90-108]-S-S- β -lg AB [f90-108], β -lg A [f90-157] and β -lg AB [f135-157/158] are also present in the aggregates. The aggregating peptide fraction, containing the above mentioned peptides, has a low solubility ($\leq 2 \text{ mg g}^{-1}$). The peptide β -lg AB [f1-45] is intrinsically insoluble. The peptides β -lg AB [f135-157/158], β -lg AB [f90-108]-S-S- α -la [f50-113] and α -la [f12-49]-S-S- α -la [f50-113] are more soluble than the parental aggregating peptide fraction. The latter peptides were in fact co-aggregated by intrinsic insoluble peptides, e.g. β -lg AB [f1-45] (Chapter 5).

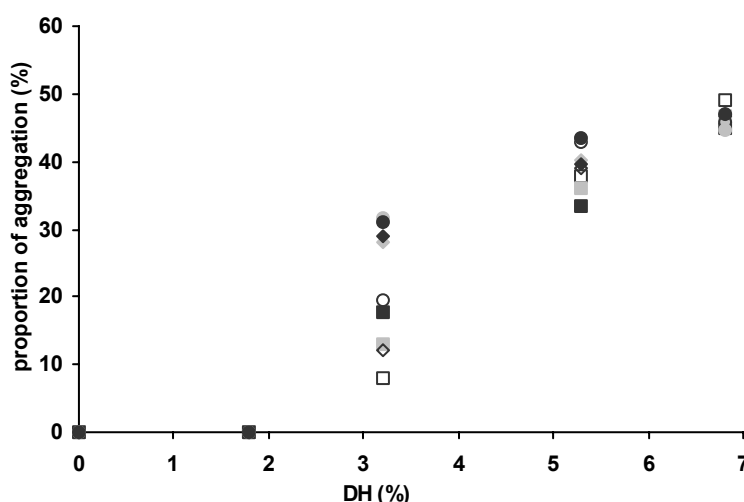


Figure 6.3. Proportion of aggregation in WPI hydrolysates as a function of the degree of hydrolysis (at pH 7.0) at temperatures of 20°C (square), 40°C (rhombus) and 60°C (circle), and at ionic strengths of 0.020 M (empty symbol), 0.075 M (gray) and 0.200 M (black).

In this study, we identified a different dominant aggregating peptide than the one identified by Otte and co-workers (Otte et al., 2000a). The latter authors reported that the peptide β -lg [f135-158] is present at high concentrations in the enzyme-induced aggregates and responsible for initiation of aggregation in β -lg hydrolysates made with BLP. This peptide is also present in the aggregates obtained in this study, but it is by far not the dominating peptide with respect to relative abundance. In fact, in the former study (Otte et al., 2000a), mass spectrometry (MALDI-TOF MS) was used as a quantitative tool to annotate the dominant peptide. It should not. High signal intensity in mass spectrometry predominantly means that the species analyzed ionizes well, but intensity should not be

confused with quantity, as ionization suppression phenomena certainly occur (Biemann, 1992).

In order to investigate if the extent of aggregation depended on the specific nature of peptides created upon hydrolysis or on the amount of aggregating peptides created, the proportion of aggregation and the amounts of aggregating peptides present in the total (non-fractionated) hydrolysates were presented as a function of DH in the **Figure 6.4**. Apparently, when considering the sum of the aggregating peptides, no relation between the total amount of aggregating peptides and the proportion of aggregation can be observed. In addition, for

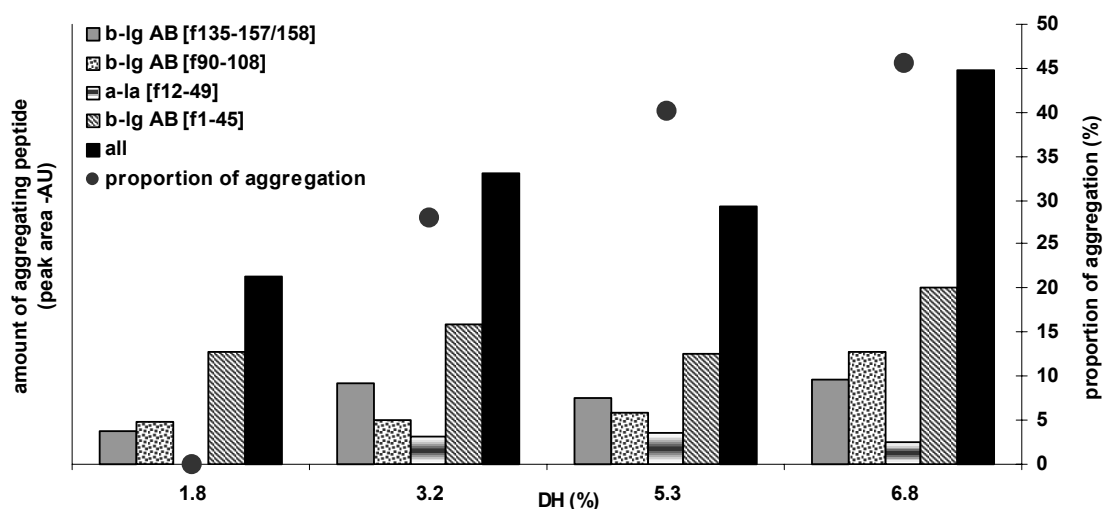


Figure 6.4. Proportion of aggregation (at 40°C and 75 mM) and amount of aggregating peptides (peak area as determined from reversed phase chromatograms, under reducing conditions) in the hydrolysates as a function the degree of hydrolysis. (AU means arbitrary unit).

the four dominant peptides present, no obvious relation between the amount of individual peptide and the proportion of aggregation was also observed. No data was presented for the peptide α -la [f50-113] because, as it coelutes with intact proteins in the reversed phase chromatograms, the amounts of this peptide were difficult to evaluate. Therefore, it cannot be excluded that the peptide α -la [f12-49]-S-S- α -la [f50-113] is responsible for peptide aggregation in the hydrolysates. If this is the case, it would mean that peptides from α -la have stronger aggregating capacity than those of β -lg. The peptide α -la [f12-49]-S-S- α -la [f50-113] can theoretically be responsible for peptide aggregation. However, we propose that aggregation of peptides in a total hydrolysate is more complex than ascribing its extent to one individual peptide. Indeed, it was shown in Chapter 5 that the peptide α -la [f12-49]-S-S- α -la [f50-113] is rather soluble and that it was co-aggregated by insoluble peptides. Our explanation for the increase in aggregation would be peptide co-aggregation, which was already shown to occur in the hydrolysate with DH 6.8% (Chapter 5). In this case, between DH 1.8 and 3.2%, the peptide composition would be suitable for co-aggregation.

Already some mechanisms of peptide-induced aggregation and (cold-) gelation have been tentatively described in literature:

- Upon hydrolysis, unfolded proteins, having exposed hydrophobic areas, could bind to hydrophobic areas of other molecules and then lead to aggregation and gelation (Otte et al., 1996);

- Upon hydrolysis, peptides containing clusters of hydrophobic amino acids are formed. If a peptide containing two hydrophobic regions is formed, it could link other peptides together by hydrophobic interaction and form aggregates (Ju et al., 1995);

- Aggregates and gels could consist of small hydrophobic peptides that have a net charge of 0 at the gelation pH (Doucet et al., 2003a);

- Upon the plastein reaction, reversible association of small peptides into aggregates occurs, at high concentration, that ultimately form a thixotropic solution stable over a wide pH range and a temperature range from at least 0 to 70°C (Andrews and Alichanidis, 1990).

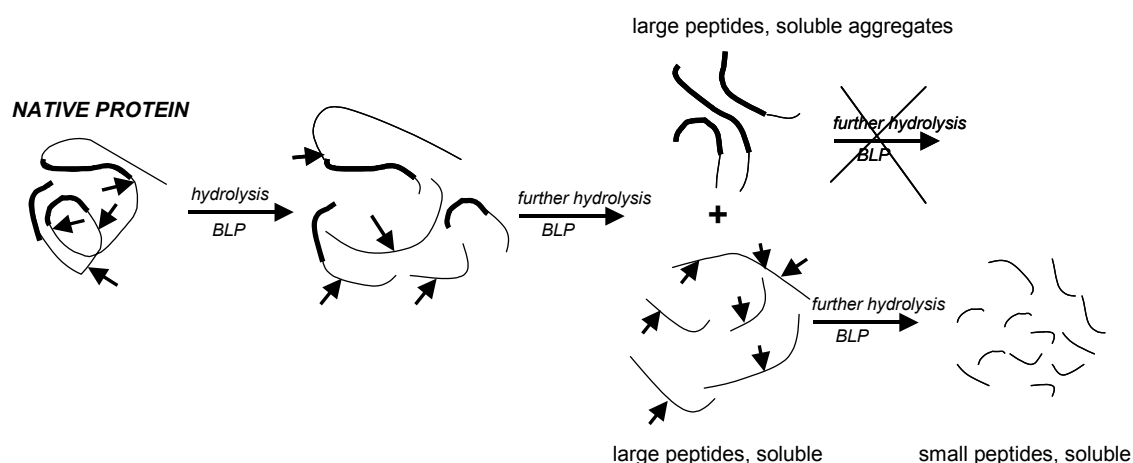


Figure 6.5. Schematic representation of the production of large insoluble and small soluble peptides upon hydrolysis at pH 8.0 of whey proteins by BLP. Arrows indicate enzymatic cleavage, bold lines indicate hydrophobic segments.

It can be stated that enzyme-induced aggregation of proteins is a matter of substrate primary structure and of enzyme specificity and selectivity (Chapter 4). BLP or glutamyl endopeptidase cleaves hydrophilic segments in a polypeptide and, therefore, preserves hydrophobic segments. BLP prefers Glu-X bonds to Asp-X bonds (Breddam and Meldal, 1992). Because of the uneven partition of the Glu residues in β -lg and α -la, large fragments containing either a high average hydrophobicity (β -lg AB [f90-108]), a specific hydrophobic segment (β -lg AB [f1-45]) or a specific folding, which exposes hydrophobic residues (α -la [f50-113]), are produced. In addition, aggregates are formed at pH 8.0 that prevent further hydrolysis of the peptides containing Asp residues. These aggregates are presumably charged enough at pH 8.0 to stay soluble, compared with pH 7.0 where they are insoluble. This explains that the peptide distribution between aggregating and non-aggregating peptides at

pH 7.0 (**Figures 6.1 and 6.5**) is such (Chapter 5) that the largest peptides of the hydrolysate are the aggregating ones (low solubility) and the smallest peptides are the non-aggregating ones (high solubility).

As a preliminary study we investigated the heat-induced gelation of WPI hydrolysates. To this end, hydrolysates of different DH's (DH 0, 1.8, 3.2, 5.3 and 6.8%) were acid treated to inhibit the enzyme (the pH was set to 2.0 with 6 M TFA), freeze-dried and suspended at a concentration of 6%, pH 7.0, and at an ionic strength of 100 mM. Next, the hydrolysates were heated at 80°C for 1 h. According to our observations (**Figure 6.6**), hydrolysis is detrimental to heat-gelation. This is in contradiction with the results of Ju and co-workers where limited hydrolysis (DH 1.1%) of WPI with BLP lead to an increased gel strength (Ju et al., 1995). Our procedure included an acid treatment of the hydrolysates, to inhibit the enzyme, before bringing the pH up to 7.0 and heat treatment. This acid treatment could denature remaining intact and truncated proteins (at low DH) and, therefore, alter their gelation properties. However, the control (DH 0%) was also acid-treated and could still gel.

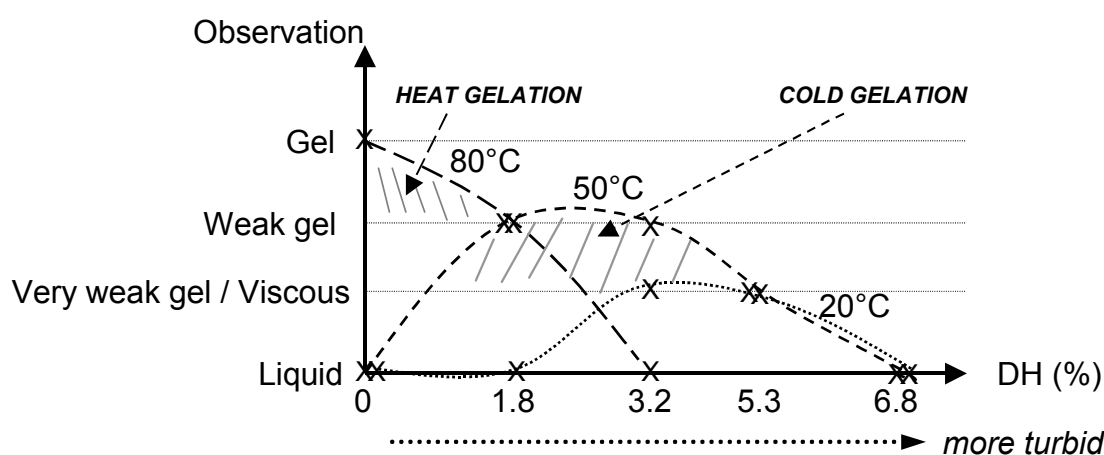


Figure 6.6. Visual observations made on hydrolysates treated for 1 h at 20, 50 and 80°C, followed by 1 h at 20°C.

In the above mentioned publication of Ju and co-workers (Ju et al., 1995), heat-induced gelation of WPI was enhanced by limited hydrolysis with BLP, at DH 1.1 and 1.5%, prior to heat-treatment (at 80°C for 30 min). Gel strength was enhanced 6 times (at a concentration of 12%, DH 1.5%) as measured by penetrometry. The gel strengthening effect decreased as the DH increased. In the publication of Ju and Kilara (1998), enhanced heat induced gelation by limited hydrolysis is much less than described previously (Ju et al., 1995), since a maximum increase in hardness of only ~2 times was observed (at a concentration of 18%, DH <1.0%). From these described results, it is not clear to which hydrolysis conditions (DH, concentration, ionic strength) the enhancement of heat-induced gelation by hydrolysis applies.

We suspected ionic strength to affect the enhancement of heat-induced gelation by hydrolysis because ionic strength, to a certain extent, also enhances the heat-induced gelation of WPI. Additional experiments were performed. WPI (concentration of 14%) was hydrolyzed at pH 8.0 to reach different DH's (DH 0, 0.4, 0.9, 1.8, 2.7 and 3.3%). Next, the pH was adjusted to 7.0 and NaCl was added to reach different concentrations (30 to 400 mM). Final proteinaceous concentration was 12%. In this case the procedure was more analogous to the ones used in literature, as the acid inactivation step was omitted. Gelation was induced by heat treatment at 80°C for 30 min. Gel strength was measured with penetrometry. The initial slope of the force measured against the distance of the resulting gels vs. NaCl concentration is plotted in **Figure 6.7**. At almost all NaCl concentrations, the control gels, without hydrolysis, gave stronger gels than the gels obtained from the hydrolysates. The minimal NaCl concentration tested was 30 mM. The enhancing effect of the ionic strength on heat-induced gelation of WPI is higher than that of a possible enhancing effect of limited hydrolysis. If an enhancement of heat-induced gelation by hydrolysis exists, it would be at a low ionic strength (<30 mM in the conditions tested). Therefore, our results show that any possible enhancement of heat-induced gelation by limited hydrolysis is overruled by ionic strength (at ionic strengths >30 mM).

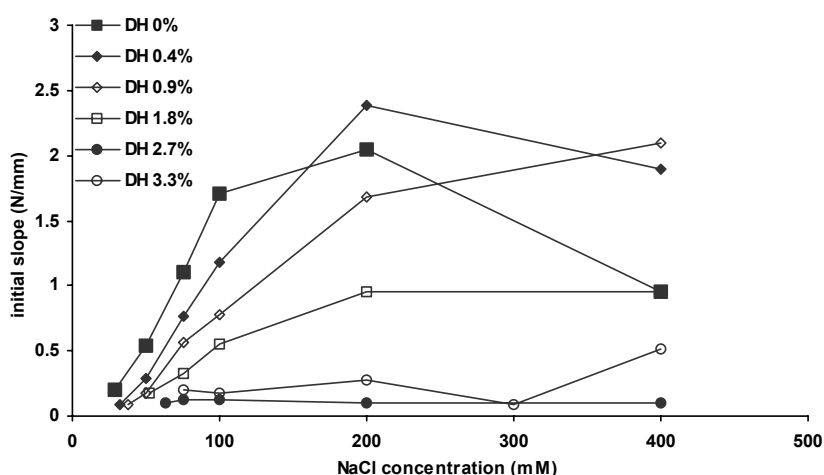


Figure 6.7. Effects of NaCl concentration on the initial slope of the force against the distance measured by penetrometry on heat-treated hydrolysates.

As it was not clear to which extent the enhancement of heat-induced gelation by hydrolysis applies and as it might be overruled by ionic strength, focus in our studies was given on peptide interactions being the basis for cold-induced gelation. In analogy to the experiments done at 80°C, we also investigated cold gelation at 50°C and 20°C, using hydrolysates of different DH's. As shown in **Figure 6.6**, cold gelation of whey protein hydrolysates, at 50°C, was optimal at intermediate degrees of hydrolysis (DH 1.8-3.2%). Assumedly too strong peptide-peptide interactions over peptide-solvent interactions prevented the formation of a gel network at DH > 3.2%. Another explanation would be that

there is not enough intact protein in the hydrolysate at $DH > 3.2\%$ to form a network. Based on the original amount of protein present, there is 29 and 16% of intact protein (both β -lg and α -la) at DH 1.8 and 3.2%, respectively. At DH 5.3 and 6.8%, there is only 9 and 5% of intact protein, respectively (**Figure 6.8**). For a hydrolysate concentration of 6%, the above mentioned proportions of intact protein corresponds to an intact protein concentration of 0.78% at DH 3.2% and 0.54% at DH 5.3%.

It was hypothesized that the remaining intact protein plays a role in the cold-gelation behavior of the hydrolysates. Peptides could act as crosslinkers between intact protein molecules and help to build up a gel network. In this case, optimal gelation is assumed to require a perfect balance between peptides and intact protein, which is not always easily met during protein hydrolysis. An alternative is the production of aggregating peptides, which could subsequently be mixed with intact protein.

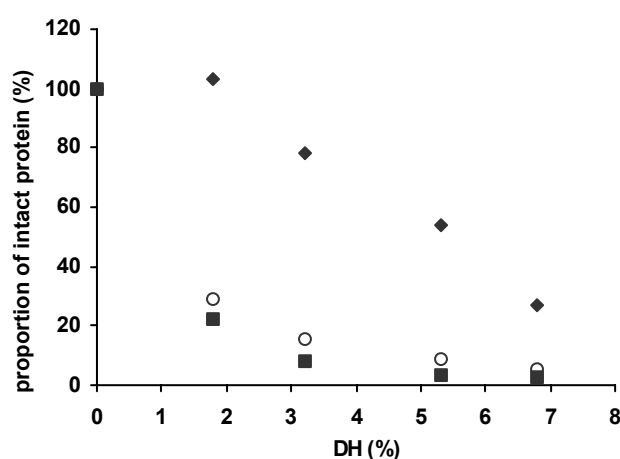


Figure 6.8. Proportion of β -lg (■) and α -la (◆) and total proportion of protein (○) remaining intact during hydrolysis of WPI with BLP (as determined with size exclusion chromatography under reducing and denaturing conditions).

6.4. Protein-peptide aggregation

Our results clearly show that the hydrolysates were able to aggregate added WPI (Chapter 2). For hydrolysates with a low DH ($DH < 6.8\%$), it was assumed that protein-peptide interactions indeed occurred. However, the concentration of aggregating peptides was apparently too low to form stable protein-peptide aggregates. For hydrolysates with a high DH (6.8%), the amount and the nature of the aggregating peptides apparently suited to hold the otherwise not aggregating proteins together in aggregates. Aggregation of protein by peptides is dominated by both hydrophobic interactions and by reduced electrostatic repulsion (Chapter 2).

Table 6.1. Sequences and characteristics of peptides originating from β -lg and α -la reported to be involved in peptide-peptide and protein-peptide interactions.

Peptide	Peptide-peptide interactions	Protein-peptide interactions	pI ^a	MW (Da)	Hf _{av} (kcal res ⁻¹) ^b	Maximum size of hydrophobic segment (aa) ^c	Net charge at pH 7.0
β -lg A			5.18 ^d	18367.3	1.23	14	-9.2
β -lg [f1-8] [*]	x		10.1	933.2	1.34	-	+1.0 (+1.0 at pH 4.0)
β -lg AB [f1-45] ^{*,\bullet}	x	x	4.14	4895.6	1.19	14	-2.0
β -lg [f15-20] [*]	x		6.0	695.8	1.46	-	0.0 (0.0 at pH 4.0)
β -lg [f41-60] [*]	x		3.97	2313.7	1.37	5	-2.9 (-2.4 at pH 4.0)
β -lg [f69-83] [□]		x	10.18	1714.2	1.68	6	+4.0
β -lg B [f75-89] [•]	x		9.86	1687.0	1.45	9	+1.0
β -lg B [f75-108] [•]	x		9.69	4003.8	1.46	9	+1.0
β -lg AB [f90-108] [•]	x		6.97	2335.2	1.46	5	0.0
β -lg AB [f90-108]-S-S- β -lg AB [f90-108] [#]	x		6.97	4668.4	1.46	5	0
β -lg AB [f90-108]-S-S- α -la [f50-113] [#]	x	x	5.20	9736.7	1.25	6	-2.9
β -lg A [f90-157] [#]	x		4.52	7868.9	1.21	9	-4.0
β -lg [f102-105] [□]		x	5.52	554.7	2.58	3	0.0
β -lg [f130-135] [□]		x	4.14	703.7	0.78	1	-2.0
β -lg AB [f135-157/158] ^{*,\bullet}	x		9.86	2826.5	1.31	5	+1.1
β -lg [f146-149] [□]		x	9.76	537.7	1.53	1	+1.1
130 peptides [*]	x		-	<2000	1.0-1.5	-	0.0 at pH 6.0
α -la B			4.30 ^d	14186.0	1.15	6	-6.7
α -la [f12-49]-S-S- α -la [f50-113] [#]	x	x	4.80	11575.5	1.10	6	-2.9
α -la [f26-37]-S-S- α -la [f50-113] [†]	x		4.7	8818.0	1.17	6	-3.7

^a Isoelectric point was determined from ExPASy Proteomics tools. ^b Average hydrophobicity was calculated according to the method of Bigelow (1967). ^c according to Kyte and Doolittle (1982) with a window of 5 residues (aa means amino acid). ^d from Walstra et al., 2006. ^{*} Groleau et al., 2003; [#] present study; [•] Otte et al., 1997; [□] Noiseux et al., 2002; [•] Doucet et al., 2003a; [†] Otte et al., 2004.

The composition of the aggregates produced by the hydrolysate with a DH of 6.8% was further investigated (Chapter 3). There was an optimal amount of added intact WPI that could interact with aggregating peptides (at 60°C and at an ionic strength of 175 mM), yielding the maximum amount of aggregated material. The peptide/protein molar ratio at this point was of around 6. Mainly β -lg was present in the aggregates. Its proportion (relative to the other intact proteins) in the aggregates was higher than that in WPI. It was assumed that the peptides were able to interact with the surface of the native β -lg (Chapter 2). Therefore, under these conditions, 6 moles of aggregating peptides were interacting with 1 mole of β -lg.

Under all conditions applied the same peptides were observed in the protein-peptide aggregates formed. The dominant peptides were β -lg AB [f1-45], β -lg AB [f90-108] and α -la [f50-113] (under reducing conditions; Chapter 3). When mixing peptides with intact protein, peptide-peptide interactions competed with protein-peptide interactions. Interestingly, the peptides involved in the peptide-peptide interactions were the same as those involved in the protein-peptide interactions.

It was hypothesised that peptides could form a kind of glue network that can include intact protein (Chapter 3). When there was an excess of protein in the system, less aggregation was observed, meaning that the glue network of peptides was disrupted into soluble protein-peptide adducts. That proves that non-covalent interactions hold the peptide network. However, it cannot be excluded that the free SH group of the peptides could form covalent protein-peptide interactions. It was hypothesized that the presence of both insoluble (e.g. β -lg AB [f1-45]) and soluble (e.g. β -lg AB [90-108]-S-S- α -la [50-113] and α -la [12-49]-S-S- α -la [50-113] peptides is required to aggregate intact protein (Chapter 5). Indeed, insoluble peptides preferred peptide-peptide interactions over protein-peptide interactions. In addition, soluble protein-peptide adducts were obtained with partly soluble peptides.

The aggregating peptides could aggregate different proteins and interestingly the same peptides were at each condition involved in the aggregation (Chapter 5). The aggregating peptide network could include intact proteins, globular or not, with a preference for the unfolded amphipathic protein β -casein that offers more possibilities for hydrophobic interactions with hydrophobic peptides.

Table 6.1 lists peptides originating from β -lg and α -la that were reported to be involved either in peptide-peptide interactions or in protein-peptide interactions, including the present study. The peptides involved in both peptide-peptide and protein-peptide interactions do not seem to possess corresponding characteristics. For example, the masses of the peptides involved in peptide-peptide interactions varied from 537.7 to 11575.5 Da, their pI's varied from 4.14 to 10.1 and their average hydrophobicities varied from 1.0 to 1.46. There are different mechanisms (Chapter 5) for peptide aggregation and for protein-peptide interactions (e.g. via hydrophobic and/or electrostatic interactions). Therefore there are various peptides involved with different characteristics. As the interacting peptides “cover” almost the entire

sequences of the β -lg and α -la molecule, the latter are good substrates for isolating interacting peptides.

It can be concluded that aggregating peptides could form a network that can include intact proteins, being not only the parental ones. Also, peptide aggregation in hydrolysates of whey protein isolate made with *Bacillus licheniformis* protease is more complex than only ascribing its extent to one individual peptide as we have shown that peptide co-aggregation occurs.

SUMMARY

Partial hydrolysis of whey protein isolate (WPI) with a seryl protease from *Bacillus licheniformis* (BLP) leads to the formation of enzyme-induced aggregates that eventually form a gel (Otte et al., 1996). This study aimed at understanding the underlying mechanism of peptide-induced aggregation of whey protein hydrolysates made with BLP. It was hypothesized that remaining intact protein in the whey protein hydrolysate plays a role in the gelation behavior of the hydrolysate. In order to evaluate if aggregating peptides could aggregate intact proteins, it was decided to uncouple hydrolysis from aggregation and to induce aggregation in presence of added intact proteins. In this way the proportions of intact protein and peptides are not interrelated as it is in the case with total hydrolysates.

The specific aims of the thesis are: 1) To understand the formation of peptide-peptide aggregates with respect to quantification, identification of the type of interactions, identification of the aggregating peptides, and proposing an aggregation mechanism. 2) To understand the formation of protein-peptide aggregates, with the same set up as the one used for the study of peptide aggregates (1).

Chapter 1 introduces the subject with a literature overview concerning the effects of enzymatic hydrolysis on globular protein gelation. Enzyme-induced aggregation and gelation of native whey proteins was shown to be rather specific. To our knowledge, for whey proteins, it was only obtained with an enzyme possessing glutamyl endopeptidase activity (e.g. BLP). This chapter also presents the current knowledge on the enzyme-induced aggregation of whey proteins with BLP and on cold- and heat-induced gelation of BLP hydrolysates in more detail.

In **Chapter 2**, the extent of aggregation in hydrolysates of WPI induced by BLP was quantified as a function of degree of hydrolysis (DH), temperature and ionic strength. The capacity of the hydrolysates to aggregate added intact protein was also studied. The amount of aggregated material and the size of the aggregating peptides were measured by nitrogen content and size exclusion chromatography, respectively. Aggregation was shown to increase with DH up to the practical end point of hydrolysis (DH 6.8 %). The hydrolysates were also able to aggregate added WPI. The additional amount of aggregated material increased with increasing DH. Peptides involved in peptide-peptide interactions were also involved in protein-peptide interactions. It was hypothesized that hydrophobic interactions dominate peptide-peptide interactions, while protein-peptide interactions depend on the balance between hydrophobic attraction and electrostatic repulsions.

The objective of the work in **Chapter 3** was to identify the dominant peptides that have the capability to aggregate intact whey proteins. This study also aimed at understanding

the underlying protein-peptide interactions leading to aggregation in mixtures of whey protein hydrolysate, made with BLP, and WPI. The hydrolysate at the end point of hydrolysis (DH 6.8%) was selected in this study as it was shown (**Chapter 2**) to aggregate the highest amount of intact protein. First, the effect of several conditions on the amount and composition of aggregates formed was investigated using response surface methodology. Next, the peptides present in the aggregates were separated from the intact protein and identified with liquid chromatography-mass spectrometry. There was an optimal amount of added intact WPI that could interact with aggregating peptides, yielding a maximal amount of aggregated material in which the peptide/protein molar ratio was of around 6. Under all conditions applied the same peptides were observed in the protein-peptide aggregates formed. The dominant peptides were β -lg AB [f1-45], β -lg AB [f90-108] and α -la [f50-113] (under reducing conditions). It was hypothesized that peptides can form a kind of glue network that can include β -lg via hydrophobic interactions at the hydrophobic binding sites at the surface of the protein.

The objective in **Chapter 4** was to identify the dominant aggregating peptides in a whey protein hydrolysate obtained with BLP without the presence of added intact protein. For that purpose, the hydrolysate with DH 6.8% was again selected as it was shown (**Chapter 2**) that aggregation reaches a maximum at the end point of hydrolysis. The aggregating peptides were isolated by centrifugation, fractionated with preparative reversed-phase chromatography and identified with liquid chromatography-mass spectrometry. The dominant aggregating peptide is β -lg AB [f1-45]. In addition, the peptides β -lg AB [f90-108]-S-S- α -la [f50-113], α -la [f12-49]-S-S- α -la [f50-113], β -lg AB [f90-108]-S-S- β -lg AB [f90-108], β -lg A [f90-157] and β -lg AB [f135-157/158] were also identified as main aggregating peptides. Aggregation, via hydrophobic interactions, prevented further digestion, thereby explaining the large size of the aggregating peptides. A mechanism for peptide aggregation in hydrolysates of WPI made with BLP was proposed. It was hypothesized that BLP breaks down hydrophilic segments in the substrate and, therefore, preserves hydrophobic segments that aggregate once exposed to the solvent.

In **Chapter 5**, the solubility behavior of the aggregating peptides, fractionated and identified in **Chapter 4**, as well as their aggregating capacities towards added intact proteins were investigated. The amount of aggregated material and the composition of the aggregates obtained were measured by nitrogen content and size exclusion chromatography, respectively. The aggregating peptides consist of insoluble peptides, mainly β -lg AB [f1-45], β -lg A [f90-157] and peptides having a partial common sequence with the fragment β -lg AB [f1-45]. It was concluded that these peptides co-aggregate, assumedly via hydrophobic interactions, other relatively more soluble peptides (β -lg AB [f90-108]-S-S- α -la [f50-113] and α -la [f12-49]-S-S- α -la [f50-113]). It was also shown that aggregating peptides can aggregate intact protein in a generic manner since the same peptides are involved in the aggregation of whey

proteins, β -casein and bovine serum albumin. In addition, we found that both insoluble and partly insoluble peptides are required for the aggregation of intact protein.

In **Chapter 6** the main results of this thesis are discussed. Peptide aggregation in hydrolysates of WPI made with BLP is concluded to be more complex than only ascribing its extent to one individual peptide as we have shown that peptide co-aggregation occurs. A mechanism for peptide-induced aggregation is discussed in comparison with other reported mechanisms. Heat- and cold-gelation of the WPI hydrolysates are discussed. From literature data, the conditions for enhancing heat-induced gelation of WPI by limited hydrolysis are not clear and are shown, in the present study, to be overruled by ionic strength. It was also shown that cold-induced gelation of the hydrolysates is optimal at intermediate DH's (DH 1.8-3.2%), while the extent of aggregation in these hydrolysates is the highest at the end point of hydrolysis. Finally, protein-peptide interactions were discussed. The sequences of the peptides involved in peptide-peptide interactions and in protein-peptide interactions were compared with literature data. The peptides involved in both interactions do not seem to possess specific characteristics.

SAMENVATTING

Gedeeltelijke hydrolyse van wei eiwit-isolaat (whey protein isolate; WPI) met een seryl-protease van *Bacillus licheniformis* (BLP) leidt tot de vorming van enzym-geïnduceerde aggregaten die een gel kunnen vormen (Otte et al., 1996). Het doel van de in dit proefschrift beschreven studie was begrip te krijgen van het onderliggende mechanisme van peptide-geïnduceerde aggregatie van wei-eiwithydrolysaten, gemaakt met BLP. De verwachting was dat intact eiwit, dat nog aanwezig is in het wei-eiwithydrolysaat, een rol speelt in het geleringsgedrag van het hydrolysaat. Om te onderzoeken of aggregerende peptiden intact eiwit kunnen aggregeren, werd besloten de hydrolyse los te koppelen van de aggregatie, om vervolgens aggregatie te induceren in de aanwezigheid van intact eiwit. Op deze manier staat de verhouding van de hoeveelheid intact eiwit en de peptiden niet met elkaar in verband zoals dit wel het geval is bij totale hydrolysaten.

De specifieke doelen van dit proefschrift zijn: 1) Het begrijpen van de vorming van peptide-peptide aggregaten aangaande de kwantificering, identificatie van het type interactie, identificatie van de aggregerende peptiden, en het opstellen van een aggregatiemechanisme. 2) Het begrijpen van de vorming van eiwit-peptide aggregaten, met dezelfde opzet als gebruikt voor de bestudering van de peptide aggregaten (1).

Hoofdstuk 1 introduceert het onderwerp met een literatuuroverzicht over de effecten van enzymatische hydrolyse op gelering van globulaire eiwitten. Enzym-geïnduceerde aggregatie en gelering van natieve wei-eiwitten bleek behoorlijk specifiek te zijn. Voorzover bekend, werd dit voor wei eiwitten alleen verkregen als een enzym met glutamyl-endopeptidase activiteit (bijvoorbeeld BLP) werd gebruikt. Verder beschrijft dit hoofdstuk de huidige kennis betreffende de enzym-geïnduceerde aggregatie van wei eiwitten met BLP en koude- en hitte-geïnduceerde gelering van BLP hydrolysaten in meer detail.

In **Hoofdstuk 2** wordt de mate van aggregatie in hydrolysaten van WPI geïnduceerd door BLP gekwantificeerd als functie van de hydrolysegraad (degree of hydrolysis; DH), temperatuur en ionsterkte. Daarnaast werd het vermogen van de hydrolysaten om toegevoegde intacte eiwitten te aggregeren bestudeerd. De hoeveelheid geaggregeerd materiaal en de grootte van de aggregerende peptiden werd gemeten aan de hand van, respectievelijk, het stikstofgehalte en gelpermeatie chromatografie. De aggregatie nam toe met oplopende DH tot aan het eindpunt van de hydrolyse (DH 6.8%). De hydrolysaten konden ook toegevoegd WPI aggregeren. De extra hoeveelheid geaggregeerd materiaal nam toe met oplopende DH. De peptiden die betrokken waren bij de peptide-peptide interacties, waren ook betrokken bij de eiwit-peptide interactie. Verondersteld wordt dat hydrofobe interacties domineren in de

peptide-peptide interacties, terwijl eiwit-peptide interacties afhangen van de verhouding tussen hydrofobe aantrekking en elektrostatistische repulsie.

Het doel van het werk in **Hoofdstuk 3** was het identificeren van de belangrijkste peptiden die de eigenschap hebben intact wei eiwit te aggregeren. Dit werk had tevens als doel het mechanisme te begrijpen van eiwit-peptide interacties, welke leiden tot de aggregatie in mengsels van wei eiwit hydrolysaat, gemaakt met BLP en WPI. Het hydrolysaat aan het einde van de hydrolyse (DH 6.8%) werd geselecteerd, omdat dit hydrolysaat de grootste hoeveelheid intact eiwit kon aggregeren, zoals gepresenteerd in **Hoofdstuk 2**. Allereerst werd het effect van verschillende condities op de hoeveelheid en samenstelling van de gevormde aggregaten bestudeerd met “response surface methodology”. Vervolgens werden de peptiden die aanwezig waren in de aggregaten gescheiden van het intacte eiwit en geïdentificeerd met behulp van vloeistof chromatografie gecombineerd met massaspectrometrie. Er is een optimale hoeveelheid toegevoegd intact WPI dat een interactie kan aangaan met aggregerende peptiden, wat resulteert in een maximale hoeveelheid van geaggregeerd materiaal waarin de molaire peptide/eiwit ratio ongeveer 6 is. Onder alle geteste condities werden dezelfde peptiden gevonden in de gevormde eiwit-peptide aggregaten. De meest voorkomende peptiden waren β -lg AB [f1-45], β -lg AB [f90-108] en α -la [f50-113] (onder reducerende condities). De gestelde hypothese is dat peptiden een soort lijm-netwerk vormen dat β -lg kan insluiten via hydrofobe interacties met de hydrofobe delen aan de buitenkant van het eiwit.

Het doel van **Hoofdstuk 4** is de identificatie van belangrijkste aggregerende peptiden in een wei-eiwit hydrolysaat, verkregen met BLP, zonder de aanwezigheid van toegevoegd intact eiwit. Hiervoor was wederom het DH 6.8% hydrolysaat gebruikt aangezien bij deze DH de aggregatie maximaal was aan het einde van de hydrolyse (**Hoofdstuk 2**). De aggregerende peptiden waren geïsoleerd door middel van centrifugatie, gefractioneerd met preparatieve reversed phase chromatografie en geïdentificeerd met vloeistof chromatografie gecombineerd met massa spectrometrie. Het belangrijkste aggregerende peptide is β -lg AB [f1-45]. Daarnaast zijn ook de peptiden β -lg AB [f90-108]-S-S- α -la [f50-113], α -la [f12-49]-S-S- α -la [f50-113], β -lg AB [f90-108]-S-S- β -lg AB [f90-108], β -lg A [f90-157] en β -lg AB [f135-157/158] geïdentificeerd als belangrijke aggregerende peptiden. Aggregatie, via hydrofobe interacties, voorkomt verdere enzymatische afbraak, wat het grote formaat van de aggregerende peptiden verklaart. Een mechanisme voor peptide aggregatie in hydrolysaten van WPI, gemaakt met behulp van BLP, is vervolgens opgesteld. De gestelde hypothese is dat BLP hydrofiele segmenten in het substraat afbreekt, waardoor de hydrofobe segmenten worden behouden die aggregeren zodra ze in aanraking komen met het oplosmiddel.

In **Hoofdstuk 5** werd de oplosbaarheid van aggregerende peptiden, gefractioneerd en geïdentificeerd in **Hoofdstuk 4**, samen met hun aggregerende vermogen, met betrekking tot van toegevoegd intact eiwit, onderzocht. De hoeveelheid aggregerend materiaal en de samenstelling van de verkregen aggregaten is respectievelijk gemeten als stikstofgehalte en gel permeatie chromatografie. De aggregerende peptiden bestaan uit onoplosbare peptiden,

voornamelijk β -lg AB [f1-45], β -lg A [f90-157] en peptiden die een gedeeltelijk overeenkomstige sequentie hebben met het fragment β -lg AB [f1-45]. Geconcludeerd wordt dat deze peptiden coaggregeren, waarschijnlijk door middel van hydrofobe interacties met andere relatief beter oplosbare peptiden (β -lg AB [f90-108]-S-S- α -la [f50-113] en α -la [f12-49]-S-S- α -la [f50-113]). Verder laten de resultaten zien dat aggregerende peptiden intact eiwit konden aggregeren op een generieke manier, aangezien dezelfde peptiden betrokken waren bij de aggregatie van wei eiwitten, β -caseïne en BSA. Daarnaast ontdekten we ook dat zowel de onoplosbare als deels onoplosbare peptiden nodig waren om intact eiwit te aggregeren.

In **Hoofdstuk 6** worden de belangrijkste resultaten van dit proefschrift bediscussieerd. Peptide-aggregatie in hydrolysaten van WPI gemaakt met BLP blijkt complexer te zijn dan de mate van aggregatie toe te kennen aan één individueel peptide, aangezien we hebben laten zien dat peptide co-aggregatie plaatsvindt. Een mechanisme voor peptide-geïnduceerde aggregatie wordt bediscussieerd in vergelijking met andere beschreven mechanismen. Hitte en koude gelering van de WPI hydrolysaten wordt bediscussieerd. Uit literatuur zijn de condities om hitte-geïnduceerde gelering van deels gehydrolyseerd WPI te verbeteren niet duidelijk. Uit deze studie is gebleken dat ionsterkte de belangrijkste parameter is. Verder is gebleken dat koude-geïnduceerde gelering van de hydrolysaten optimaal is bij een hydrolysegraad van DH 1.8-3.2%, terwijl de mate van aggregatie in deze hydrolysaten het hoogste is bij het eindpunt van de hydrolyse (DH 6.8%). Tenslotte worden eiwit-peptide interacties bediscussieerd. De sequenties van de peptiden betrokken bij de peptide-peptide interacties en de eiwit-peptide interacties werden vergeleken met literatuur data. De betrokken peptiden in beide interacties lijken geen specifieke kenmerken te hebben.

RÉSUMÉ

L'hydrolyse limitée d'un isolat de protéines de lactosérum par une sérine protéase de *Bacillus licheniformis* (BLP) conduit à la formation d'agrégats peptidiques qui peuvent former un gel (Otte et al., 1996). Le but de cette étude était de comprendre le mécanisme d'agrégation peptidique induite par l'hydrolyse d'un isolat de protéines de lactosérum par la BLP. Il est supposé que la protéine intacte, encore présente dans l'hydrolysate, joue un rôle dans le comportement de gélification de l'hydrolysate. Dans le but d'évaluer si les peptides agrégateurs peuvent aussi agréger la protéine intacte, l'hydrolyse et l'agrégation ont été découplées afin d'induire l'agrégation en présence de protéine intacte ajoutée. De cette façon, les proportions de protéine intacte et de peptides ne sont pas associées, comme c'est le cas dans les hydrolysats complets.

Plus spécifiquement, les objectifs de cette thèse sont : 1) comprendre la formation d'agrégats peptidiques par quantification ainsi qu'en identifiant le type d'interactions et les peptides impliqués ; ces informations permettront de proposer un mécanisme d'agrégation. 2) comprendre la formation d'agrégats contenant à la fois des peptides et de la protéine intacte, suivant le même procédé que celui utilisé pour l'étude des agrégats peptidiques (1).

Le **Chapitre 1** présente une vue d'ensemble de la littérature concernant les effets de l'hydrolyse enzymatique sur la gélification des protéines globulaires. L'agrégation et la gélification de protéines de lactosérum natives par voie enzymatique sont spécifiques. A notre connaissance, l'agrégation et la gélification de protéines de lactosérum ne sont obtenues qu'avec une enzyme possédant une activité glutamyl endopeptidase (par exemple la BLP). Ce chapitre présente aussi les connaissances actuelles sur l'agrégation des protéines de lactosérum induite par la BLP, ainsi que la gélification à chaud et à froid de ces hydrolysats.

Dans le **Chapitre 2**, la proportion d'agrégation dans les hydrolysats de protéines de lactosérum réalisés par la BLP a été quantifiée en fonction du degré d'hydrolyse (DH), de la température et de la force ionique. La capacité des hydrolysats à agréger la protéine intacte ajoutée a également été étudiée. La quantité de matériel agrégé et la taille des peptides agrégateurs ont été mesurées par la teneur en azote et par chromatographie d'exclusion de taille, respectivement. L'agrégation augmente avec le DH jusqu'à la fin pratique de l'hydrolyse (DH 6.8%). Les hydrolysats sont aussi capables d'agréger la protéine de lactosérum ajoutée. La quantité supplémentaire de matériel agrégé augmente avec l'augmentation du DH. Les peptides impliqués dans les interactions peptides-peptides sont aussi impliqués dans les interactions protéine-peptides. Il est supposé que des interactions hydrophobes dominent les interactions peptides-peptides alors que les interactions protéine-

peptides dépendent d'un équilibre entre des attractions hydrophobes et des répulsions électrostatiques.

Dans le **Chapitre 3**, l'objectif était d'identifier les peptides ayant la capacité d'agréger les protéines de lactosérum intacte. Cette étude avait aussi pour but la compréhension des mécanismes d'interactions protéine-peptides menant à l'agrégation dans des mélanges d'hydrolysats de protéines de lactosérum, réalisés avec le BLP, et de protéines de lactosérum intactes. L'hydrolysate obtenu en fin d'hydrolyse (DH 6.8%) a été sélectionné pour cette étude car il agrège la plus grande quantité de protéine intacte (**Chapitre 2**). Tout d'abord, l'effet de différentes conditions sur la quantité et la composition des agrégats formés a été étudié par la méthode des surfaces de réponse. Ensuite, les peptides présents dans les agrégats ont été séparés de la protéine intacte et ont été identifiés par chromatographie liquide-spectrométrie de masse. Une quantité optimale de protéines de lactosérum ajoutée peut interagir avec les peptides agrégateurs, produisant une quantité maximale de matériel agrégé dans lequel le ratio peptide/protéine est de 6 environ. Dans toutes les conditions, les mêmes peptides sont présents dans les agrégats protéine-peptides. Les peptides dominants sont β -lg AB [f1-45], β -lg AB [f90-108] et α -la [f50-113] (en conditions réductrices). Il est supposé que ces peptides forment une sorte de réseau capable d'inclure la β -lg par interactions hydrophobes avec des sites d'interaction hydrophobes à la surface de la protéine.

L'objectif du **Chapitre 4** était d'identifier les peptides agrégateurs dominants dans un hydrolysate de protéines de lactosérum réalisé avec la BLP, sans ajout de protéine intacte. A cette fin, l'hydrolysate avec DH 6.8% a été sélectionné à nouveau car l'agrégation est maximale à la fin de l'hydrolyse (**Chapitre 2**). Les peptides agrégateurs ont été isolés par centrifugation, fractionnés par chromatographie de phase inverse préparative et identifiés par chromatographie liquide-masse spectrométrie. Le peptide agrégateur dominant est β -lg AB [f1-45]. En plus, les peptides β -lg AB [f90-108]-S-S- α -la [f50-113], α -la [f12-49]-S-S- α -la [f50-113], β -lg AB [f90-108]-S-S- β -lg AB [f90-108], β -lg A [f90-157] et β -lg AB [f135-157/158] ont également été identifiés parmi les principaux peptides agrégateurs. L'agrégation peptidique, par interactions hydrophobes, empêche l'hydrolyse de se poursuivre, expliquant de ce fait la grande taille des peptides agrégateurs. Un mécanisme d'agrégation des peptides issus de l'hydrolyse des protéines de lactosérum par la BLP est proposé. Il est supposé que la BLP hydrolyse les segments hydrophiles dans le substrat et, par conséquent, préserve les segments hydrophobes qui agrègent une fois exposés au solvant.

Dans le **Chapitre 5**, la solubilité des peptides agrégateurs, fractionnés et identifiés dans le **Chapitre 4**, ainsi que leur capacité d'agrégation vis à vis de protéines intactes ajoutées, ont été étudiés. La quantité de matériel agrégé ainsi que la composition des agrégats obtenus ont été mesurées par la teneur en azote et par chromatographie d'exclusion de taille, respectivement. Les peptides agrégateurs consistent en des peptides insolubles, principalement β -lg AB [f1-45], β -lg A [f90-157] et des peptides ayant partiellement une séquence en commun avec le peptide β -lg AB [f1-45]. Il en est conclu que ces peptides coagrègent,

probablement par interactions hydrophobes, d'autres peptides relativement plus solubles (β -lg AB [f90-108]-S-S- α -la [f50-113] et α -la [f12-49]-S-S- α -la [f50-113]). Il est montré que les peptides agrégateurs peuvent également agréger une protéine intacte de façon générique car les mêmes peptides peuvent agréger à la fois des protéines de lactosérum, la β -caséine et l'albumine de sérum bovin. De plus, il est montré que la présence simultanée de peptides insolubles et partiellement insolubles est requise pour agréger la protéine intacte.

Dans le **Chapitre 6**, les principaux résultats de cette thèse sont discutés. L'agrégation des peptides dans les hydrolysats de protéines de lactosérum réalisés avec la BLP est plus complexe que la simple attribution de l'étendue de ce phénomène à un seul peptide puisque nous avons montré que la co-agrégation a lieu. Un mécanisme d'agrégation peptidique est discuté en comparaison avec d'autres mécanismes rapportés dans la littérature. La gélification à chaud et à froid d'hydrolysats de protéines de lactosérum est aussi discutée. D'après les connaissances actuelles, les conditions requises pour obtenir une activation de la gélification à chaud de protéines de lactosérum par l'hydrolyse ne sont pas claires. Dans cette étude, il semble que ces conditions soient dominées par l'effet de la force ionique. Il est aussi montré que la gélification à froid de ces hydrolysats est optimale à DH intermédiaires (DH 1.8-3.2%), alors que la proportion d'agrégation de ces hydrolysats est maximale en fin d'hydrolyse. Finalement, les interactions protéine-peptide sont discutées. Les séquences des peptides impliqués dans les interactions peptide-peptide et dans les interactions protéine-peptide sont comparées avec les données rapportées dans la littérature. Les peptides impliqués dans les deux types d'interactions ne semblent pas posséder de caractéristiques spécifiques.

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Curriculum Vitae

Nathalie Creusot was born on January 22nd 1977 in Remiremont (Vosges, France). In 1999 she completed her “Maîtrise” (BSc.) in Biochemistry at Université Henri Poincaré, Nancy, France. During summer 1998, she performed a stage at Laboratoire des BioSciences de l’Aliment, Université Henri Poincaré, Nancy, that aimed at studying the structural features of camel and mare component-3 of proteoses peptones and lactoferrin. She graduated in 2000, obtaining a “Diplôme d’Etudes Approfondies” (MSc.) in Food Sciences at ENSBANA, Université de Bourgogne, Dijon, France. During her Master’s thesis at the Laboratoire d’ingénierie moléculaire et sensorielle de l’aliment, ENSBANA, Dijon, she studied the interfacial properties of α_{S1} -casein and its tryptic peptides in mixtures with the surfactant Tween 20.

From January 2001 until July 2005 she was a PhD student at the Laboratory of Food Chemistry, Wageningen University, The Netherlands. The results obtained during this period are described in this thesis.

Since August 2005 she works as Junior Scientist at the Laboratory of Food Chemistry, Wageningen University, on a project dealing with the techno-functional properties of plant proteins.

List of publications

Creusot, N., Gruppen, H., van Koningsveld, G.A., de Kruif, C.G., Voragen, A.G.J. (2006) Peptide-peptide and protein-peptide interactions in mixtures of whey protein isolate and whey protein isolate hydrolysates. *International Dairy Journal*, 16, 840-849.

Creusot, N., Gruppen, H. Protein-peptide interactions in mixtures of whey peptides and whey proteins. *Submitted*.

Creusot, N., Gruppen, H. Hydrolysis of whey protein isolate with *Bacillus licheniformis* protease. 1. Fractionation and identification of aggregating peptides. *To be submitted*.

Creusot, N., Gruppen, H. Hydrolysis of whey protein isolate with *Bacillus licheniformis* protease. 2. Aggregating capacities of peptide fractions. *To be submitted*.

Girardet J-M., Humbert G., Creusot N., Chardot V., Campagna S., Courthaudon J-L., Gaillard J-L. (2001) Dilational rheology of mixed β -casein/Tween 20 and β -casein (f114–169)/Tween 20 films at oil–water interface. *Journal of Colloid and Interface Science*. Vol. 243, no. 2, 515-522(8).

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Overview of completed training activities

Discipline specific activities:

Courses:

Statistics course, PE&RC (Wageningen, 2001)

Advanced Food Analysis, VLAG (Wageningen, 2002)

Mass spectrometry in biology, BioCentrum (Amsterdam, 2002)

Rheological Measurements, European Society of Rheology and Belgian Group of Rheology (Leuven, 2003)

Industrial Proteins, VLAG and Senter (Wageningen, 2003)

Food Enzymology, VLAG (Wageningen, 2004)

Meetings:

Food Colloids Congress (Wageningen, 2002)

Symposium “Advanced methods to probe molecular properties of proteins”, Senter (Wageningen, 2002)

Conference on milk proteins, Agricultural University of Norway (Vinstra, 2003)

Symposium Industrial Proteins, Senter (Ede, 2003)

Nizo Dairy Conference (Arnhem, 2003)

Scientific exchange (Hambourg, 2004)

Symposium “Transition towards sustainable protein supply chains”, VLAG (Wageningen, 2004)

44th Thematic Meeting: Enzymatic conversion of biopolymers, VLAG (Wageningen, 2004)

B-Basic Symposium Protein based materials, NWO ACTS (Wageningen, 2005)

General courses:

Techniques for writing and presenting a scientific paper, Vrij Universiteit (Amsterdam, 2001)

VLAG PhD week (Bilthoven, 2002)

Presentation skill course, Senter (Oosterbeek, 2002)

Food Chemistry PhD trip (USA, 2002)

Food Chemistry PhD trip (Japan, 2004)

Additional activities:

Preparation PhD research proposal

Food Chemistry Seminars (2001-2005)

Food Chemistry Colloquia (2001-2005)

Food Chemistry Protein meetings (2001-2005)

