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Hydrolysis of Whey Protein Isolate with *Bacillus licheniformis* Protease: Aggregating Capacities of Peptide Fractions

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In a previous study, peptides aggregating at pH 7.0 derived from a whey protein hydrolysate made with *Bacillus licheniformis* protease were fractionated and identified. The objective of the present work was to investigate the solubility of the fractionated aggregating peptides, as a function of concentration, and their aggregating capacities toward added intact proteins. The amount of aggregated material and the composition of the aggregates obtained were measured by nitrogen concentration and size exclusion chromatography, respectively. The results showed that of the four fractions obtained from the aggregating peptides, two were insoluble, while the other two consisted of 1:1 mixture of low and high solubility peptides. Therefore, insoluble peptides coaggregated, assumedly via hydrophobic interactions, other relatively more soluble peptides. It was also shown that aggregating peptides could aggregate intact protein nonspecifically since the same peptides were involved in the aggregation of whey proteins, β-casein, and bovine serum albumin. Both insoluble and partly insoluble peptides were required for the aggregation of intact protein. These results are of interest for the applications of protein hydrolysates, as mixtures of intact protein and peptides are often present in these applications.

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

INTRODUCTION

In our previous study (*1*), 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 (*2*) and Doucet and co-workers (*3*) earlier reported about aggregating peptides from β-lactoglobulin (β-lg). It was shown (*2*) that in hydrolysates of 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 (*4*) in the aggregation of the 6−7 major peptides present in the aggregates (*2*). Interestingly, in hydrolysates of β-lg made with subtilisin Carlsberg, the same peptides were present in the supernatant and the precipitate (*3*) 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 (*5*). In addition, peptide−peptide interactions leading to aggregation were already reported in tryptic hydrolysates of β-lg at acidic conditions (*6, 7*). 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 (*7*).

We have already shown that the extent of aggregation within hydrolysates of whey proteins made with BLP increased with increasing degrees of hydrolysis (DH) (*8*). 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 (*8*). After further investigating the protein−peptide interactions (*9*), 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 our previous 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 peptide fractions and also at understanding their aggregation capacities toward the aggregation of intact protein. For that,

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we have chosen β-casein (β-cn), an amphipathic protein, and bovine serum albumin (BSA), a large globular protein.

MATERIALS AND METHODS

Materials. A commercial WPI powder (trade name Bipro, Davisco Foods International Inc., Le Sueur, MN) was used for the experiments. According to the manufacturer, it consisted of 74.0% (w/w) β-lg, 12.5% (w/w) α-lactalbumin (α-la), 5.5% (w/w) BSA, 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-cn was purchased from Eurial (Nantes, France), and BSA was from Sigma (product number A-4503; Sigma Chemical Co., St. Louis, MO). The enzyme used was a serine proteinase from BLP (product name NS-46007, batch PPA 6219; EC 3.4.21.19), specific for Glu-X bonds and to a lesser extent for Asp-X bonds (*10*). The enzyme was kindly provided by Novozymes (Novozymes A/S, Bagsvaerd, Denmark). All reagents were of analytical grade and were purchased from Sigma or Merck (Darmstadt, Germany).

Hydrolysis of WPI. WPI (50 mg/g) was hydrolyzed by BLP at pH 8.0 and 40 °C to DH 6.8% as described previously (*1*). After hydrolysis, the pH of the hydrolysate, diluted to 20 mg/g, was adjusted to pH 2.0 with a 6.5 M trifluoroacetic acid (TFA) solution to permanently inactivate the enzymatic reaction of BLP (*8*). Part of the hydrolysate was freeze-dried while the other part was used to induce peptide aggregation at pH 7.0. Next, peptides aggregating at pH 7.0 (pellet) were isolated from nonaggregating peptides (supernatant). Part of the aggregating peptides was further fractionated into four fractions (P1, P2, P3, and P4) with preparative reversed-phase chromatography as described in ref *1*. Subsequently, all materials (hydrolysate, supernatant, pellet, and the fractions P1−P4) were freeze-dried.

Solubility and Aggregation Experiments. Solubility experiments were performed with the total hydrolysate and peptide fractions 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 μL 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 23.2 mg/g. Each sample was divided into two parts. After incubation for 1 h at 40 °C, one part was centrifuged (20 min at 19000*g*, at 20 °C), while the other part was not centrifuged. To determine the extent of aggregation, the nitrogen concentrations of the supernatant (N) and of the noncentrifuged part (N_0) 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−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*g*, 20 °C), while the other part (0.45 g) was left noncentrifuged (further denoted "total"). The extent of aggregation was next determined. 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 noncentrifuged part was freeze-dried. The "total" and pellets samples were further analyzed with size exclusion chromatography.

Nitrogen Concentration Determination. The Dumas method (*11*) was used, with a NA 2100 Protein nitrogen analyzer (CE Instruments, Milan, Italy). For WPI, a 6.38 conversion factor was used to convert nitrogen concentration to proteinaceous (intact and degraded protein) concentration. For β-cn and BSA, conversion factors used were 6.30 and 6.07, respectively. The obtained proteinaceous concentration was in mg/g.

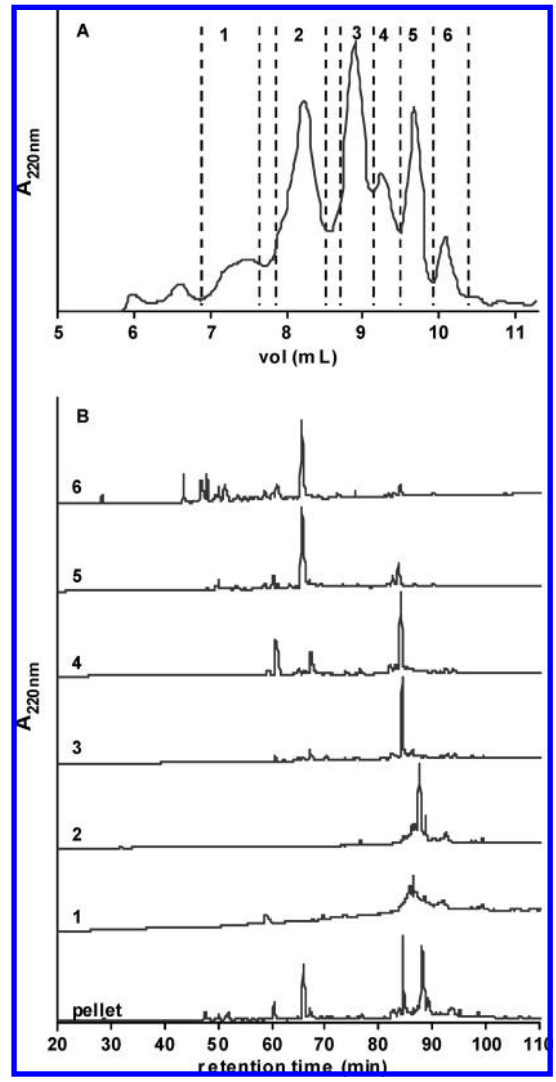

Figure 1. (**A**) Size exclusion chromatogram, under reducing conditions, and fractionation of the aggregating peptide fraction; (**B**) reversed-phase chromatograms of the fractionated peptide peaks.

Analytical Size Exclusion Chromatography. Size exclusion experiments were performed as previously described (*8*). The column Shodex Protein KW-802.5 (300 mm × 8 mm; particle size, 7 μm; pore size, 500 Å; Showa Denko K. K., Kanagawa, Japan) was equilibrated with 6 M urea containing 30% (v/v) acetonitrile and 0.1% (v/v) TFA. Sample preparation was as follows. The freeze-dried pellets and "totals" from the mixing experiment (peptides mixed with intact protein) were dissolved in 550 and 250 μL of 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), the proportion of intact protein in the aggregates and, therefore, the amount of intact protein in the aggregates, was calculated.

The pellet fraction was additionally fractionated into six fractions with size-exclusion chromatography (**Figure 1A**). This fractionation was performed to further determine the peptide composition in every peak, with reversed-phase chromatography, so that the peptides present

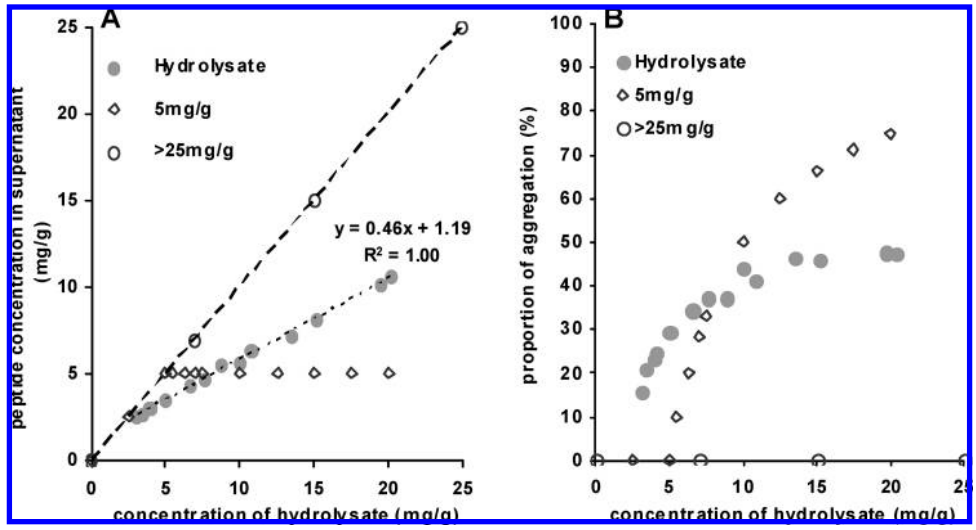

Figure 2. Solubility data of the hydrolysate and of fictive peptides that have saturation concentrations of 5 and >25 mg/g expressed as (**A**) peptide concentration in the supernatant and (**B**) proportion of aggregation, after centrifugation, as a function of the total hydrolysate concentration.

in the protein−peptide aggregates of the experiment "protein aggregation capacity" could be identified. Therefore, this analytical scale fractionation has no relation to the large scale fractionation earlier described in the section "Hydrolysis of WPI". The freeze-dried pellet fraction was dissolved in 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1 M DTT and 8 M guanidinium hydrochloride at peptide concentration of 14.3 mg/mL. 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. A volume of 100 μL of 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 (*8*).

Analytical Reversed-Phase Chromatography. Samples (supernatants and pellets) were analyzed with reversed-phase chromatography as previously described (*1*). Sample preparation was as follows. The pellets and supernatants were first dissolved in 8 M guanidinium hydrochloride to a concentration of 3 mg/mL. 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. A volume of 30 μL of sample was injected onto the column. 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 analytical scale fractionation of the pellet with size-exclusion chromatography (**Figure 1A**) were analyzed with reversed-phase chromatography (**Figure 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 (*1*).

RESULTS

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, and at an ionic strength of 100 mM. The results are given in **Figure 2A**. First, to facilitate the comparison, an example of solubility data is given for two fictive peptides, with saturation concentrations of 5 and >25 mg/g. 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 mg/g. Upon increasing peptide concentration, concentration in the supernatant would be constant (plateau) (**Figure 2A**). In the hydrolysate, the lowest concentration measured was 3 mg/g. 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). 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, of which around 46% have a relative high saturation concentration (≥11 mg/g; within the experimental range) because the peptide concentration in the supernatant (11 mg/g) did not reach a plateau at the highest concentration tested (20 mg/g).

The proportion of aggregation in the hydrolysate with DH 6.8% was also expressed as a function of hydrolysate concentration (**Figure 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 mg/g. Next, the proportion of aggregation would increase asymptotically from 0 to 100% as total peptide concentration increased, following this equation:

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

where T is the total peptide concentration and s is the concentration of soluble peptides. In the hydrolysate, the proportion of aggregation started to increase at an estimated hydrolysate concentration of 2.5 mg/g. Next, the proportion of aggregation increased asymptotically from 0 to around 50% until a hydrolysate concentration of 15 mg/g, indicating again that the hydrolysate contained 50% peptides with high and 50% 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. Combining results of **Figure 2A**,**B**, it can be stated that the hydrolysate contained around 50% of peptides with low (2.5 mg/g) and 50% peptides with high saturation concentrations (≥11 mg/g).

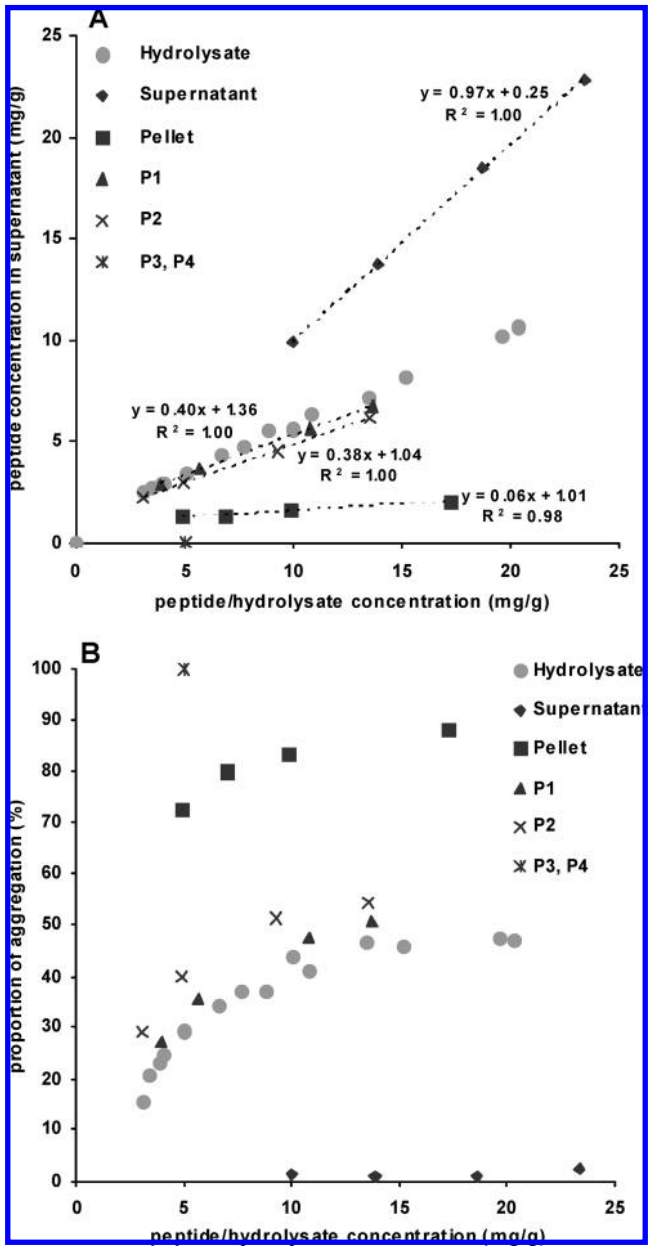

Figure 3. Solubility data of the hydrolysate and of the peptide fractions supernatant, pellet, P1, P2, P3, and P4 expressed as (**A**) peptide concentration in the supernatant and (**B**) proportion of aggregation, after centrifugation, as a function of the total hydrolysate concentration. (The same symbol is attributed to P3 and P4 because they display the same results.)

Effects of Peptide Concentration on the Solubility of Different Peptide Fractions. The hydrolysate was fractionated (*1*) into different fractions to compare their solubilities as a function of concentration. **Figure 3A** shows that the supernatant fraction was almost completely soluble until at least a concentration of 23 mg/g (slope of 0.97) and that the pellet fraction was almost completely insoluble. Indeed, the saturation concentration of all of the peptides in the pellet fraction was reached at a concentration of around 2 mg/g.

The solubilities of the four fractions isolated from the pellet fraction were also measured. **Figure 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 around 0.4, between peptide concentration in the supernatant of fractions P1 and P2 and their total concentrations (**Figure 3A**) indicated that these fractions contained a relatively high proportion of soluble peptides at total peptide concentrations between 4 and 14 mg/g. The saturation concentration of all of the peptides in the fractions P1 and P2 was not reached at a concentration of 14 mg/g (being the highest concentration tested), since the peptide concentration in the supernatant (7 mg/g) of these fractions did not reach a plateau. **Figure 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 90% as the pellet fraction concentration increased. The same was shown for the fractions P1 and P2 (**Figure 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 (estimated at 1−2 mg/g) and 50% of peptides with relatively high saturation concentration (≥7 mg/g).

The peptide distribution between the soluble and the insoluble material in fractions P1 and P2 was further investigated with reversed-phase chromatography at a total peptide concentration of 9 mg/g. The chromatograms are shown in **Figure 4**. The peptide β-lg 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.

Intact Protein Aggregating Capacities of the Peptide Fractions. It was shown in ref *9* that an optimal amount of aggregated material, at pH 7.0, was obtained when a mass of 10 mg of hydrolysate (DH 6.8%) was mixed with a mass of 20 mg of intact WPI, at 100 mM, whatever the temperature. In the present study, 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−P4) with 20 mg of intact WPI, because the hydrolysate contains around 50% of aggregating peptides (peptides with low solubility). 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 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−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 < P < P4.

The protein−peptide aggregates induced by the peptide fractions P1−P4 were analyzed with size-exclusion chromatography, under reducing conditions. Peptide peaks were annotated after identification from analytical peak fractionation (**Figure 1**) and LC-MS (*1*). The relative abundance of peptides (after quantitative analysis using calculated extinction coef-

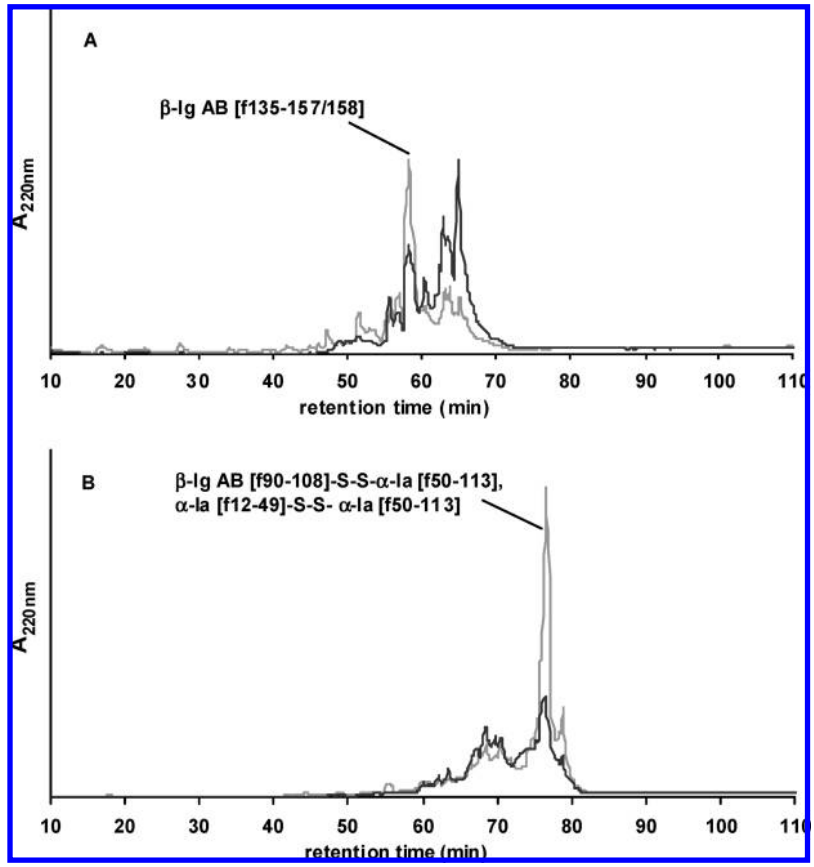

Figure 4. Reversed-phase chromatograms of the pellets (black line) and supernatants (gray line) isolated, by centrifugation, from the peptide fractions P1 (**A**) and P2 (**B**) at a concentration of 9 mg/g.

Table 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

ficients following the method developed by ref *12*) in the different fractions in the peptide aggregates and in the WPI−peptide aggregates is summarized in **Table 2**. It was shown that in the protein−peptide aggregates induced by the peptide fraction P1, the peptide β-lg AB [f90−108] was mainly involved in aggregation of intact protein. The peptide β-lg 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-lg AB [f90−108]) 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 and P4 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.

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 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 the 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 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 the pellet fraction and fraction P4, the same peptides were involved in protein−peptide aggregates with the different proteins.

DISCUSSION

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. We 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 (*13*). When solution saturation

Table 2. Total Amount of Peptides Precipitated and Relative Abundance of Peptides (as Compared with the Rest of the Peptide Material in a Fraction and after Quantitative Analysis Using Calculated Extinction Coefficients Following the Method Developed by Ref *12*) from the Different Fractions in Peptide and WPI−Peptide Aggregates, Analyzed with Size-Exclusion Chromatography, Under Reducing Conditions

		without intact WPI		with intact WPI	
fraction	peptide	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-lg AB [f135−157/158]		++++++		++
	β-lg AB [f90−108]		++++++++++		++++++++++++
	β-lg 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-lg AB [f1−45]		+++		++++
	α-la [f12−49]		+++		++
	β-lg AB [f90−108]		+++++++++		++++++++++
	β-lg AB [f115−127], α-la [f26−37/46/49]		++		−
P3		5.00		5.00	
	β-lg AB [f54−114], α-la [f50−113]		+++		+++
	β-lg AB [f1−45]		++++++++++		++++++++++
	β-lg AB [f90−108]		+++++++		+++++++
P4		5.00		5.00	
	β-lg A [f90−157], β-lg B [f66−129]		+		+
	α-la [f50−113]		+++++		+++++
	β-lg AB [f1−45]		+++++		+++++
	β-lg AB [f90−114], α-la [f12−49]		++++		++++
	β-lg AB [f90−108]		+++++		+++++

[a] Peptide peak: not detectable (−), detectable (+, . . ., ++++++++++) high intensity.

Table 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

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 (≤2 mg/g; they were separated as the pellet fraction), and the other 50% have high saturation concentration (≥23 mg/g; 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 (*1*). The fraction P3 contains mainly the peptide β-lg AB [f1−45], which 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 (*7*). Therefore, peptide coaggregation 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 (1−2 mg/g fraction concentration) and, unexpectedly, around 50% peptides with relatively high saturation concentration (≥7 mg/g 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 coaggregation 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 (*8*). It was shown in ref *1* that upon hydrolysis of whey proteins by BLP, at pH 8.0, soluble 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 (**Figure 5**). The nonaggregating 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**).

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 1** and **2**) to have poor intact protein aggregating capacities. This indicated that insolubility is not enough for a peptide to aggregate intact protein. It

Table 4. Relative Abundance of Peptides (as Compared with the Rest of the Peptide Material in a Fraction and after Quantitative Analysis Using Calculated Extinction Coefficients Following the Method Developed by Ref *12*) from the Aggregating Peptides Fraction and from the Fraction P4 in Peptide Aggregates and in Protein (WPI, β-cn, and BSA)−Peptide Aggregates, Analyzed with Size-Exclusion Chromatography, Under Reducing Conditions

		without intact WPI		with intact WPI		with intact β-cn		with intact BSA	
fraction	peptide	amount of aggregated peptides (mg)	relative abundance of peptides[a]	amount of aggregated peptides (mg)	relative abundance of peptides[a]	amount of aggregated peptides (mg)	relative abundance of peptides[a]	amount of aggregated peptides (mg)	relative abundance of peptides[a]
pellet		3.60		3.60		1.90		3.60	
	α-la [1−123], α-la [12/15−113/116], β-lg AB [90−157], β-lg B [66−129]		++		++		++		++
	α-la [50−113]		++		++		++		++
	β-lg AB [1−45]		++++		++++		++++		++++
	β-lg AB [135−157/158], β-lg AB [54−114], β-lg AB [90−114], α-la [12−49]		+++		+++		+++		+++
	β-lg AB [90−108]		++++++		+++++++		+++++++		++++++
	β-lg AB [115−127], α-la [26−37/46/49]		++		++		++		++
P4		5.00		5.00		5.00		5.00	
	β-lg AB [90−157], β-lg B [66−129]		+		+		+		+
	α-la [50−113]		+++++		+++++		+++++		+++++
	β-lg AB [1−45]		+++++		+++++		+++++		+++++
	β-lg AB [90−114], α-la [12−49]		++++		++++		++++		++++
	β-lg AB [90−108]		+++++		+++++		+++++		+++++

[a] Peptide peak: not detectable (−), detectable (+,..., ++++++++++) high intensity.

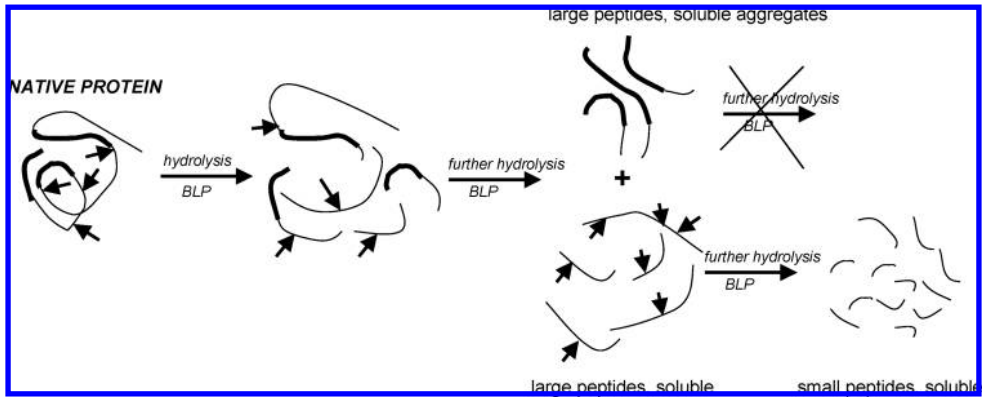

Figure 5. Schematic representation of the production of large and small peptides upon hydrolysis, at pH 8.0, of whey proteins by BLP. Arrows indicate enzymatic cleavage, and bold lines indicate hydrophobic segments.

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, which could aggregate more WPI than the fraction containing all of the aggregating peptides, consisted of 25% of the peptides of the latter fraction (*1*). It was shown that the fractions P2 and P4 have a quite similar peptide composition (*1*) 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 α-la [f12−49] and β-lg AB [f90−108], which was further shown to be the dominant peptide in the protein−peptide aggregates induced by the peptide fractions P2. 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. On this basis, 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 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 (*14, 15*). 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.

ABBREVIATIONS USED

α-la, α-lactalbumin; β-cn, β-casein; β-lg, β-lactoglobulin; BLP, *Bacillus licheniformis* protease; BSA, bovine serum albumin; DH, degree of hydrolysis; DTT, dithiothreitol; N,

nitrogen concentration; TFA, trifluoroacetic acid; WPI, whey protein isolate.

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