JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Hydrolysis of Whey Protein Isolate with *Bacillus licheniformis* Protease: Fractionation and Identification of Aggregating Peptides

NATHALIE CREUSOT AND HARRY GRUPPEN*

Laboratory of Food Chemistry, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

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 *B. licheniformis* protease breaks down hydrophilic segments in the substrate and, therefore, preserves hydrophobic segments that aggregate once exposed to the solvent.

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

INTRODUCTION

Hydrolysis is usually used to improve the solubility of proteinaceous material. Whey protein hydrolysates are usually more heat stable than intact protein. For example, trypsin (specific for Lys and Arg residues) hydrolysis inhibits the heat-induced gelation of whey proteins (*1*). As well, hydrolysis of soy proteins usually enhances their solubility (*2*). For example, trypsin and Alcalase 0.6L (a nonspecific commercial protease preparation) generate soy hydrolysates that show good solubility (around 50%) at pH 4.5, a pH at which intact soy proteins are insoluble (*3*).

However, both limited and extensive hydrolysis of globular proteins can also lead to aggregation and gelation. Much research has been performed on the hydrolysis of whey proteins with *Bacillus licheniformis* protease (BLP; glutamyl endopeptidase) since Otte and co-workers (*4*) showed that limited hydrolysis, at a degree of hydrolysis (DH) of approximately 2%, led to the 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 (*5*). The aggregates formed upon hydrolysis of β-lactoglobulin (β-lg), the main protein in bovine whey, consisted of six to seven major peptides [2–6 kDa; (*6*)]. According to the work of the same authors, the fragment β-lg [f135–158] was the initiator of aggregation, because it was present at high concentrations in the aggregates, as determined with matrix-assisted laser desorption–ionization time of flight (MALDI-TOF) mass spectrometry.

In addition, Doucet and co-workers (*7*) observed that aggregation and gelation of whey protein isolate (WPI) 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 the main proteolytic component. As reported later (*8*) 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 (*9*). Spellman and co-workers (*10*) 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.

Complementary to these findings, we reported in a former study (*11*) that, within whey protein isolate hydrolysates produced with BLP, the extent of aggregation increased with increasing DH, 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 of 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

* Author to whom correspondence should be addressed (telephone +31-317 482888; fax +31-317 484893; e-mail Harry.Gruppen@wur.nl.

10.1021/jf071584s CCC: $37.00 © 2007 American Chemical Society
Published on Web 09/29/2007

at DH = 6.8% and to understand the peptide aggregation. To this end, aggregating peptides were isolated from the hydrolysate and fractionated with preparative reversed-phase chromatography. Upon subsequent peptide identification, hypotheses on a mechanism of enzyme-induced aggregation of whey proteins were postulated.

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-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 *B. 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 (*12*). 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) or Merck (Darmstadt, Germany).

Hydrolyses. A 5% (w/w) WPI solution, prepared as previously described (*11*), was preheated at 40 °C for 15 min. The pH was adjusted to pH 8.0, 5 min before addition of the enzyme solution, as supplied by the manufacturer. The enzyme/substrate ratio (v/v) used was 1:100. During hydrolysis (3 h), the reaction mixture, incubated at 40 °C, was maintained at pH 8.0 by the addition of a 0.4 M NaOH solution in a pH-STAT (719 S Titrino, Metrohm Ion Analysis, Metrohm Ltd., Herisau, Switzerland). When a volume of NaOH corresponding to a DH of 6.8%, which is the experimental end point of hydrolysis (*11*), was reached (as calculated using a total number of peptide bonds in the protein–substrate of 8.79 mequiv/g and an average degree of dissociation of the α-NH groups of 0.833), the hydrolysis was stopped. For that, the pH of the hydrolysate, diluted to 20 mg/mL, was adjusted to pH 2.0 with a 6.5 M trifluoroacetic acid (TFA) solution and kept for 1 h 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 7.0 with 2.5 M NH_4HCO_3. After 1 h at room temperature, the hydrolysate was centrifuged (19000*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 four fractions (P1, P2, P3, and P4) using preparative reversed-phase chromatography.

WPI and fraction P3 were also hydrolyzed in the presence of 4 M urea. A WPI solution, at a concentration of 20 mg/g in 75 mM Tris-HCl buffer containing 4 M urea (pH 8.0), 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. Fraction P3 was suspended at 3.5 mg/g in 75 mM Tris-HCl buffer, with and without 4 M urea, at 40 °C and pH 8.0, and was hydrolyzed 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 further analyzed with reversed-phase chromatography.

Preparative Reversed-Phase Chromatography. Fractionation of the aggregating peptides was performed with a preparative HPLC system (Waters, Milford, MA) controlled by MassLynx version 4.0 software using an XTerra Prep MS C_{18} OBD column (50 × 100 mm; bead diameter = 5 μm, Waters). The flow rate was 82.7 mL/min and the column temperature, 20 °C; eluent A was 0.07% (v/v) TFA in 5% (v/v) aqueous acetonitrile, and eluent B was 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/mL. A volume of 10 mL of sample was injected onto the column. After 5 min of 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 3**.

Desalting of the supernatant, containing the nonaggregating 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/mL. A volume of 10 mL of sample was injected onto the column. After 5 min of 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 of the eluates with a rotative evaporator, and samples were subsequently freeze-dried.

Analytical Size Exclusion Chromatography. Size exclusion experiments were performed as previously described (*11*). 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. Samples were also prepared under nonreducing 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 because the proportion of aggregating material in the hydrolysate was around 50% (*11*).

The column was calibrated as previously described (*11*).

Analytical Reversed-Phase Chromatography. Samples were separated on an analytical Vydac C8 column (208MS52; 250 × 2.1 mm; bead diameter = 5 μm; porosity = 300 nm; Dionex, Sunnyvale, CA) by HPLC (Thermo Separation Products Inc., San Jose, CA) with ChromQuest software. The flow rate was 0.2 mL/min and the column temperature, 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. A volume of 30 μL of sample was injected onto the column. After 10 min of 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. Samples obtained from WPI hydrolysis made at a WPI concentration of 50 mg/g were analyzed with the following sample preparation. 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. Samples were also prepared under nonreducing 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.

The hydrolysates of WPI and P3, obtained in the presence or absence of urea during hydrolysis, were analyzed as follows. A volume of 25 μL of WPI hydrolysate (20 mg/g) and 71 μL of P3 hydrolysate (3.5 mg/g) were mixed with 450 and 404 μL, respectively, 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, 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, respectively.

Mass Spectrometry Analysis. *Electrospray Mass Spectrometry.* Mass spectra were recorded with an electrospray ionization mass spectrometer (LCQ ion-trap, Finnigan MAT 95, San Jose, CA) connected to the reversed-phase chromatography unit. It was operating as previously described (*13*). 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 Corp., Waltham, MA), with a FASTA database containing the β-lg A, β-lg B, and α-la B sequences.

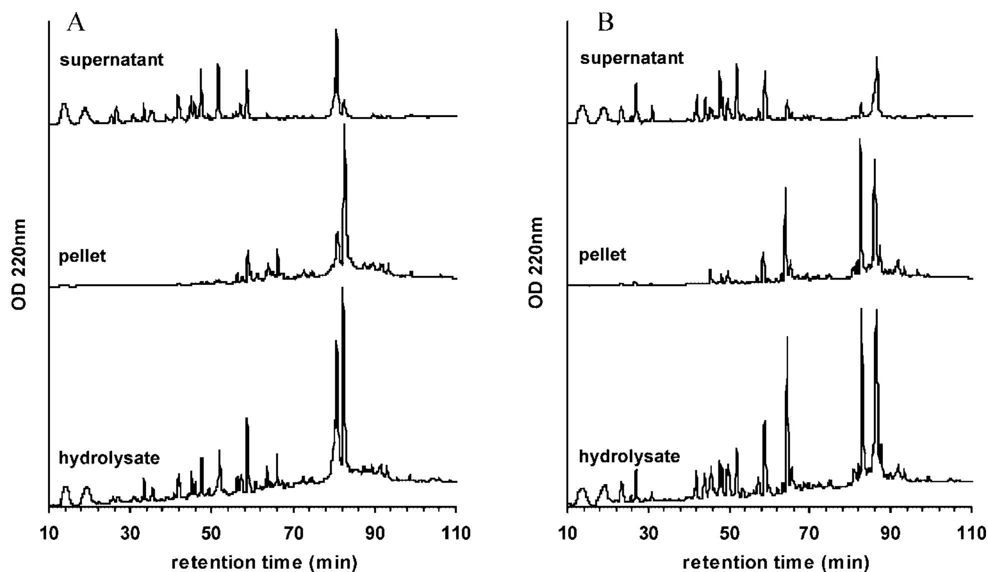

Figure 1. Reversed-phase chromatograms, under nonreducing (**A**) and reducing (**B**) conditions, of the complete hydrolysate (DH 6.8%) and of the pellet and supernatant isolated from the complete hydrolysate by centrifugation.

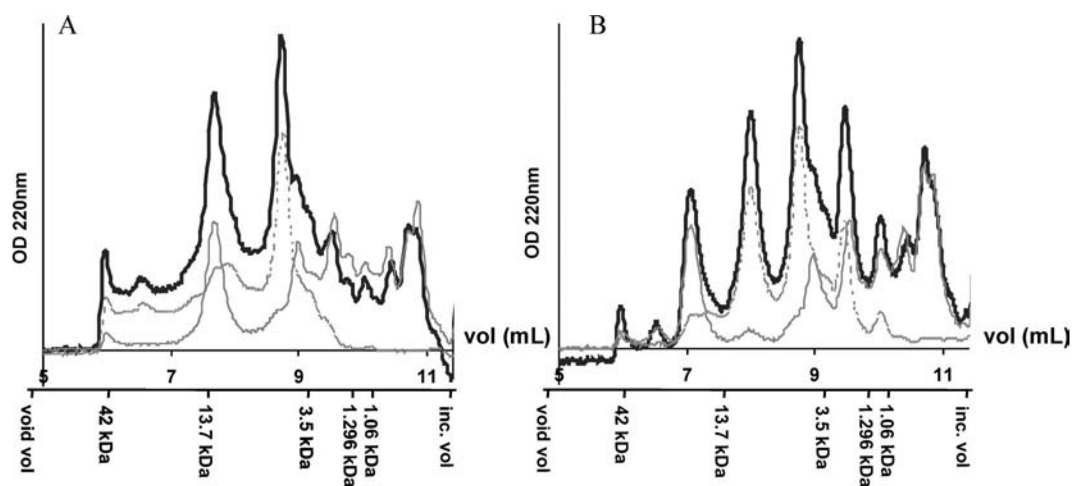

Figure 2. Size exclusion chromatograms, under nonreducing (**A**) and reducing (**B**) conditions, of the complete hydrolysate (DH 6.8%, solid line) and of the pellet (dashed gray line) and supernatant (gray line) isolated from the complete hydrolysate by centrifugation.

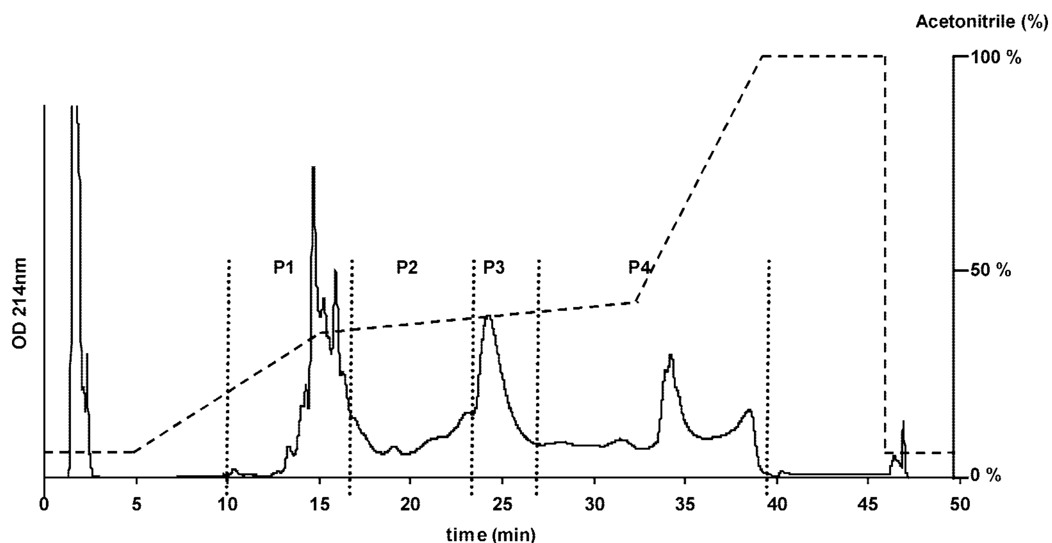

Figure 3. Preparative reversed-phase chromatogram, under nonreducing conditions, of the pellet isolated from the complete hydrolysate by centrifugation. Four fractions were denoted P1, P2, P3, and P4.

MALDI-TOF Mass Spectrometry. MALDI-TOF mass spectrometry (MALDI-TOF MS) was performed using an UltraFlex workstation (Brucker Daltonics, Bremen, Germany) equipped with a nitrogen laser of 337 nm and operated in the positive mode. A volume of 1 μL of

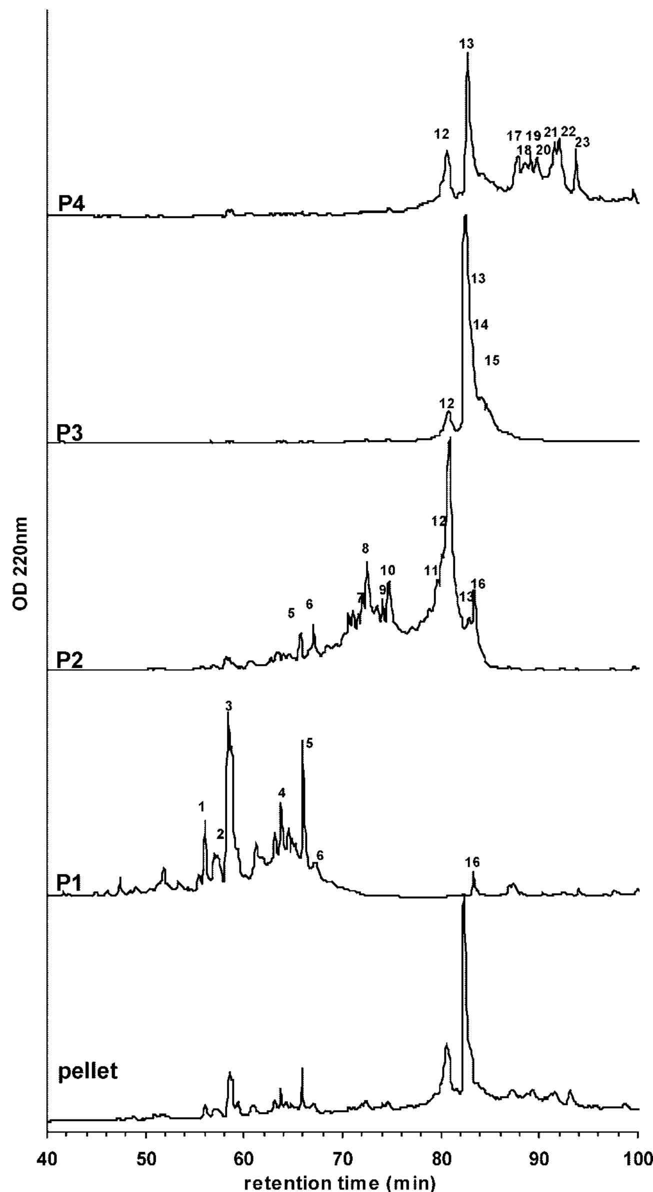

Figure 4. Reversed-phase chromatograms, under nonreducing conditions, of the pellet and the four fractions isolated from it. Codes for the peaks refer to **Table 1**.

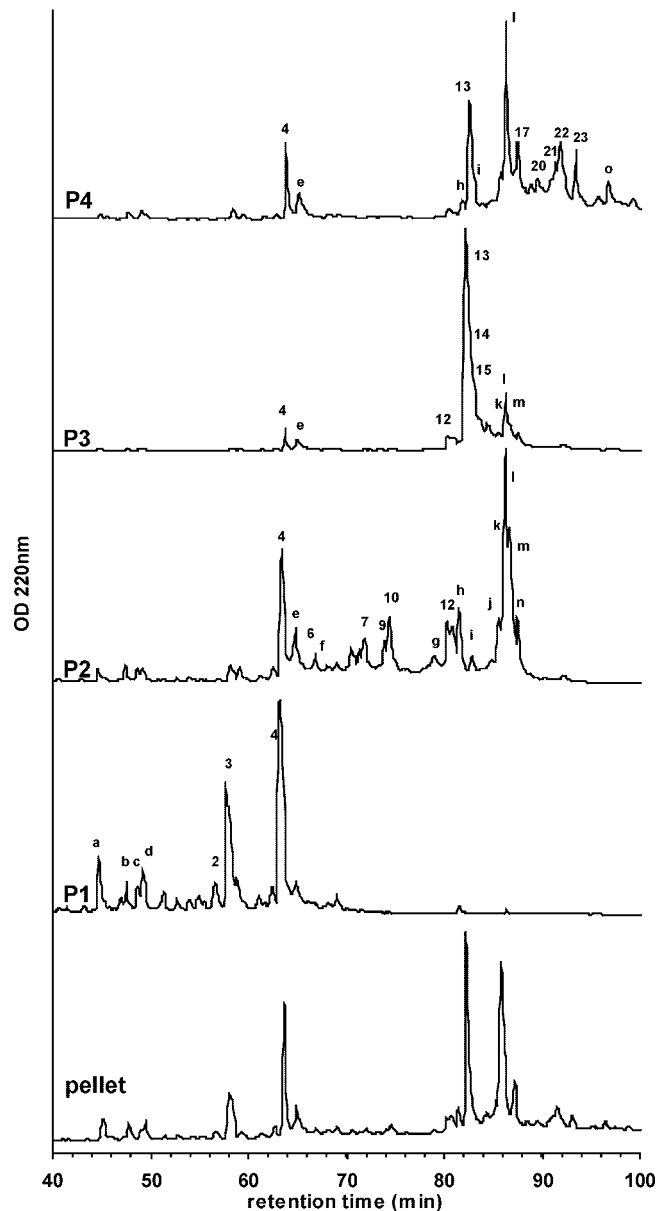

Figure 5. Reversed-phase chromatograms, under reducing conditions, of the pellet and the four fractions isolated from it. Codes for the peaks refer to **Tables 1** and **2**.

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 of acetonitrile, 100 μL of 3% (v/v) TFA, and 400 μL of water. The SA matrix was used to detect masses from 5 to 100 kDa (with linear detector), and the α-CN matrix was used 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 and 6181.05 Da for single- and double-protonated molecules, respectively), and horse myoglobin (16952.55 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).

Nitrogen Concentration Determination. Nitrogen concentrations were measured using the combustion or Dumas method (*14*) with a NA 2100 protein nitrogen analyzer (CE Instruments, Milan, Italy). A $6.38 \times N$ conversion factor was used to convert nitrogen concentration to proteinaceous (intact and degraded protein) concentration.

RESULTS

Fractionation of Aggregating Peptides. The hydrolysate was first fractionated into aggregating (pellet) and nonaggregating peptides (supernatant) by centrifugation under nonreducing conditions at pH 7.0. The proportion of the peptides that aggregated was around 45% in the hydrolysate with a DH of 6.8% (*11*). The complete hydrolysate, the aggregating and the nonaggregating peptides, was analyzed with reversed-phase chromatography and size exclusion chromatography, under both reducing and nonreducing conditions (**Figures 1** and **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 on if the BLP would cleave only after 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,

Table 1. Mass Spectrometry Results for the Peptides Present under Nonreducing Conditions Only (Boldface) and under both Nonreducing and Reducing Conditions (Lightface) in the Fractions Separated by Reversed-Phase Chromatography (**Figures 4** and **5**)

RP-HPLC peak	RT (min)	measd mass (Da)	possible fragment	theor 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][a]	
7	72.1	3608.5	*b*	
8	72.4	**5580.0**	**β-lg AB [f90–108]-S-S-*b* (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**	*b*	
		8404.3	**α-la [f1–49]-S-S-α-la [f12–37]**	**8408.0**
		b	*b*	
		b	*b*	
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][c]	3750.8
		4276.5	*b*	
		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
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
		b	*b*	
16	83.5	*b*	*b*	
17	87.9	7706.2	β-lg A [f90–157]	7868.9
18	88.7	*b*	*b*	?
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	*b*	
		6137.1	β-lg A [f12–65]	6044.1
		4083.2	*b*	
		b	*b*	
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]	

[a] Peptide may result from nonspecific cleavage. [b] No identification based on mass (no fragmentation pattern available). [c] Peptide present or mass present, but not identified.

according to reversed-phase chromatography under reducing conditions (and at least 23 peptide peaks under nonreducing conditions), it can already be assumed that BLP could cleave after both Glu and Asp residues, although BLP is more specific for Glu–X bonds than for Asp–X bonds (*12*), but missed cleavage of some of these.

Under nonreducing conditions, the aggregating peptides (pellet) eluted later in the reversed-phase chromatogram (**Figure 1**) compared to the nonaggregating 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 nonreducing conditions, and contained mainly three other peptide peaks (eluting at 58, 66, and 81 min) under nonreducing conditions.

According to the size exclusion patterns shown in **Figure 2**, remaining intact α-la, which eluted at 7.0 mL (*11*) under reducing conditions, was present in the supernatant after fractionation of the hydrolysate into supernatant and pellet. The hydrolysate contained mainly eight peptide fractions, with an average mass around 2 kDa, under reducing conditions (*11*). Under both reducing and nonreducing conditions, it was noted that the pellet was enriched in larger peptides (eluting early) than the supernatant. The aggregating peptides consisted of four peptide fractions, under reducing conditions.

Preparative reversed-phase chromatography (**Figure 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 nonreducing 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.

The four fractions were subsequently analyzed with analytical reversed-phase chromatography (**Figure 4**) under both reducing and nonreducing 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–P4 eluted at different retention time ranges (**Figures 4** and **5**). When going from nonreducing to reducing conditions, peptide peaks with high intensity appeared in the pellet, at 64 min (no. 4) and

Table 2. Mass Spectrometry Results[a] for the Peptides Present in Only Reducing Conditions under the Fractions Separated by Reversed-Phase Chromatography (**Figure 5**)

RP-HPLC peak	RT (min)	measd mass (Da)	possible fragment	theor 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	*b*	
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
		b	*b*	
		b	*b*	
n	87.5	5880.2	β-lg B [f34–85] or β-lg B [f45–96][c]	5877.2/5872.2
		6924.6	*b*	
		5052.6/8420	β-lg A [f115–158][c]	5054.6
		b	*b*	
o	96.8	7215.6	β-lg A [f66–129]	7285.8

[a] See **Table 1**.

at 86 min (letters j–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 variable length (the enzyme can miss cleavage of the substrate at some positions) because fractions P1, P2, and P4 did not have one common peak under nonreducing conditions. The peptide peak eluting at 84 min (no. 13) was the major component of fraction P3 and was also present in fraction P4. Peptide fraction P3 seemed to be rather pure and did not originate from disulfide-bridged fragments because the peptide peak had the same retention time under reducing and nonreducing conditions.

Identification of Aggregating Peptides. Table 1 lists the peptides present at both reducing and nonreducing conditions (peptide peaks denoted by numbers in **Figures 4** and **5**), and **Table 2** lists the peptides present at only reducing conditions (peptide peaks denoted by letters in **Figure 5**). The identification was based on the masses determined with both MALDI-TOF MS (nonreducing conditions) and LC-MS (reducing and nonreducing 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 in the identification of peptides present at the nonreduced conditions.

In total, 23 peaks were considered under nonreducing conditions, containing 26 peptides identified deriving from β-lg, 2 peptides identified deriving from α-la, and 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 nonreducing conditions, the masses of the non-disulfide-linked peptides ranged from around 1.4 to 14.2 kDa, and those of the disulfide-linked peptides ranged from around 3.3 to 11.6 kDa.

It was remarkable that the fragment β-lg AB [f90–108] (peptide 4 under reducing conditions) was present in the aggregates either non-disulfide-linked or linked via a disulfide 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 (*6*)) and peptides from α-la.

According to **Figures 4** and **5** and **Tables 1** and **2**, fraction P1 consisted mainly of the peptides β-lg AB [f135–157/158] and β-lg AB [f90–108]-S-S-β-lg AB [f90–108]. 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]. Fraction P3 contained mainly the peptide β-lg AB [f1–45] (**Figure 6**). Fraction P4 contained β-lg AB [f1–45] and other peptides with higher retention times (peaks 20–23) having a partial common sequence with the fragment β-lg AB [f1–45] (see next section); it 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].

Peptide β-lg AB [f1–45]. Peptide β-lg AB [f1–45] was the main aggregating peptide in a WPI hydrolysate made with BLP at high DH, because it made up about 22% of the total peak area of the aggregating peptides present in the chromatogram of the pellet (**Figure 3**). Besides β-lg AB [f1–45], the aggregating peptides comprised also peptides that had partial common sequences with it (**Tables 1** and **2**) such as β-lg AB [f12–45] and β-lg AB [f1–33], which eluted before β-lg AB [f1–45], and such as 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], which eluted after β-lg AB [f1–45] in the reversed-phase chromatogram. For this reason, the hydrophobicity profile [according to Kyte and Doolittle (*15*)] could be studied and interpreted solely on the amino acid sequence of the peptide β-lg AB [f1–45] (**Figure 7**). It could be seen that within the peptide β-lg AB [f1–45], the segment β-lg AB [f20–34] is

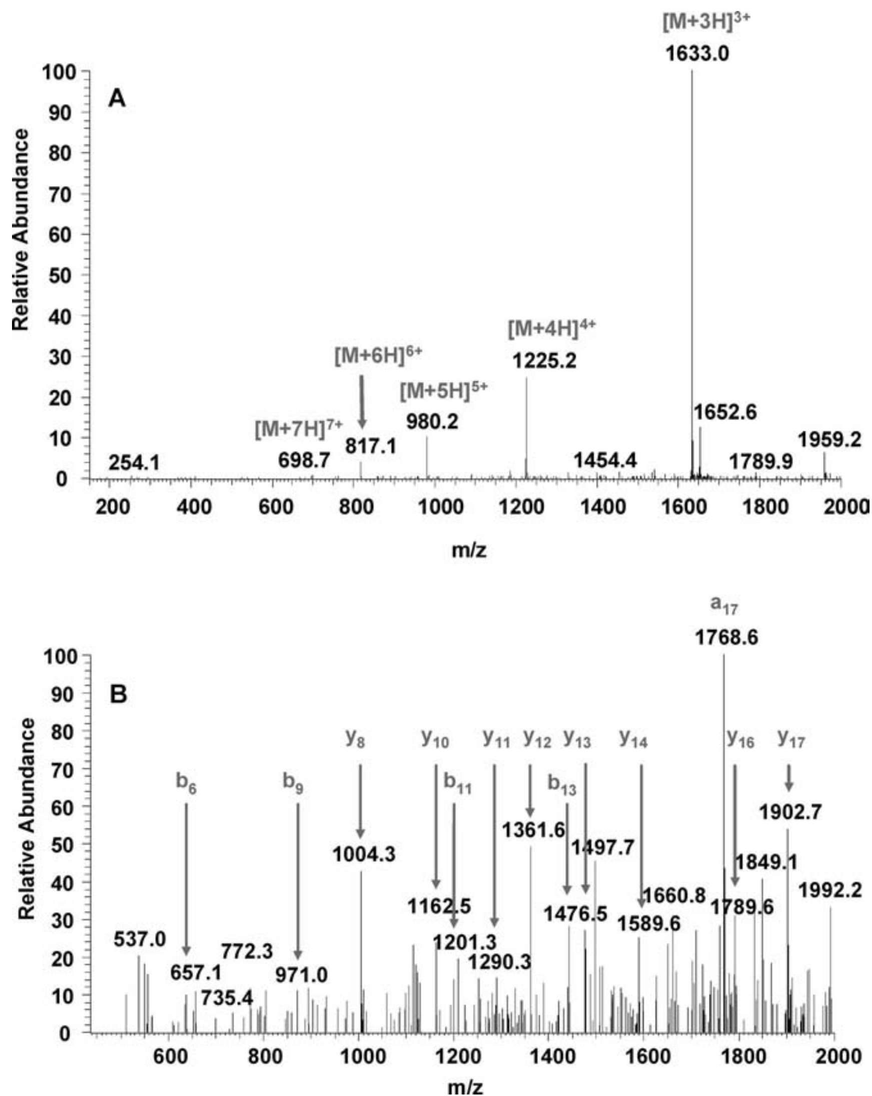

Figure 6. Mass spectrometry results for the identification of the peptide β-lg AB [f1–45] (peak 13 in Figure 4): (**A**) full MS scan; (**B**) MS/MS scan of the ion *m/z* 1633.0 ([M + 3H]$^{3+}$).

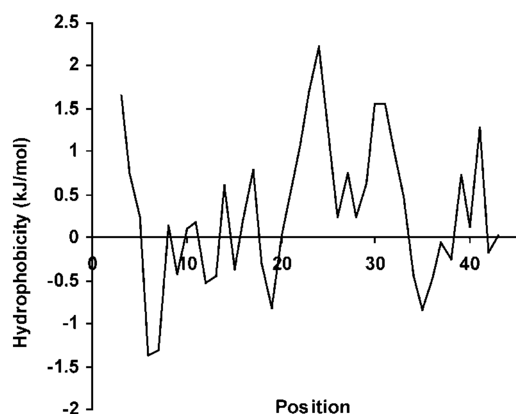

Figure 7. Schematic representation of hydrophobicity in the peptide β-lg AB [f1–45], according to Kyte and Doolittle (*19*), with a window of five residues.

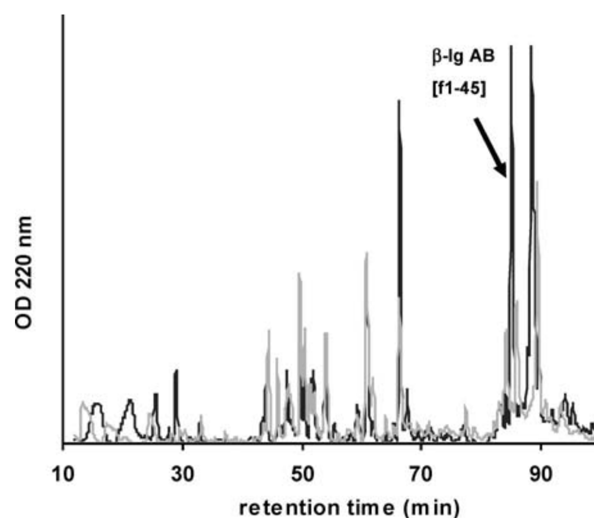

Figure 8. Reversed-phase chromatograms, under reducing conditions, of hydrolysates of WPI made with BLP, in the absence (black) and presence of 4 M urea (gray).

indeed relatively hydrophobic and, therefore, could strongly contribute to peptide aggregation.

Fragment β-lg AB [f1–45] resisted digestion by BLP at pH 8.0, although the enzyme can 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 fragment β-lg AB [f1–45] was created, it aggregated, thereby preventing further hydrolysis. To verify this assumption, hydrolysis of both WPI and fraction P3 in the presence of urea

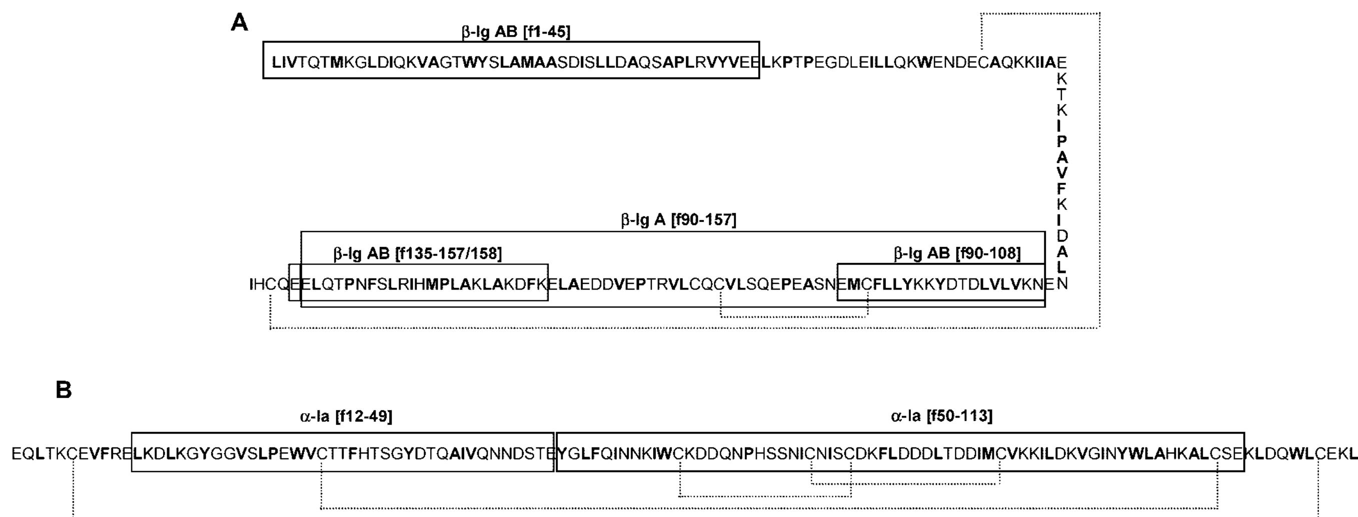

Figure 9. Amino acid sequences of bovine β-lg A (**A**) and bovine α-la B (**B**). The dotted lines indicate the native disulfide bonds. Hydrophobic residues are in bold. The sequences of the main aggregating peptides are framed.

Table 3. Characteristics of the Main Aggregating Peptides in a Hydrolysate of WPI Made with BLP

peptide	p*I*[a]	mol mass (Da)	Hf_{av} (kcal res^{-1})[b]	max size of hydrophobic segment (aa)[c]	net charge at pH 2.0	net charge at 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 (*21*). [c] According to Kyte and Doolittle (*15*) with a window of five residues (aa = amino acid). [d] From Walstra and co-workers (*22*).

was performed. The hydrolysates were subsequently analyzed with reversed-phase chromatography. It was first positively verified that the BLP was active in the presence of 4 M urea as WPI could be digested into many peptides (**Figure 8**). As shown in the reversed-phase chromatograms in **Figure 8**, fragment β-lg AB [f1–45] could be digested by BLP in the 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, because the peptide β-lg AB [f34–45] was produced upon hydrolysis of fraction P3 (not shown). Although a change in specificity of the enzyme in 4 M urea could not be excluded, it was confirmed, from the last result, that peptide aggregation occurred during hydrolysis at pH 8.0, despite not being detected after centrifugation of the hydrolysate at this pH value. As well, it was confirmed that aggregation of peptide β-lg AB [f1–45] prevented its further digestion.

DISCUSSION

Characteristics of the Main Aggregating Peptides. Peptides originating from only β-lg substrate or from only α-la substrate can aggregate because enzyme-induced aggregation and gelation of β-lg (*5*) and α-la (*16*) was reported. According to the present results, all of the peptides listed in **Table 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, 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 9**).

The masses of the aggregating peptides largely deviate from the estimated average masses of the peptides in the hydrolysate [2 kDa; (*11*)] because 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 such that the largest peptides of the hydrolysate are the aggregating ones and the smallest peptides are the nonaggregating ones.

Experiments demonstrated that hydrophobic interactions dominate peptide aggregation (*11*). Peptide β-lg AB [f90–108] and its covalent dimer possess the highest average hydrophobicity (1.46 kcal/res) of the main aggregating peptides, which, together with a net charge of 0 at neutral pH (**Table 3**), might contribute to aggregation. For comparison, the average hydrophobicity of β-lg is 1.23 kcal/res and that of α-la is 1.15 kcal/res. It is assumed that fragment β-lg AB [f90–108] is an aggregating peptide that could act as an "anchor", because it is present in the aggregates linked via a disulfide bond to various peptides. Peptide β-lg AB [f1–45] has a lower average hydrophobicity than intact β-lg (**Table 3**), but contains hydrophobic amino acid residues belonging to an assumed exposed hydrophobic segment at the region β-lg AB [f20–34] (**Figure 7**) that could contribute to aggregation. According to reversed-

phase chromatography, fragment α-la [f50–113] was more hydrophobic than its parental nonreduced peptides (α-la [f12–49]-S-S-α-la [f50–113] and β-lg AB [f90–108]-S-S-α-la [f50–113]), because the latter eluted earlier under nonreducing conditions. Although no hydrophobic segment was observed in the sequence of α-la [f50–113] (**Table 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 (*6*) reported that 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** and **5**), but it is not the dominating peptide when its abundance is compared with that of the other peptide peaks, assuming an equal weight based response factor at 220 nm of the different peptides. In addition, peptide β-lg AB [f135–157/158] is present in both the pellet and the supernatant, as shown in **Figure 1**, whereas peptide β-lg AB [f1–45] is not. This indicates that peptide β-lg [f135–157/158] is more soluble than peptide β-lg AB [f1–45]. In fact, in the former study of Otte and co-workers (*6*), 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 (*17*).

The presence of 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 because thiol groups have a pK_a of 9.0–9.5. As enzyme-induced aggregation of WPI was reported upon hydrolysis at pH 7.0 (*18*), with assumedly fewer 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 more quickly when disulfide-linked peptides are involved.

Mechanism of Enzyme-Induced Aggregation of WPI. 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 cleaves the substrate after Glu residues because it is more specific for Glu than for Asp residues (*12*). 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. The structure of the fragment β-lg AB [f90–108] in the β-lg protein is known as two 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 (*19*). The structure of the fragment β-lg AB [f1–45] in the β-lg protein is known as three β-strands, belonging to the same β-barrel (*19*). Peptide α-la [f50–113] belongs to both α- and β-subdomains of α-la (*20*). However, the three-dimensional structure of these peptides in solution and in aggregates is not known. 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 because in a hydrolysate made with glutamyl endopeptidase activity (BLP), aggregation occurs at relatively limited hydrolysis (DH ∼2–7%, pH 7.0) and involves rather large peptides (2–10 kDa), whereas 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; (*8*)). When aggregation occurs at limited hydrolysis, it possibly requires an enzyme with narrow specificity for polar residues and a substrate containing hydrophobic segments. When aggregation occurs after extensive hydrolysis, it possibly requires several enzymes with specificity for both polar and nonpolar residues to cleave most hydrophilic and hydrophobic segments so that resulting oligopeptides, having a net charge of 0 at pH 6.0 (*8*), at high concentration, physically aggregated. This hypothesis is supported by the fact that Spellmann and co-workers (*10*) showed that a hydrolysate of whey proteins made with only subtilisin activity 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.

ABBREVIATIONS USED

α-CN, α-cyano-4-hydroxycinnamic acid; α-la, α-lactalbumin; β-lg, β-lactoglobulin; BLP, *Bacillus licheniformis* protease; DH, degree of hydrolysis; DTT, dithiothreitol; MALDI-TOF MS, matrix-assisted laser desorption–ionization time of flight mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; RT, retention time; SA, dimethoxy-4-hydroxycinnamic acid; TFA, trifluoroacetic acid; WPI, whey protein isolate.

ACKNOWLEDGMENT

We thank Novozymes for supplying the *Bacillus licheniformis* protease.

LITERATURE CITED

(1) Ju, Z. Y.; Otte, J.; Madsen, J. S.; Qvist, K. B. Effects of limited proteolysis on gelation and gel properties of whey protein isolate. *J. Dairy Sci.* **1995**, *78* (10), 2119–2128.

(2) Inouye, K.; Nagai, K.; Takita, T. Coagulation of soy protein isolates induced by subtilisin Carlsberg. *J. Agric. Food Chem.* **2002**, *50*, 1237–1242.

(3) Kim, S. Y.; Park, P. S. W.; Rhee, K. C. Functional properties of proteolytic enzyme modified soy protein isolate. *J. Agric. Food Chem.* **1990**, *38*, 651–656.

(4) Otte, J. J.; Ju, Z. Y.; Faergemand, M.; Lomholt, S. B.; Qvist, K. B. Protease-induced aggregation and gelation of whey proteins. *J. Food Sci.* **1996**, *61* (5), 911915, 923.

(5) Otte, J.; Lomholt, S. B.; Ipsen, R.; Stapelfeldt, H.; Bukrinsky, J. T.; Qvist, K. B. Aggregate formation during hydrolysis of β-lactoglobulin with a Glu and Asp specific protease from *Bacillus licheniformis*. *J. Agric. Food Chem.* **1997**, *45*, 4889–4896.

(6) Otte, J.; Lomholt, S. B.; Halkier, T.; Qvist, K. B. Identification of peptides in aggregates formed during hydrolysis of β-lactoglobulin B with a Glu and Asp specific microbial protease. *J. Agric. Food Chem.* **2000**, *48*, 2443–2447.

(7) Doucet, D. D.; Gauthier, S. F.; Foegeding, E. A. Rheological characterization of a gel formed during extensive enzymatic hydrolysis. *J. Food Sci.* **2001**, *66* (5), 711–715.

(8) Doucet, D.; Otter, D. E.; Gauthier, S. F.; Foegeding, E. A. Enzyme-induced gelation of extensively hydrolyzed whey proteins by alcalase: peptide identification and determination of enzyme specificity. *J. Agric. Food Chem.* **2003**, *51*, 6300–6308.

(9) Doucet, D.; Gauthier, S. F.; Otter, D. E.; Foegeding, E. A. Enzyme-induced gelation of extensively hydrolyzed whey proteins by alcalase: comparison with the plastein reaction and character-

ization of interactions. *J. Agric. Food Chem.* **2003**, *51*, 6036–6042.

(10) Spellman, D.; Kenny, P.; O'Cuinn, G.; FitzGerald, R. J. Aggregation properties of whey protein hydrolysates generated with *Bacillus licheniformis* proteinase activities. *J. Agric. Food Chem.* **2005**, *53*, 1258–1265.

(11) Creusot, N.; Gruppen, H.; van Koningsveld, G. A.; de Kruif, C. G.; Voragen, A. G. J. Peptide–peptide and protein–peptide interactions in mixtures of whey protein isolate and whey protein isolate hydrolysates. *Int. Dairy J.* **2006**, *16* (8), 840–849.

(12) Breddam, K. K.; Meldal, M. Substrate preferences of glutamic-acid-specific endopeptidases assessed by synthetic peptide substrates based on intramolecular fluorescence quenching. *Eur. J. Biochem.* **1992**, *206* (1), 103–107.

(13) Creusot, N.; Gruppen, H. Protein–peptide interactions in mixtures of whey peptides and whey proteins. *J. Agric. Food Chem.* **2007**, *55*, 2474–2481.

(14) AOAC Method 990.03. *Official Methods of Analysis of AOAC*, 16th ed.; Association of Official Analytical Chemists: Washington, DC, 1995; Chapter 4, pp 5–7.

(15) Kyte, J.; Doolittle, R. F. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **1982**, *157* (1), 105–132.

(16) Ipsen, R. R.; Otte, J.; Qvist, K. B. Molecular self-assembly of partially hydrolysed α-lactalbumin resulting in strong gels with a novel microstructure. *J. Dairy Res.* **2001**, *68* (2), 277–286.

(17) Biemann, K. Mass spectrometry of peptides and proteins. *Annu. Rev. Biochem.* **1992**, *61* (1), 977–1010.

(18) Otte, J.; Ju, Z. Y.; Skriver, A.; Qvist, K. B. Effects of limited proteolysis on the microstructure of heat-induced whey protein gels at varying pH. *J. Dairy Sci.* **1996**, *79* (5), 782–790.

(19) Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of β-lactoglubulin and its similarity to plasma retinol-binding protein. *Nature* **1986**, *324* (6095), 383–385.

(20) Demarest, S. J.; Boice, J. A.; Fairman, R.; Raleigh, D. P. Defining the core structure of the α-lactalbumin molten globule state. *J. Mol. Biol.* **1999**, *294* (1), 213–221.

(21) Bigelow, C. C. On the average hydrophobicity of proteins and the relation between it and protein structure. *J. Theor. Biol.* **1967**, *16* (2), 187–211.

(22) Walstra, P.; Wouters, J. T. M.; Geurts, T. J. In *Dairy Science and Technology*, 2nd ed.; CRC Press, Taylor and Francis Group: Boca Raton, FL, 2006; Chapter 2, p 75.

Received for review May 30, 2007. Revised manuscript received July 17, 2007. Accepted August 5, 2007. The Dutch Ministry of Economic Affairs supported this research through the program IOP-Industrial Proteins.

JF071584S