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Protein−Peptide Interactions in Mixtures of Whey Peptides and Whey Proteins

NATHALIE CREUSOT AND HARRY GRUPPEN*

Laboratory of Food Chemistry/Center for Protein Technology, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

The effects of several conditions on the amounts and compositions of aggregates formed in mixtures of whey protein hydrolysate, made with *Bacillus licheniformis* protease, and whey protein isolate were 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. Increasing both temperature and ionic strength increased the amounts of both intact protein and peptides in the aggregates. There was an optimal amount of added intact WPI that could aggregate with peptides, yielding a maximal amount of aggregated material in which the peptide/protein molar ratio was 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-lactoglobulin via hydrophobic interactions at the hydrophobic binding sites at the surface of the protein.

KEYWORDS: ***Bacillus licheniformis*** **protease; aggregation; protein isolate hydrolysate**

INTRODUCTION

Specific interactions between protein domains and peptides play an important role in a wide range of biological processes, for example, peptide interaction with a protein membrane receptor for propagation of information through a signaling system, inhibition of an enzyme with a peptide, and formation of molecular complexes. Nonspecific 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 (*1*, *2*), the major whey protein, and that such an interaction could increase the resistance of β-lactoglobulin to thermodenaturation (*2*). 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 (*3*) such as melittin, a 26-residue cytolytic peptide from bee venom (*4*). The binding of α-lactalbumin to the synthetic peptide WHWRKR (*3*) was even used to develop a purification strategy of the protein.

We have shown recently (*5*) that hydrolysates of a whey protein isolate (WPI) made by a seryl protease from *Bacillus licheniformis* (BLP) could aggregate nonhydrolyzed 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. Because partial hydrolysis of WPI with BLP induces the formation of aggregates that eventually form a gel (*6*), information on protein−peptide interactions could increase understanding of 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 coprecipitation of intact WPI with peptides. In this respect, intact WPI is defined as nonhydrolyzed WPI, regardless of the extent of denaturation of WPI. For that purpose, first temperature, ionic strength, and amount of added intact protein were varied, according to an experimental design, 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 intact aggregated protein in the aggregates were investigated using response surface methodology 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 peptide identification, hypotheses on protein−peptide interactions 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-lacto-

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

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Table 1. Central Composite Design: Variables and Levels

variable	level of the variables				
	–1.68 (-α)	–1	0	+1	+1.68 (+α)
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

globulin (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 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 (*7*). The enzyme was kindly provided by Novozymes A/S (Bagsvaerd, Denmark).

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

Hydrolysis of WPI. A 5% (w/w) WPI solution, prepared as previously described (*5*), was preheated at 40 °C for 15 min. The pH was adjusted to pH 8.0, 5 min before the 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 degree of hydrolysis (DH) of 6.8%, which is the experimental end point of hydrolysis (*5*), 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 by acidification to pH 2.0 with a 6 M HCl solution (*5*). The nitrogen concentration of the hydrolysate was measured using the Dumas method. The hydrolysate was stored at 4 °C prior to further analysis and use.

Mixing of Hydrolysate with Intact WPI. An experimental design was applied to study 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 α, −1, 0, +1, +α (**Table 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 (−α or + α) and the other variables at 0 level ($2 \times k$ experiments) (*8*).

Figure 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 strength 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 and WPI concentrations of 0, 8.1, 20, 31.9, and 40 mg/g. 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*g*, 20 °C), whereas the other part was left noncentrifuged (further denoted "total"). To determine the extent of aggregation, the nitrogen concentration (*N*) in the supernatant and in the noncentrifuged part (N_0) 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

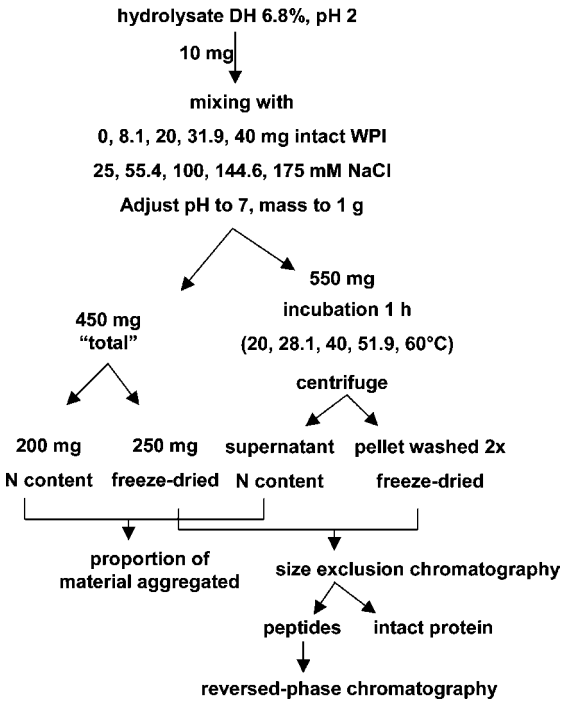

Figure 1. Outline of the experiments performed.

at 20, 28.1, 40, 51.9, or 60 °C and freeze-dried. As well, 0.25 g of the noncentrifuged part was freeze-dried.

Size Exclusion Chromatography. Size exclusion experiments were performed as previously described (*5*) except that a volume of 100 μL of sample was injected onto the column and detection was performed both at 220 (for peptides) and at 280 nm (for proteins).

Sample preparation was as follows. The freeze-dried pellets and "total" samples were dispersed in 550 and 250 μL of 8 M guanidinium hydrochloride, respectively. All material dissolved as no precipitates were observed after centrifugation. 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 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 of TFA was added per milliliter 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). 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 by subtraction (**Figure 3**). 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.

The column was calibrated as previously described (*5*).

Liquid Chromatography−Mass Spectrometry. Samples were separated on an analytical reversed-phase Vydac C8 column (208MS52; 2.1 × 250 mm; bead diameter = 5 μm; porosity = 300 nm; Dionex, Sunnyvale, CA) by HPLC (Thermo Separation Products Inc., San Jose, CA), with ThermoQuest software. The flow rate was 0.2 mL/min, 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

Table 2. Central Composite Design: Arrangements and Responses with (*A*) Temperature, (*B*) Ionic Strength, and (*C*) Amount of Added WPI

		responses			
run	coded variable levels: *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

of 75 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. 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 v 1.1 by N.-P. V. Nielsen was used to align the peaks by correlation optimized warping (*9*, *10*). Mass spectra were recorded with an electrospray ionization mass spectrometer (LCQ ion-trap, Finnigan MAT 95, San Jose, CA), 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 *m*/*z* 1.5−2 and a 30−35% relative collision energy. The apparatus and data were controlled by Xcalibur software. The accuracy of the mass determination was ±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 Corp., Waltham, MA), with a FASTA database containing the β-lg A, β-lg B, and α-la B sequences. Peptide identification was considered valid when Bioworks cross-correlation scores were above 3 for +2 and +3 peptides and above 2 for +4 peptides.

Nitrogen Concentration Determination. Nitrogen concentrations were measured using the combustion or Dumas method (*11*) with a NA 2100 protein nitrogen analyzer (CE Instruments, Milan, Italy). A 6.38 × N conversion factor was used to convert nitrogen concentration to protein concentration.

Statistical Analysis. Statistical analysis was performed with The Unscrambler program (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 eq 1

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i<j=2}^{3} b_{ij} X_i X_j + e \qquad (1)$$

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 a 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. 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 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 $SS_{residual} - SS_{pure\ error}$. The significance of the lack of fit was tested with the Student t test with a level of statistical significance defined as $p \leq 0.05$ (*8*, *12*).

Table 3. Average, Standard Deviation, and Range of All Points Compared with the Central Points for All Responses in the Central Composite Design

response		all samples	center samples
amount of aggregated material (mg)	range	4.56–7.98	6.12–6.06
	average	5.83	6.09
	SD[a]	0.98	0.04
amount of WPI in the aggregates (mg)	range	0.00–3.84	2.14–2.17
	average	2.08	2.16
	SD	1.02	0.02
amount of peptides in the aggregates (mg)	range	2.29–5.60	3.89–3.98
	average	3.81	3.94
	SD	0.98	0.06
WPI/peptide molar ratios in the aggregates	range	0.000–0.220	0.099–0.103
	average	0.110	0.101
	SD	0.064	0.003

[a] Standard deviation.

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 aggregate composition and to select representative aggregates to study the peptide composition in further detail.

Statistical Analysis of Data. The composition and the amount of aggregates were determined upon variation of temperature, ionic strength, and addition of WPI. Results or responses (amounts of aggregated material, intact WPI, and peptides in the aggregates as well as WPI/peptide molar ratios) are given in **Table 2**. Using starting amounts of intact WPI of 0−40 mg, 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. We chose to express WPI/peptide molar ratio and not peptide/WPI molar ratio (which would be more expressive) because a peptide to WPI molar ratio of infinity (run 1) cannot be entered in the program.

Table 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

Table 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				
R^2	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.

specified separately because these samples are the only replicates. This provides an indication of 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 and the amounts of intact WPI and peptides in the aggregates as well as the WPI/peptide molar ratios, which makes regression analysis interesting.

Response Surface Modeling. Regression coefficients and quadratic response surfaces provide information about the effects 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, that is, 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 aggregates, 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 4**. The initial regression analysis performed with all regression parameters ("complete model") revealed that several terms were not significant (p value > 0.2). To simplify the model, nonsignificant 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 seven regression terms (**Table 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 = 0.052) or in the simplified model (p value = 0.062).

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 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 (**Figure 2A−C**). An optimum amount of aggregated material is found when 20 mg of WPI is added to the hydrolysate (10 mg), whatever the temperature, at an ionic strength of 100 mM (**Figure 2A**). A maximum amount of aggregated material was obtained at combinations of high ionic strength with high temperature (**Figure 2B**) and a high amount of WPI added with high ionic strength (**Figure 2C**).

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 2D**) a maximum amount of intact protein in the aggregates is observed at high temperature (around 60 °C) and for a 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 (**Figure 2E**,**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 2G**) a maximum WPI/peptide molar ratio in the aggregates is observed at high temperature (around 60 °C) and for a high amount of WPI added.

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**) in order to separate intact protein from peptides. The peptides were collected and afterward analyzed with reverse phase chromatography (**Figure 4**).

Interestingly, the 16 reversed-phase chromatograms obtained were similar with respect to the number of peaks and 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.

The peptides present in the three major peaks (**Figure 4**) were identified with mass spectrometry (**Table 5**), from both full mass

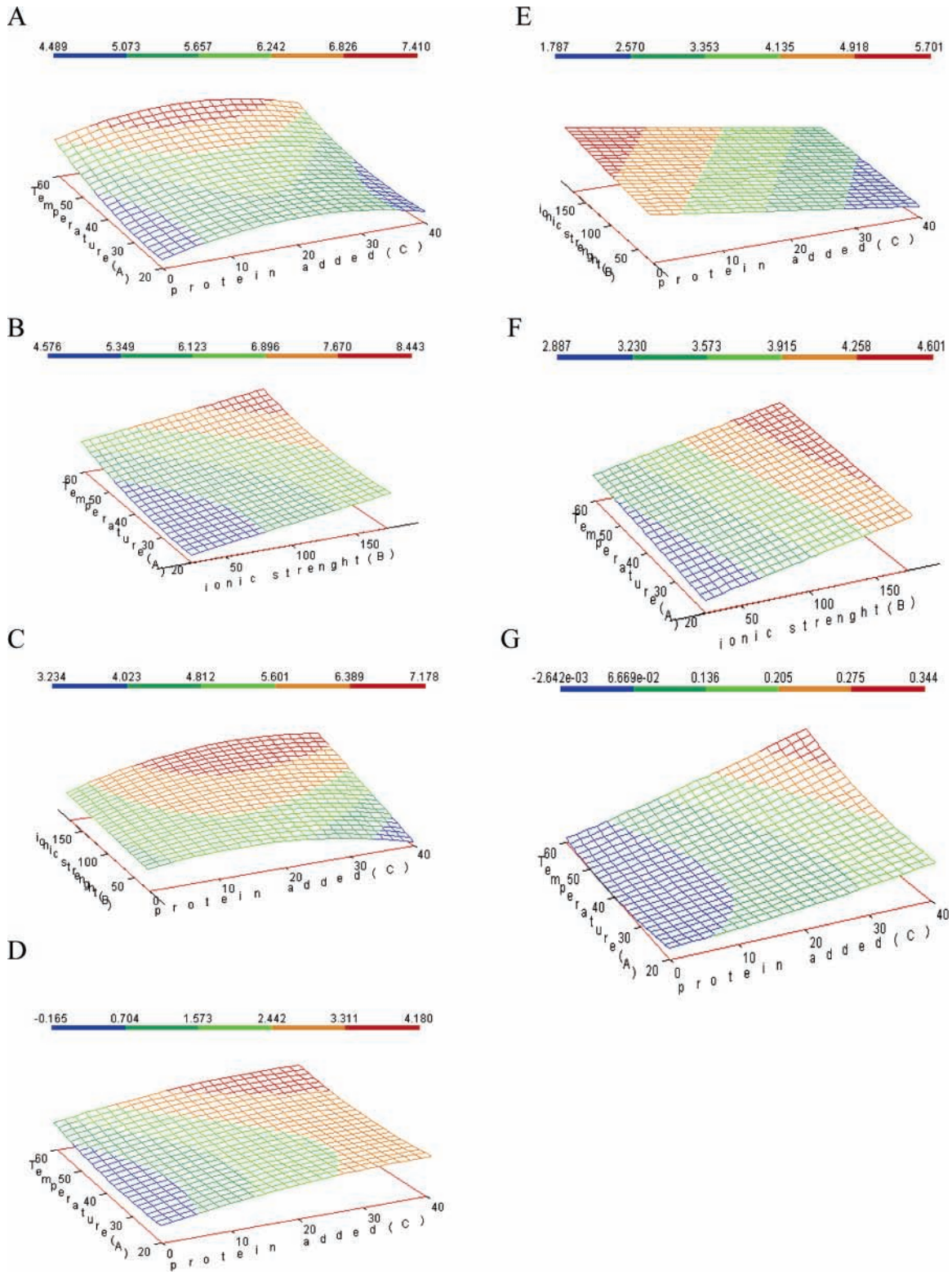

Figure 2. Effects of selected variables on the amount of aggregated material in the aggregates (**A**–**C**), on the amount of WPI in aggregates (**D**), on the amount of peptides in the aggregates (**E**, **F**), and on the WPI/peptide ratio in the aggregates (**G**). (In every figure, the third variable is at its center value.)

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-lg AB [f90−108]) and peak 3 (α-la [f50−113]) contain peptides originating from disulfide-bridged fragments. Peak 2 contained the peptide β-lg AB [f1−45], which corresponds to the N-terminal extremity of β-lg.

DISCUSSION

It has been shown that hydrolysis of WPI with BLP leads to the formation of peptide aggregates (*13*). We have recently shown that these WPI hydrolysates have the capacity to aggregate intact WPI (*5*). To further investigate aggregate composition and interactions in the protein−peptide system, we

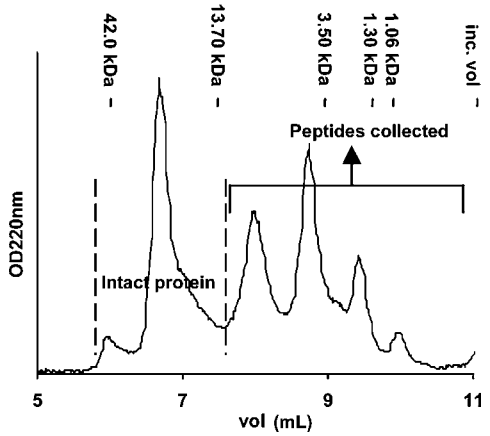

Figure 3. Size exclusion chromatogram, under reducing conditions, of aggregates obtained from the mixture of a hydrolysate with intact WPI (run 6 of **Table 2** in this case).

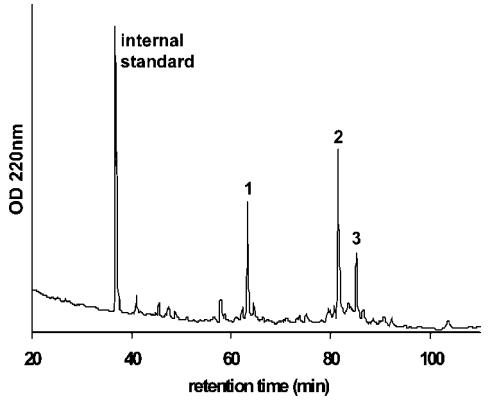

Figure 4. Reverse phase chromatogram, under reducing conditions, of peptides isolated from intact protein with size exclusion chromatography (run 6 of **Table 2** in this case).

used response surface methodology to analyze the effects of mixing conditions on aggregate composition. In addition, the peptides present in the aggregates were separated from the intact protein and identified with liquid chromatography−mass spectrometry.

Composition of the Aggregates. The results obtained with respect to the effects of temperature, ionic strength, and amount of added WPI are schematically summarized in **Figure 5**. Increasing both temperature and ionic strength increased the amounts of both intact protein and peptides in the aggregates and, therefore, the total amount of aggregated material. There were protein−peptide interactions leading to the formation of stable protein−peptide aggregate because WPI alone did not aggregate under these conditions. Because 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 (*5*).

In addition, a change in the composition of the aggregates toward less peptide and more intact protein occurred upon WPI addition (**Figure 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 2A**) in which the WPI/peptide molar ratio was around 0.166 (**Figure 2G**; 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 (*5*), and accounts for a proportion of around 45% (w/w) in the aggregates. Therefore, in these conditions, 6 mol of aggregating peptides was interacting with 1 mol of β-lg. Upon further WPI addition, the WPI/peptide ratio in the aggregates increased. There was too much protein per aggregating peptide to form stable protein−peptide aggregates. Protein−peptide interactions were assumed to give soluble adducts because the amount of aggregated material decreased.

Table 5. Mass Spectrometry Results for the Peptide Peaks 1–3 Separated by Reverse Phase Chromatography (see **Figure 4**)

peak	measured mass (Da)	possible fragment	theoretical mass (Da)
1	2335.2	β-lg AB [f90–108]	2336.8
2	4895.6	β-lg AB [f1–45]	4898.7
3	7403.5	α-la [f50–113]	7408.5

In addition, it was shown that variables (temperature, ionic strength, and amount of added WPI) have no effect on peptide composition because the same peptides were present in the aggregates in all of the conditions tested (**Figure 4**) at the same relative proportions. However, the variables have an effect on the total amount of peptides present in the aggregates (**Figure 2E**,**F**).

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 p*I* of 5.2, and contains two stabilizing disulfide bridges and a thiol function on Cys 121. Whereas the disulfide bond between residues 66 and 160 is on the outer surface, the disulfide bond between residues 106 and 119 and the free thiol group 121 are buried in the interior of the native molecule (*14*). The monomer of β-lg has a calyx fold consisting of an eight-stranded antiparallel β-barrel, typical of the lipocalin protein superfamily (*15*); this structure confers a hydrophobic pocket to the β-lg (*16*).

The peptide β-lg AB [f90−108] has some hydrophobic nature because 53% of the amino acid residues are hydrophobic. According to the sequence of β-lg and the specificity of the enzyme, the peptide β-lg AB [f90−108] would be linked via a disulfide bridge, between residues Cys 106 and 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 (*17*) 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 if this bond also took part in the reshuffling (*18*).

As the fragment β-lg AB [f1−45] is resistant to enzymatic cleavage of the three aspartic acid residues that it contains, it is assumed to adopt a compact structure 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. Otte and co-workers (*19*) also found the two fragments of β-lg in aggregates formed from β-lg hydrolysates made with the same enzyme. However, the primary aggregating fragment identified by Otte and co-workers (β-lg [f135−158] (*19*)) was not found.

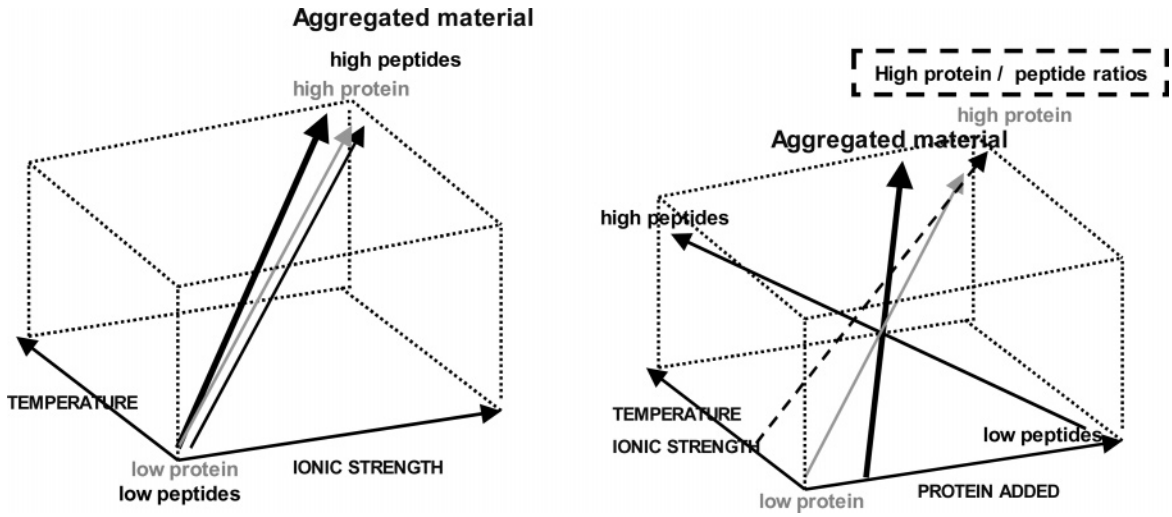

Figure 5. Schematic representation of the variations of composition in the aggregates as a function of temperature, ionic strength, and amount of intact WPI added.

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 p*I* of 4.8. α-La is composed of two subdomains (α and *β*). The α-subdomain consists of helices in the amino- and carboxyl-terminal regions of the polypeptide chain (residues 1−34 and 86−123). The *β*-subdomain consists of the remaining protein, residues 35−85, which are a three-stranded antiparallel *β*-sheet, a small 3_{10} helix, and some loops and coils (*20*).

The peptide α-la [f50−113] belongs to both α- and *β*-subdomains of α-la. According to the sequence of α-la and the specificity of the enzyme, the peptide α-la [f50−113] could be linked via a disulfide bridge with the minimum sequence α-la [f26−37]. However, the latter peptide was not found in the aggregates. The peptide α-la [f50−113] is rather large (7.408 kDa; around 50% of mass of the intact α-la). As it is resistant to enzymatic cleavage of the nine aspartic acid residues that it contains, it is also assumed to adopt a compact structure to hide the cleavable peptide bonds. Otte and co-workers (*21*) also found this fragment, disulfide linked with α-la [f26−37], as the primary aggregating one after hydrolysis of α-la with the same enzyme.

Peptide−*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. 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 (*16*): 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 (*1*) and promote a more compact structure to the protein (*2*). In addition, charged peptides could also bind to *β*-lg (*1*). 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 (*1*). However, in the present study, peptides involved in protein−peptide interactions are larger than the ligands described in the 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) 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.

ABBREVIATIONS USED

α-La, α-lactalbumin; ANOVA, analysis of variance; *β*-lg, *β*-lactoglobulin; BLP, *Bacillus licheniformis* protease; CCRD, central composite rotatable design; DTT, dithiothreitol; lof, lack of fit; SS, sum of squares; TFA, trifluoroacetic acid; WPI, whey protein isolate.

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