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ARTICLE

Isolation and Characterization of an Aggregating Peptide from a Tryptic Hydrolysate of Whey Proteins

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Spontaneous precipitation of a peptide mixture has been observed during the concentration by reverse osmosis of a tryptic hydrolysate of whey protein. The precipitated material collected by centrifugation could not be solubilized by urea, mercaptoethanol, or sodium dodecyl sulfate. However, a complete solubilization of the aggregates was observed when the pH of the solution was lowered to 2.0. Analysis of the insoluble fraction has allowed the identification of β-lactoglobulin (β-lg) fragment 1−8 as the major peptide involved in the formation of aggregates. Peptide β-lg f1−8 accounted for >94% of the peptide content in the precipitate washed twice with distilled water. The investigation of the secondary structure using circular dichroism evidenced that the peptide β-lg f1−8 isolated from the flocculated peptide mixture is under random coil conformation at acidic and neutral pH and tends to adopt a β-sheet conformation at basic pH. The findings of this study provide evidence that peptide β-lg f1−8 forms aggregates via an efficient self-assembly process.

KEYWORDS: Whey peptides; enzymatic hydrolysis; reverse osmosis; aggregation; circular dichroism

INTRODUCTION

Enzymatic hydrolysis of whey proteins yields peptides having functional and biological properties (*1*, *2*). For example, some peptides resulting from the hydrolysis of β-lactoglobulin (β-lg) with trypsin, namely, β-lg f41−60 and β-lg f21−40, are known to have emulsifying properties (*3*). More recently, it has also been shown that tryptic hydrolysis of β-lg generates various bioactive peptides such as bactericide peptides (β-lg f15−20, f92−100) (*4*) or ACE-inhibitory peptides (β-lg f15−20, f102−105, f142−148) (*5*, *6*).

The aggregation of peptides during enzymatic hydrolysis of whey proteins has been observed in several studies. Otte and co-workers (*7*) have shown that hydrolysis of α-lactalbumin (α-la) and β-lg isolates by a Glu and Asp specific protease from *Bacillus licheniformis* (BLP) led to the formation of aggregates and gels. These aggregates are composed of a number of peptides of 2−6 kDa mainly associated together by electrostatic and hydrophobic interactions. Otte and co-workers (*8*) identified peptide β-lg f135−158 as the initiator of the aggregation process.

Likewise, Doucet and co-workers (*9*, *10*) studied the aggregation process leading to the gelation of whey proteins extensively hydrolyzed by a liquid preparation from *Subtilisin carlsberg*. The gel formed showed stability over a wide range of pH and ionic strength. Over 130 different peptide sequences were identified, but it was suggested that the gel formed consisted of short peptides of approximately four residues and that the peptides were held together by non-covalent forces such as hydrophobic interactions and, to a lesser extent, hydrogen and electrostatic interactions.

Groleau and co-workers (*11*, *12*) studied the formation of aggregates in a tryptic hydrolysate of β-lg under different physicochemical conditions. The most important aggregation was observed at pH 4.0. Aggregates consisted of β-lg f1−8, f15−20, and f41−60, and aggregation was assumed to result from hydrophobic interactions between peptides.

In more recent studies of the aggregation phenomena occurring during enzymatic hydrolysis of whey proteins by *B. licheniformis*, Creusot and co-workers (*13*−*16*) have evidenced the occurrence of a number of β-lg peptide sequences aggregating at pH 7.0. Peptide fragments β-lg AB f1−45, β-lg AB f90−108, and α-la AB f50−53 were found in aggregates that also contained β-lg. Overall, peptide fragment β-lg AB f1−45 was identified as the dominant aggregating peptide of the hydrolysate.

An aggregation phenomenon has been recently observed during reverse osmosis (RO) concentration of tryptic hydrolysates of whey proteins (*17*). The peptide mixtures were prepared as previously described by Lapointe and co-workers (*18*) with the only difference being that the peptide mixture was extensively diafiltered before final RO concentration. The aggregated material collected from the peptide mixture was insoluble in aqueous media, and adding 8 M urea, β-mercaptoethanol, or sodium dodecyl sulfate (SDS) could not solubilize the aggregates.

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The present work was undertaken to characterize the insoluble peptide aggregates formed in a tryptic hydrolysate of β-lg. The solubility of the peptide mixture was estimated as a function of pH using turbidity measurements at 600 nm. The peptide aggregates were isolated and analyzed with respect to peptide composition by reverse-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry (MS). In addition, circular dichroism (CD) spectrometry was used to characterize the secondary structure of the aggregating peptide as a function of pH.

MATERIALS AND METHODS

Materials. A commercial whey protein isolate (WPI) (BiPRO, Davisco Food International Inc., Le Sueur, MN) was used as substrate for enzymatic hydrolysis. The enzyme used was trypsin VI, a serine-type endopeptidase that cleaves preferentially the carboxylic side of basic amino acid residues (Arg and Lys) and was purchased from Inovatech Inc. (Abbotsford, BC, Canada). This commercial preparation contained 2400 U mg^{-1} of trypsin activity, but also 400 U mg^{-1} of chymotrypsin activity.

Preparation of the Tryptic Hydrolysate. The tryptic hydrolysate was prepared according to the method of Chay Pak Ting and co-workers (*17*) as schematized in **Figure 1**. A 10% (w/v) aqueous solution was made with 120 kg of WPI, adjusted to pH 8.0, and heated to 40 °C. The enzymatic reaction was then initiated by adding 100 mL of a 10% (w/v) trypsin solution dissolved in 0.001 N HCl (final enzyme/substrate ratio of 1:800). During the hydrolysis, the pH of the reaction mixture was maintained at 8.0 by adding 2 N sodium hydroxide (NaOH), according to the pH-stat technique (*19*). When the degree of hydrolysis (DH) reached 5.6%, the reaction was stopped by ultrafiltering the solution on a UF membrane system (GEA Niro, Hudson, WI) with a 10 kDa molecular weight cutoff (MWCO) membrane (Osmonics, Vista, CA) to separate peptides from the enzyme and nonhydrolyzed proteins. This filtration step was performed at 40 °C at a transmembrane pressure (P_T) of 4.5 bar. The permeate was then concentrated by RO using the same system with a RO polyamide membrane (Desal RO membrane, Osmonics) operated at an average pressure of 13.5 bar. The concentrated mixture of tryptic peptides was freeze-dried and stored at −20 °C. The preparation of peptide mixtures was performed in triplicate.

Turbidity. The tryptic peptide mixtures were rehydrated to 5% (w/v) in HPLC water. Then the pH was adjusted to 2.0, 4.0, 6.0, 8.0, 10.0, or 12.0 using HCl or NaOH. Solutions were agitated during 1 h at room temperature. Turbidity was estimated by the optical density measured at 600 nm (A_{600}) using a Hewlett-Packard (Palo Alto, CA) model 8453 UV−visible spectrophotometer. Data acquisition was performed using UV−visible Chem Station software version A.06.01 (Agilent Technologies, Palo Alto, CA). All of the measurements were performed on triplicates of solutions.

Isolation of the Aggregating Peptides. The freeze-dried flocculated peptide mixtures were rehydrated to 5% (w/v) in HPLC water and centrifuged at 5000*g* for 12 min at room temperature. The precipitate was resuspended in HPLC water and centrifuged under the same conditions twice. The final precipitate was freeze-dried and stored at −20 °C.

Reverse-Phase Chromatography (RP-HPLC) and Mass Spectrometry (MS). The RP-HPLC analyses were performed using an HPLC Agilent system equipped with a variable UV−visible detector operating at 214 nm (series 1100, Agilent Technologies). Data acquisition and chromatography analyses were performed using Chem Station software version B.01.03 (Agilent Technologies). Peptides were chromatographed on a Luna 5 μm C_{18} column (2 i.d. × 250 mm, Phenomenex, Torrance, CA). The column was operated at a flow rate of 0.2 mL/min at 40 °C. Solvent A was composed of 0.11% (v/v) trifluoroacetic acid (TFA) in water, and solvent B was composed of 90% (v/v) acetonitrile, 10% (v/v) water, and 0.1% (v/v) TFA. The freeze-dried samples were rehydrated to 5% (w/v) in HPLC water and then diluted to a ratio of 1:5 with solvent A. The samples (20 μL) were injected and eluted using a linear gradient of solvent B from 1 to 50 vol % over 60 min.

Peptide identification was determined using liquid chromatography−mass spectrometry (LC-MS) using an LC-MSD QUAD Agilent 1100 series (Agilent Technologies) consisting of an autosampler (G1329A), two pumps (bin G1323A), and a diode array detector (DAD G1315A) monitoring at 214 nm. Mass spectra were acquired in a positive ion mode using a 90 V fragmentation with a scan range of 0 m/z 250−300. Nitrogen was used as drying gas (13.0 L/min, 350 °C) and nebulizer gas at 35.0 psi. The capillary voltage was set at 4000 V. The instrument was calibrated using ES tuning mix (G2431A, Agilent). Data acquisition was processed by LC-MSD Chem Station software version A.08.04. Assignment of sequence from molecular masses was based on the known sequence of β-lg variant B using Peptide Tools software version 8.03 (Agilent Technologies).

Circular Dichroism (CD). Analyses were performed using a Jasco J-710 instrument (upgraded to a J-715) with 1.08.01 spectral management software on freshly prepared 2 mg/mL (2.144 mM) aqueous solution of β-lg f1−8 peptide, rehydrated during 1 h. Solubilization of the aggregating peptide was improved by combining the addition of 10% (v/v) of 2,2,2-trifluoroethanol (TFE) with a 10 min ultrasonic treatment. Spectra were recorded at 22 °C in a 0.02 cm path length quartz cylindrical cell. Twenty scans were collected from 250 to 190 nm with a data pitch of 0.2 nm and a scanning speed of 100 nm/min. The pH adjustments between pH 2 and 11 were done using NaOH and HCl, and amounts added for pH adjustments were taken into account in the calculation of the final peptide concentration. The data were baseline corrected and smoothed.

RESULTS

Effect of pH Adjustment on the Solubility of the Peptide Mixture. The effect of pH adjustment on the flocculated peptide mixture was investigated in an attempt to improve the solubility of the aggregating peptide and allow its characterization by LC-MS. **Figure 2** reports the turbidity ($A_{600\ nm}$) of a 5% (w/v) solution of the peptide at different pH values between 2.0 and 12.0, at room temperature. Decreasing the pH of the peptide solutions to pH 2.0 decreased their turbidity to very low values ($A_{600} < 0.01$), whereas much higher turbidity values ($A_{600} > 1.5$) were obtained at any other pH value.

Characterization of the Aggregating Peptide. The completely soluble peptide mixture at pH 2.0 was used to further characterize its total peptide content by RP-HPLC and also to characterize the composition of the aggregating material. **Figure 3** shows the RP-HPLC chromatograms of the complete peptide mixture (**Figure 3a**), the precipitate after a first centrifugation (**Figure 3b**), and the precipitate washed twice with HPLC water (**Figure 3c**). The peak numbers in **Figure 3** correspond to the peptides listed in **Table 1**, which also contains their amino acids composition, molecular weight, p*I*, and hydrophobicity. A total of 24 peptide sequences from β-lg (variant B) were identified in the complete peptide mixture, whereas 16 of them were identified in the precipitate collected upon a first centrifugation, mostly the peptides β-lg f15−20, f1−8, and f142−148. A first water wash of the precipitate allowed the purification of the aggregates, but a second water wash was necessary to isolate the peptide β-lg f1−8 as some traces of the peptide β-lg f15−20 were found in the precipitate washed only once. Peptide β-lg f1−8 accounted for >94% of the total area of the chromatogram of the precipitate washed twice. Given the high degree of purity of the precipitate washed twice, a larger quantity of

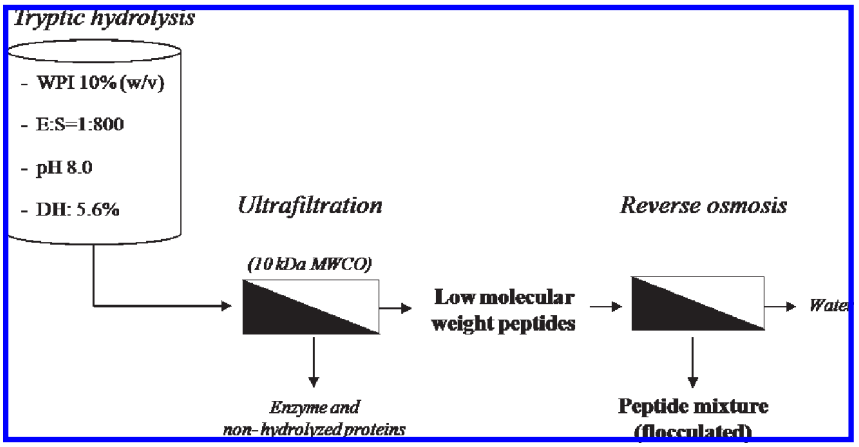

Figure 1. Schematic of the processing steps involved in the preparation of the flocculate peptide mixture from a whey protein isolate.

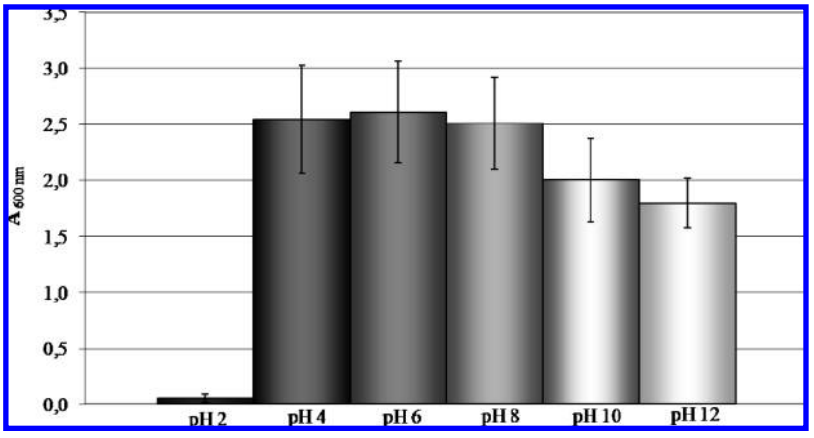

Figure 2. Turbidity (A_{660}) of the flocculated peptide mixture (5% w/v) at room temperature as a function of pH between 2.0 and 12.0. Values are mean ± SD of triplicate measurements.

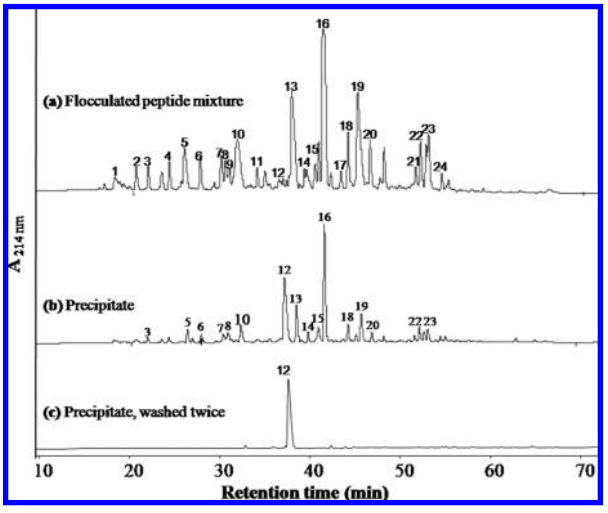

Figure 3. RP-HPLC chromatograms of the flocculated peptide mixture (**a**), of the precipitate collected after a first centrifugation and solubilized at pH 2.0 (**b**), and of the precipitate collected after washing twice with HPLC water (**c**).

this material was prepared and freeze-dried for further characterization by CD.

Structural Changes of Peptide β-Lg f1−8 as a Function of pH. Structural characteristics of peptide β-lg f1−8, obtained from the washed precipitate of the flocculated peptide mixture, were investigated using CD spectropolarimetry. More specifically, the effect of pH was studied in the range between pH 2.0 and 11.0.

Figure 4 shows the CD spectra of the peptide β-lg f1−8 as a function of pH ranging from 2.0 to 11.0. The results show that varying the pH induces significant conformational changes. At pH 2.0, 4.0, and 6.0, the CD spectrum shows a negative maximum, around 195 nm, typical of random coil conformation. However, peptide β-lg f1−8 adopts a β-sheet conformation at pH 8.0, 9.0, and 10.0, as shown in the CD spectrum by the positive maximum around 200 nm and the negative maximum around 218 nm. Finally, the conformation returns to random coil at pH 11.0. Thus, the isolated peptide β-lg f1−8 exists mainly under a random coil conformation in acidic and neutral pH, whereas it tends to adopt a β-sheet conformation at alkaline pH. Such conformation behavior is likely relevant to the aggregation ability of peptide β-lg f1−8.

DISCUSSION

The tryptic peptide mixture prepared in the present work has a peptide composition similar to that of previous recent studies (*17*, *18*). However, none reported aggregation phenomena occurring at pH 8.0. Some differences in the procedure used to prepare the peptide mixture, compared to previous studies, must be considered. To explain the difference observed, first, Lapointe and co-workers (*18*) used purified β-lg (>95% of total proteins) in contrast to a commercial WPI containing a lower proportion of β-lg (70−75% of total proteins) utilized in the present work. Nevertheless, despite this compositional difference, all of the peptides identified in the peptide mixture and aggregating material originated from

Table 1. Physicochemical Characteristics of the Peptides Identified by LC-MS in the Tryptic Peptide Mixture

peak[a]	peptide sequence	amino acid composition (β-lg variant B)	MW[b] (g/mol)	p*I*[c]	hydrophobicity[d] (kcal/residue)
1	139−141	ALK	331.2	8.80	1.55
2	146−148	HIR	424.3	9.76	1.23
3	136−138	FDK	409.2	5.84	1.38
4	41−42	VY	280.2	5.49	2.28
5	71−75	IIAEK	573.4	6.00	1.63
6	33−40	DAQSAPLR	856.5	5.84	0.91
7	84−91	IDALNENK	916.5	4.37	0.95
8	83−91	KIDALNENK	915.5	6.07	1.18
9	33−39	DAQSAPL	700.3	3.80	0.93
10	9−14	GLDIQK	673.4	5.84	1.14
11	125−135	TPEVDDEALEK	1245.6	3.83	0.85
12	1−8	LIVTQTMK	933.5	8.75	1.34
13	142−148	ALPMHIR	837.5	9.80	1.54
14	92−101	VLVLDTDYKK	1193.2	5.93	1.45
15	125−138	TPEVDDEALEKFDK	1635.4	4.02	0.97
16	15−20	VAGTWY	695.4	5.49	1.46
17	92−100	VLVLDTDYK	1065.6	4.21	1.44
18	61−70 + 149−162	WENGECAQKK + LSFNPTQLEEQCHI	2928.4	4.57	0.89
19	76−82	TLIPAVFK	774.4	8.41	1.88
20	78−82	IPAVF	545.3	5.52	2.13
21	43−60	VEELKPTPEGDLEILLQK	2052.6	4.25	1.13
22	21−40	SLAMAASDISLLDAQSAPLR	2029.7	4.21	1.05
23	41−60	VYVEELKPTPEGDLEILLQK	2323.3	4.25	1.24
24	21−32	SLAMAASDISLL	1190.7	3.80	1.14

[a] Corresponds to peak numbering of chromatograms in **Figure 3**. [b] Mass values are from LC-MS analyses by Lapointe and co-workers (*15*). [c] Calculated by Expasy Molecular biology server. [d] Calculated according to the method of Bigelow (*22*).

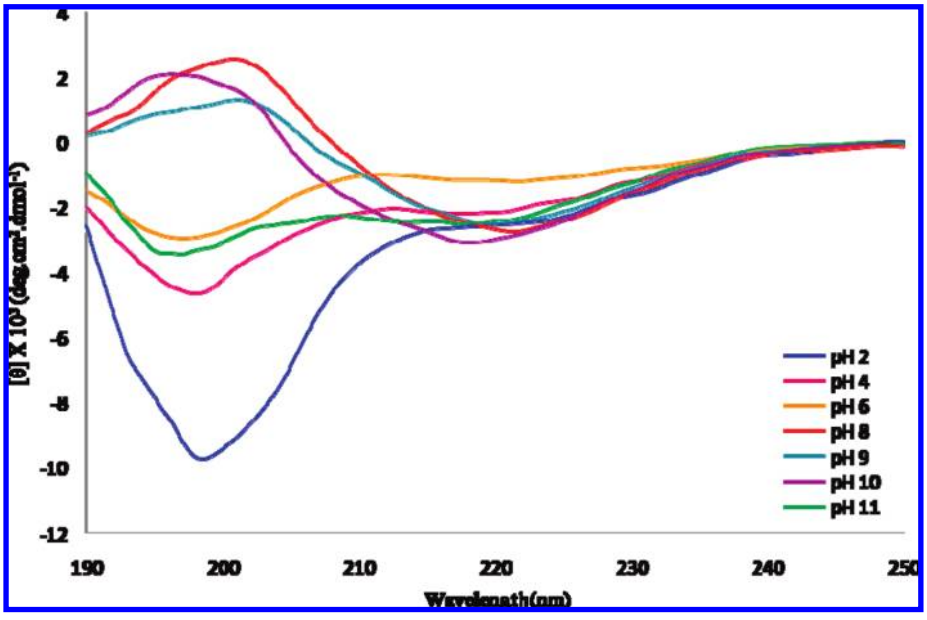

Figure 4. Circular dichroism spectra of the isolated peptide β-lg f1−8 dispersed in water containing 10% TFE at a concentration of 2 mg/mL solution (2.144 mM), after a 10 min ultrasound treatment at pH 2.0, 4.0, 6.0, 8.0, 10.0, or 11.0.

β-lg. Second, in the present study, the peptide mixture was preconcentrated by RO prior to freeze-drying and the aggregation occurred in the course of this RO concentration. However, it is not possible at this point to establish if aggregation results directly from the concentration process.

The data reported in the present study are in good agreement with previous observations on solubility problems of tryptic peptide mixtures at pH 4.0 by Groleau and co-workers (*ref12*). Although their acid precipitates consisted of β-lg f1−8, f15−20, and f41−60, it was not possible to ascribe a predominant effect to any of these species. The precipitate collected after a first centrifugation in the present study contained mainly the peptides β-lg f15−20, f1−8, and f142−148 but also at least 12 other peptides. The peptides β-lg f15−20, f1−8, and f142−148 do not have similar molecular weight or p*I* but are all relatively hydrophobic, which can explain their presence in the precipitate. However, other more hydrophobic peptides also present in significant amount in the flocculated peptide mixture, such as β-lg f76−82 and f78−82, are eliminated from the precipitate after a first water wash, whereas peptides β-lg f15−20 and f1−8 remain in the precipitate after the first water wash. Furthermore, the second water wash led to the elimination of the peptide β-lg f15−20 from the precipitate. After two washing steps with HPLC water, the degree of purity of the washed precipitate in terms of β-lg f1−8 increased sharply (>94%). Hence, it can be assumed that

the peptide β-lg f1−8 is the only initiator of peptide aggregation in the flocculated peptide mixture. This also leads to the hypothesis that peptide fragments other than β-lg f1−8 were found entrapped in the precipitate but were not interacting strongly and specifically with β-lg f1−8.

The effect of pH on the CD spectra of peptide β-lg f1−8 revealed a shift from random coil to β-sheet conformation; as the pH was increased from 2.0 to 11.0, the solubility of the peptide decreased sharply. These observations on conformational change as a function of pH led to the hypothesis that peptide β-lg f1−8 can form well-defined aggregates via a self-assembly process. It is already known in the literature that such change in secondary structure of peptides from random coil to β-sheet conformation is associated with a self-assembling phenomenon (*20*−*23*). Peptide aggregation is favored thermodynamically when a state of minimum free energy can be reached via the establishment of non-covalent intermolecular forces, such as hydrogen bonding, electrostatic interactions, hydrophobic interactions, and van der Waals interactions (*24*). The amino acid sequence of peptide β-lg f1−8 (see **Table 1**) consists of a hydrophobic sequence terminated by a C-terminal basic amino acid (Lys). Its isoelectric point is 8.75, which indicates that the peptide is near neutral at pH 8.0, but its cationic character increases as the pH is decreased. This may contribute to peptide solubilization as a result of increased peptide−water interactions. However, further CD investigations and other structural studies of the aggregating peptide β-lg f1−8 are required to establish a hypothetical model for peptide aggregation.

Our observations on the aggregating behavior of peptide fragment β-lg f1−8 are to some extent consistent with the work of Creusot and co-workers (*16*) that revealed peptide fragment β-lg AB [f1−45] as a dominant aggregating peptide. It can be hypothesized that the fragment β-lg f1−8 is strongly involved as the main hydrophobic domain of the larger sequence.

Finally, our observations on the aggregation of peptide β-lg f1−8 provide new clues for the development of large-scale methods of peptide purification using membrane separations (RO) under specific physicochemical conditions. There is a need to better characterize the aggregation process of peptide β-lg f1−8 including the effect of some other key factors such as peptide concentration, ionic strength, and temperature.

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