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Effect of Physicochemical Conditions on Peptide–Peptide Interactions in a Tryptic Hydrolysate of *β*-Lactoglobulin and Identification of Aggregating Peptides

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The objective of this study was to characterize the changes in peptide solubility resulting from changing some physicochemical conditions in a tryptic hydrolysate of *β*-lactoglobulin (*β*-LG). The turbidity (500 nm) of a 1% solution of tryptic peptides was measured at pH 3−10, at 5, 25, and 50 °C, in the presence of different salt concentrations (0, 0.5, and 1 M NaCl), in the presence of denaturing and reducing agents (6 M urea, 5% SDS, or 5% *β*-mercaptoethanol), and under an electric field (isoelectric focusing). The results reveal an increase in turbidity of the peptide solution at pH 4, but a slight increase in turbidity was also observed at pH 8, which is attributable to peptides linked by disulfide bridges. The effect of temperature and ionic strength on the turbidity occurring at pH 4 indicates that mainly hydrophobic interactions are involved in the aggregation process. The material in the precipitate at pH 4 was identified as the peptides *β*-LG 1−8, 15−20, and 41−60 and non-hydrolyzed α-lactalbumin. These results suggest that a limited number of peptides are involved in the aggregation process observed at pH 4, some of which having bioactive (*β*-LG 15−20, ACE inhibitor, and opioid) or emulsifying properties (*β*-LG 41−60). Aggregation of these peptides at acidic pH indicates that a simple acidification step could represent an easy process for isolating peptidic fractions enriched in bioactive or functional peptides.

KEYWORDS: ***β*-Lactoglobulin; tryptic hydrolysate; peptide−peptide interaction; aggregation**

INTRODUCTION

The hydrolysis of whey proteins such as *β*-lactoglobulin (*β*-LG) generates peptides of various functional and biological properties. For instance, the hydrolysis of *β*-LG with trypsin is known to generate peptides with emulsifying properties, such as *β*-LG 41−60 (*1*, *2*) and *β*-LG 21−40 (*2*). A number of bioactive peptides are also obtained from the tryptic hydrolysis of *β*-LG. These include the ACE-inhibitory peptides *β*-LG 15−20, 102−105, and 142−148 (*3*, *4*), the bactericidal peptides *β*-LG 92−100 and 15−20 (*5*), and the hypocholesterolemic peptide *β*-LG 71−75 (*6*). With the emergence of bioactive peptides in the nutraceutical market, it becomes relevant to find techniques that can selectively separate peptides to produce concentrated fractions. Although nanofiltration has been used to fractionate such hydrolysates, a high degree of selectivity has not been achieved (*7*, *8*). The separation mechanism involved in using nanofiltration membranes is, in theory, based on a molecular sieve effect and/or a charge effect, but many discrepancies in peptide transmission have been observed. For example, the transmission of a given peptide varies with the type of hydrolysate (*7*). The addition of salt is also known to have markedly different effects on the transmission of two peptides that differ only by one amino acid (*8*). Little is known about the behavior of peptides in mixtures, but peptide−peptide interactions are suspected to impair fractionation (*7*, *8*). Although electrically-assisted membrane filtration systems have been developed to improve fractionation (*9*, *10*), the impact of electric fields on the occurrence of peptide−peptide interactions has never been studied.

The aggregation of peptides during enzymatic hydrolysis of whey proteins has been observed in several studies (*11*, *13*) in which aggregates were shown to consist of peptides of 2−6 kDa, predominantly linked together by non-covalent interactions (*13*). Otte et al. (*14*) identified peptide *β*-LG 135−158 as one of the peptides involved in the aggregation process. This peptide was proposed to be the initiator of the aggregation process. The aggregation of hydrophobic peptides has also been studied by isolating acid-precipitated material in a tryptic hydrolysate of casein (*15*).

This work was undertaken to characterize peptide aggregates formed in a tryptic hydrolysate of *β*-LG under different physicochemical conditions, and to demonstrate the occurrence of peptide−peptide interactions in a peptide mixture. Turbidity (500 nm) was used as a tool to follow the aggregation process,

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and peptide aggregates were isolated and identified by chromatography and mass spectroscopy. Also, the impact of electric fields on the aggregation process was studied by isoelectric focusing (IEF).

MATERIALS AND METHODS

Materials. Bovine β-lactoglobulin (97% protein, dry basis) was obtained from Davisco Food International Inc. (Le Sueur, MN). In this product, β-LG and α-lactalbumin (α-LA) account for 95% and 2.1% of total proteins, respectively. Trypsin VI (porcine pancreas) was purchased from Inovatech Inc. (Abbotsford, BC, Canada). This commercial preparation contained 2800 U/mg of trypsin activity, but also 490 U/mg of chymotrypsin activity. All chemicals used to prepare the buffers were of analytical grade.

Preparation of the β-LG Tryptic Hydrolysate. Tryptic hydrolysate was prepared as described by Pouliot et al. (*7*). A 10% (w/v) aqueous solution was made with 12 kg of β-LG, and then the solution was adjusted to pH 8.0 with 2 N NaOH and heated to 40 ± 1 °C. Hydrolysis was initiated by adding 115 mL of enzyme solution (8% w/v in 0.001 N HCl) to give an E:S ratio of 1:1265 (grams of enzyme:grams of substrate). During hydrolysis, the solution was maintained at pH 8.0 by adding 2 N NaOH using the pH-Stat technique of Adler-Nissen (*16*). When the degree of hydrolysis (DH) reached 5.6%, the reaction was stopped by ultrafiltering the solution on a 10-kDa MWCO membrane (PM10, Romicon Inc., Woburn, MA) to separate peptides from the enzyme and the non-hydrolyzed proteins. This DH was previously used to efficiently produce tryptic peptides (*7*, *8*). Filtrations were carried out at 45 °C at a transmembrane pressure of 25 psi. The permeate was then concentrated by reverse osmosis (50 °C, 200 psi) on a Lab Unit 1812 (Filtration Engineering, Champlin, MN) using a TW30-1812-50 membrane. Concentrated tryptic hydrolysate was freeze-dried and stored at −20 °C for further analysis. The protein content of the final hydrolysate was 97%, as determined by the Kjeldahl method (*17*), and the final degree of hydrolysis was 11.7%, as determined by the OPA method (*18*).

Turbidity Measurements. The tryptic hydrolysate was rehydrated (1% w/v) in the buffer prepared from pH 3 to 10. McIlvain buffers of pH 3, 4, and 5 were prepared with 0.1 M citric acid and 0.2 M Na_2HPO_4. Phosphate buffers of pH 6, 7, and 8 were prepared from 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4, while carbonate buffers of pH 9 and 10 were prepared with 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$. For turbidity measurements, buffered hydrolysate solutions were analyzed at 5, 25, and 50 °C, but also in the presence of NaCl (0.5 and 1 M), urea (6 M), SDS (5%), or a mixture of SDS (5%)/β-mercaptoethanol (5%). All solutions were allowed to react for 90 min at room temperature, and solutions with SDS and β-mercaptoethanol (β-ME) were boiled for 2 min. Aggregation in the different solutions was estimated by turbidity measurements at 500 nm using an Agilent 8453 UV−Visible Spectroscopy System (Agilent Technologies, Waldbronn, Germany). Means of absorbance were calculated from triplicate analyses, and absorbances of all buffers were measured and estimated as nil.

Separation of Peptide Aggregates. At pH 4 and 8, aggregates were observed in tryptic hydrolysate solutions and isolated by centrifugation (10000*g*, 20 min). The precipitate obtained at pH 4 was rehydrated in a phosphate buffer (pH 8) and analyzed by RP-HPLC and mass spectrometry (MS). The precipitate formed at pH 8 could not be solubilized by gentle conditions (i.e., pH change or ultrasound bath). It was then analyzed in reduced form, after the addition of dithiotreitol (DTT, 70 mM).

Isoelectric Focusing (IEF). The tryptic hydrolysate was rehydrated in water (6.25 mg/mL) and fractionated by liquid-phase IEF in a preparative Rotofor cell (Bio-Rad Laboratories, Hercules, CA) at 4 °C and constant power (12 W) for 2 h. Initial voltage and current were in the ranges of 400−500 V and 23−27 mA, respectively. Given the amphoteric nature of the peptides generating the pH gradient, no ampholytes were added to the focusing chamber (*19*). Electrolytes in the anode and cathode compartments were 0.1 M H_3PO_4 and 0.1 M NaOH, respectively. Under these conditions, acidic peptides are attracted to the anode, while basic peptides migrate toward the cathode until they reach their zwitterionic state and stabilize at a pH corresponding to their isoelectric point (p*I*). Twenty peptide fractions were collected from each run, and their pH was measured. Fractions were then pooled according to pH similarities and freeze-dried. Turbidity was measured for solutions (1% w/v in water) prepared with each pooled fraction named A−H. During fractionation by IEF, a precipitate was observed in the acidic region of the focusing chamber. This precipitate was collected, solubilized in phosphate buffer (pH 8), and analyzed by RP-HPLC and MS. To demonstrate the occurrence of peptide−peptide interactions, each pooled fraction (A−H) was also paired two-by-two (1:1), and turbidity was measured in the solutions (1% w/v) after 1 h of reaction time. To evaluate the impact of combining two IEF fractions, a turbidity index was calculated as follows:

$$\text{turbidity index} = T_{AB}/(T_A + T_B/\ 2)$$

where T_{AB} is the turbidity measured for the paired fraction AB, while T_A and T_B are the turbidity values of the individual fractions A and B. With this turbidity index, a value of 1.0 indicated no impact of fraction pairing on turbidity, whereas values lower or higher than 1.0 reflected positive or negative effects on peptide solubility, respectively.

RP-HPLC Analysis. RP-HPLC analyses were performed using a HPLC system from Waters (Milford, MA) consisting of an injector (Rheodyne model 7725i, Cotati, CA), two pumps (model 600E), and a UV/visible detector (model 486) adjusted to 220 nm. Data acquisition and analysis were done using the Millennium 2.1 chromatography software. The peptide compositions of aggregates formed at pH 4 and 8, IEF fractions (A−H), and IEF precipitate were analyzed with a Nova-Pak C_{18} column (3.9 i.d. × 150 mm, Waters) using the following conditions: injection volume, 20 μL; flow rate, 1 mL/min; column temperature, 39 °C; solvent A, trifluoroacetic acid (TFA) 0.11% (v/v in water); solvent B, acetonitrile (60%)/water (40%)/TFA (0.1%). Elution was obtained with a linear gradient of solvent B from 0 to 60% over 30 min. DTT (70 mM) was used as reducing agent to solubilize peptide aggregates formed at pH 8.

Mass Spectrometry Analysis. Electrospray mass spectroscopy was performed using a LC-MSD Trap Agilent 1100 Series (Agilent Technologies) in a positive ion mode, using a direct infusion method at 0.6 mL/min. Peptides were collected from HPLC analysis and mixed with propionic acid (2%). Nitrogen was used as drying gas (10.0 L/min, 300 °C) and nebulizing gas at 7.0 psi. The capillary voltage was set at −3500V and the end plate offset at −500 V. The instrument was calibrated using ES tuning mix (G2431A, Agilent). Full-scan mass spectra were acquired in the mass range m/z 50−2200 in the multichannel analyzer mode (five spectra). Data acquisition was processed by DataAnalysis Version 2.1 (Bruker Daltonik GmbH, Agilent).

RESULTS AND DISCUSSION

Peptide Aggregation in the Tryptic Hydrolysate of β-LG. **Figure 1** shows the turbidity measured in the tryptic hydrolysate solutions (1% w/v) as a function of pH, and under different physicochemical conditions. Turbidity is herein used as an estimation of visible peptide aggregation in the tryptic hydrolysate. In **Figure 1A**, an important increase in turbidity was observed at pH 4, but a small increase in turbidity was also detected at pH 8.0. However, the temperature influenced the solution only at acidic pH, increasing the turbidity at a higher temperature. **Figure 1B** shows the same increases in turbidity at pH 4 and 8. As for temperature, higher ionic strength raised the turbidity level only at acidic pH. The effect of urea, SDS, and SDS + β-ME (**Figure 1C**) was different since these denaturing/reducing agents decreased the apparent turbidity at all pH values.

At acidic pH, higher temperatures (5−50 °C) or ionic strengths (0−1 M NaCl) significantly increased the turbidity, emphasizing the contribution of hydrophobic interactions in the aggregation process. The majority of the peptides in the tryptic hydrolysate of β-LG have a p*I* around pH 4 (*19*), where the

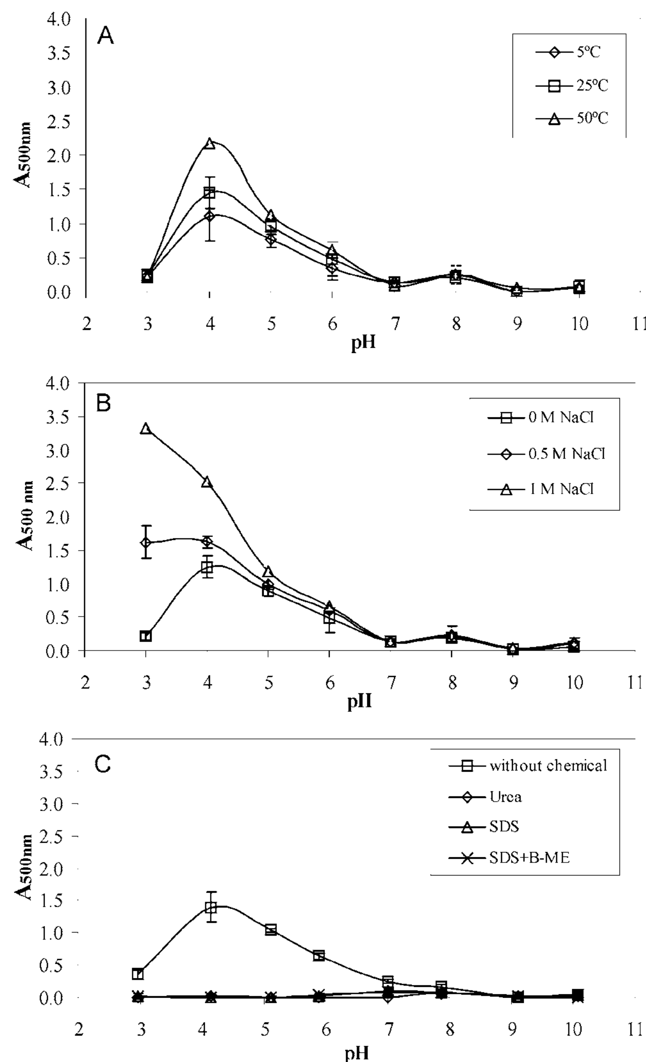

Figure 1. Turbidity ($A_{500\ nm}$) measured in the tryptic hydrolysate of β-LG (solutions at 1% w/v) as a function of pH, and under different conditions of temperature (A), salt concentration (B), and denaturing/reducing agents (C).

charges are minimized in favor of hydrophobic interactions. Caessens et al. (*20*) also observed that most of the peptides issued from a tryptic hydrolysate of β-LG were participating in the hydrophobic interactions, as determined by gel permeation chromatography. Otte et al. (*13*) also showed that hydrophobic interactions play a significant role in the aggregates formation during β-lactoglobulin hydrolysis with protease from *Bacillus licheniformis.*

At pH 8, the slight increase in turbidity is independent of temperature and salt concentration, indicating that electrostatic or hydrophobic interactions are not involved in the aggregation process. However, the effect of denaturing/reducing agents suggests that the slight increase in turbidity at pH 8 could be the result of covalent interactions, such as disulfide bridges. In fact, the reactivity of the free thiol residue (Cys_{121}) of β-LG is increased at pH 8, around the pK_a of the SH group, where new disulfide bridges are likely to form (*2*, *21*). This effect was not observed at higher pH, indicating that this covalent aggregation does not occur at higher pH, probably due to increasing repulsions.

Peptide Aggregates Formed at pH 4. Aggregates formed at pH 4 were isolated by centrifugation and analyzed by RP-HPLC and MS in order to determine their peptide composition.

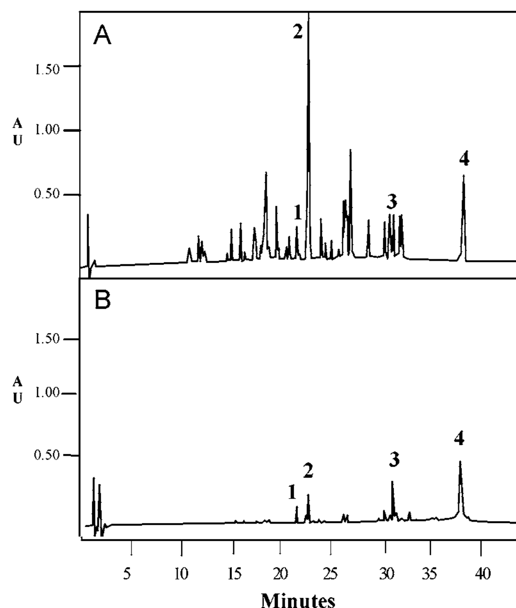

Figure 2. RP-HPLC profiles of the tryptic hydrolysate of β-LG (A) and peptide aggregates formed at pH 4 (B). Peak numbers refer to peptides described in **Table 1**.

These aggregates represent about 10% of the total peptide material. **Figure 2** shows the RP-HPLC profiles of the tryptic hydrolysate of β-LG (A) and aggregates formed at pH 4 after their solubilization in phosphate buffer at pH 8 (B). As presented in **Table 1**, the four main peaks observed in **Figure 2B** were assigned to peptides β-LG 1−8 (peak 1), β-LG 15−20 (peak 2), β-LG 41−60 (peak 3), and non-hydrolyzed α-LA (peak 4). Not all of these peptides aggregated, since they partially remained in the supernatant of the solution, as emphasized by the HPLC profiles (not shown). The proportion of peptides found in the precipitate was thus estimated from the peak area of aggregating peptides relative to the peak area in the total hydrolysate and accounts for 5.97%, 2.32%, and 6.17% for the peptides β-LG 1−8, 15−20, and 41−60, respectively. These three peptides are highly hydrophobic, which is consistent with the statement of hydrophobic interactions at pH 4. Peptide β-LG 1−8 has one positive charge at pH 4, but its N-terminal end is highly hydrophobic, and this peptide can theoretically participate in both electrostatic and hydrophobic interactions. Peptide β-LG 15−20, a bioactive peptide issued from the chymotryptic cleavage of the tryptic peptide β-LG 15−40 (*22*), is neutral and highly hydrophobic. Peptide β-LG 41−60 is a large fragment made up of charged and hydrophobic residues, which are responsible for its emulsifying properties (*1*, *2*). This peptide can also participate in both electrostatic and hydrophobic interactions, as was suggested for peptide β-LG 135−158 (*14*). This peptide was identified as the initiator of aggregation through non-covalent interactions in a microbial protease (*B. licheniformis*) hydrolysate of β-LG. However, hydrophobic interactions seem to be predominant, as emphasized by our turbidity measurements under different physicochemical conditions (**Figure 1**). This is consistent with the work of Léonil et al. (*15*), in which they separated hydrophobic peptides from a tryptic hydrolysate of casein by acid precipitation.

Intact α-LA was also detected in aggregates formed at pH 4, indicating that this protein was not hydrolyzed by trypsin at pH 8 and permeated through the filtration membrane (MWCO, 10 kDa). This result also indicates that intact α-LA could precipitate or interact with itself or with peptides at acidic pH. α-LA is a small protein (14 180 Da) that adopts a fully folded

Table 1. Physicochemical Characteristics of the Peptides Identified by Mass Spectrometry in Peptide Aggregates Formed at pH 4

peak no.[a]	mass (Da)[b]	assigned peptide	amino acid sequence	charge at pH 7.0	isoelectric point[c]	$H\varphi$av[d] (kcal/residue)
1	933	β-LG 1–8	LIVTQTMK	+1	8.75	1.34
2	696	β-LG 15–20	VAGTWY	0	5.77	1.61
3	2 313	β-LG 41–60	VYVEELKPTPEGDLEILLQHK	+2, –5	5.40	1.37
4	14 180	α-LA		+17, –34	4.8	1.12

[a] Referred to in **Figure 2**. [b] Determined by mass spectrometry. [c] Isoelectric point was calculated using the ExPASy Molecular Biology Server. [d] Average hydrophobicity was calculated according to the method of Bigelow (*29*).

and rigid structure at pH 8 (*23*, *24*), and this protein was reported to be resistant to tryptic hydrolysis (*25*). This could explain its presence in the filtered tryptic hydrolysate. Also, the low p*I* (4.8) of α-LA and its slightly unfolded state (molten globule) at acidic pH (*24*, *26*) could explain its precipitation at pH 4. Aggregation of α-LA at this pH would also be explained by its interaction with hydrophobic peptides. In fact, this property was used by Gurgel et al. (*27*) for the isolation of α-LA from a whey protein isolate using bioselective adsorption of the protein to hydrophobic peptide ligands. From those results, it is impossible to confirm if α-LA self-aggregated or interacted with peptides, but it will be interesting to study the potential of using this protein to isolate specific peptides at acidic pH from a complex hydrolysate.

Peptide Aggregates Formed at pH 8. Aggregates causing the slight turbidity increase at pH 8 were also isolated by centrifugation, but a much smaller precipitate (~1.5% of total peptide material), insoluble in mild conditions (pH variation and ultrasound bath), was obtained. DTT (70 mM) was thus added to the precipitate. Reduced peptides were analyzed by RP-HPLC and MS (result not shown). Small amounts of peptides β-LG 15−20, 61−69, 61−70, and 149−162 were found in the precipitate, reflecting the possible occurrence of peptides 61−69 + 149−162 and 61−70 + 149−162; both of these fragments contain a disulfide bridge. The slight increase in turbidity of the hydrolysate at pH 8 thus seems to be the result of peptides linked by a disulfide bridge. However, peptide β-LG 102−124, containing the free thiol group (SH_{121}) of β-LG, was not found in the aggregates. It is therefore impossible to relate the aggregation process at pH 8 to SH/SS interchange reactions. This large fragment contains both an intramolecular disulfide bond and the free thiol group known to be very reactive at pH 8 (i.e., this pH value corresponds to the pK_a of the SH group). However, it was suggested in previous studies that this peptide creates large aggregates that are retained on the filtration unit prior to the HPLC analysis (*2*). Therefore the peptide β-LG 102−124 could be responsible for aggregate formation at pH 8, even if its presence in aggregates was not detected by HPLC analysis.

Influence of Electric Fields on Peptide Solubility. The tryptic hydrolysate of β-LG was fractionated by isoelectric focusing (IEF) to evaluate the impact of electrical fields on peptide solubility. Twenty fractions were collected from the IEF chamber and pooled according to their pH similarities. Pooled fractions were named A−H. These fractions were analyzed by RP-HPLC to determine their content in α-LA and peptides β-LG 1−8, 15−20, and 41−60, identified previously as aggregating material at pH 4 (**Figure 2**). The content of these four components was estimated as the percentage of the total surface area of the chromatogram, and represents the amount of soluble components in each pooled fractions. **Figure 3** shows the pH for each pooled fraction (A−H) and their composition (%) of α-LA and peptides β-LG 1−8, 15−20, and 41−60. None of the fractions contained soluble α-LA. The amount of peptide

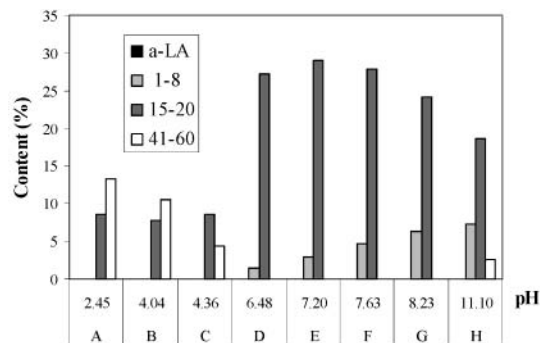

Figure 3. Content (%) of α-LA and peptides β-LG 1–8, 15–20, and 41–60 in IEF fractions as determined by RP-HPLC analysis.

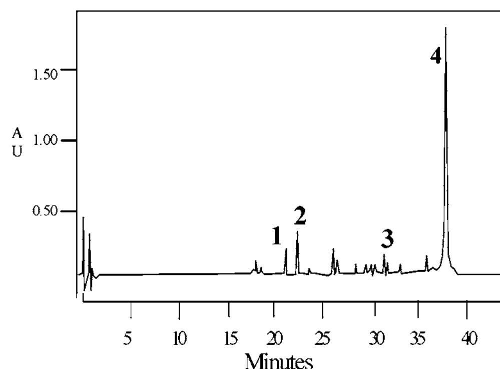

Figure 4. RP-HPLC profile of the precipitate collected in the acidic region of the IEF membrane core. Peak numbers refer to peptides described in **Table 1**.

β-LG 1−8 (**Figure 3**) was found to increase from fractions D to H (pH 6.48−11.1), which is normal, considering that this peptide has a p*I* of 8.75 (**Table 1**). Peptide β-LG 15−20 (**Figure 3**) was detected in all fractions, with higher amounts in fractions D−H. This peptide's electrophoretic mobility was expected as previously observed (*19*), since it is neutral at any pH (**Table 1**). Peptide β-LG 41−60 (**Figure 3**) was found in decreasing amounts in fractions A, B, and C, having pH values from 2.45 to 4.36, while the p*I* of this peptide is 5.4 (**Table 1**). A very low amount of this peptide was also detected in fraction H at pH 11.10, which is possibly the result of peptide−peptide interactions.

During IEF experimentation, a precipitate was observed in the region of fractions B and C. This precipitate was collected directly from the membrane core and was characterized by RP-HPLC and MS. As shown in **Figure 4**, this precipitate was composed of mainly non-hydrolyzed α-LA (peak 4), with some traces of peptides β-LG 1−8 (peak 1) and β-LG 15−20 (peak 2), and a very low amount of peptide β-LG 41−60. A very low proportion of the three peptides compared with the concentration of α-LA was detected in the precipitate collected in the acidic region (pH 4.0−4.4) of the IEF chamber (**Figure 4**), compared to those measured in aggregates obtained at pH 4

Table 2. Turbidity Index Calculated for Individual and Paired Fractions Isolated from Isoelectric Focusing (IEF); the pH Value of Individual IEF Fractions Is Also Indicated

pH	2.45	4.04	4.36	6.48	7.20	7.63	8.23	11.10
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
A	1.0	0.5	0.5	2.3	3.1	2.7	4.2	1.3
B		1.0	1.3	0.6	0.4	0.4	0.3	0.6
C			1.0	0.7	0.5	0.6	0.4	0.3
D				1.0	2.1	0.9	0.6	0.4
E					1.0	1.0	1.4	0.7
F						1.0	0.8	0.4
G							1.0	0.8
H								1.0

(**Figure 2B**). This result could reflect the higher solubility of the peptides under an electrical field. In fact, an electrical field can induce a polarization in the ionic sphere of peptides and hence increase their affinity for water molecules, which in turn improves their solubility (*28*). The overall solubility is usually increased with an electric field, which supports the efficiency of coupling electric fields with filtration systems. For instance, Daufin et al. (*9*) used electrofiltration to selectively separate casein tryptic peptides in the permeate and retentate according to the direction of the applied electric field. Bargeman et al. (*10*) obtained comparable results for the separation of lactoferrin peptides.

An inverse effect of electric field was observed for α-LA, because more of this protein was detected in the precipitate obtained by IEF (**Figure 4**) compared to aggregates isolated at pH 4 (**Figure 2B**). This result could reflect the impact of the electric field on calcium solubility leading to apo-α-LA, which is more susceptible to aggregation.

Peptide−Peptide Interactions in IEF Fractions. To demonstrate the occurrence of peptide−peptide interactions, turbidity was measured in the peptide solutions (1% w/v) prepared from each IEF fractions (A−H), but also after pairing the fractions two-by-two (AB, AC, etc.). For this experiment, the pH of paired fractions was not adjusted to avoid the effect of salts on peptide−peptide interactions. The turbidity index (TI) was calculated (see Materials and Methods), and the results are presented in **Table 2**. To facilitate their interpretation, TI $<$ 0.5 was considered to indicate higher peptide solubility in paired than in individual (1.0) fractions, whereas TI $>$ 2.0 suggested lower peptide solubility, leading to aggregation in paired fractions. Fraction A, with pH 2.45, caused an important turbidity increase when it was paired with fractions D, E, F, and G at pH 6.5−8.2. Hence, there could be some electrostatic interactions formed between very low p*I* peptides and neutral or basic peptides, leading to aggregate formation. However, the turbidity increase may also be the result of a pH change in the paired fractions. Pairing the fractions D (pH 6.48) and E (pH 7.2) also provoked an important increase in the turbidity index ($>$2). For these fractions of similar pH values, peptide−peptide interactions leading to aggregation could be the result of hydrophobic interactions. Peptides from fractions A and B behave differently when mixed with the neutral/basic fractions (D−G). In fact, pairing fraction A with the neutral/basic fractions led to high TI values ($>$2), indicating a decrease in the overall peptide solubility, while the inverse was observed with fraction B. Because the pH change was approximately the same upon pairing fraction A or B with fractions D−G, the effect on turbidity is hence the result of different aggregating properties of peptides. Fraction C, of pH 4.36, also improved the solubility (TI of 0.3−0.6) of peptides contained in neutral/basic fractions, as observed for fraction F (pH 7.63) when it was mixed with fraction H (pH 11.1). The improvement of peptide's solubility could indicate the presence in some fractions of peptides initiating the aggregation process, as demonstrated by Otte et al. (*14*) for peptide β-LG 135−158. In fact, specific peptide interactions with peptides initiating the aggregation process could prevent their action and contribute to the higher overall peptide solubility.

As observed in **Figure 3**, the neutral/basic fractions (D−H) contained the highest amounts of peptides β-LG 1−8 and 15−20, previously identified in the precipitate formed at pH 4 (**Figure 2B**) and in the one collected in the acidic region of the IEF chamber (**Figure 4**). On the other hand, fractions A−C contained the highest concentrations of peptide β-LG 41−60, also identified in the precipitate formed at pH 4 (**Figure 2B**). This peptide was previously reported to have both negatively charged and hydrophobic zones in its primary structure (*2*). This could explain its interaction with the positive/hydrophobic peptide β-LG 1−8 and the neutral/hydrophobic peptide β-LG 15−20 (**Table 1**). These interactions probably explained the aggregation (TI $>$ 2) observed when fraction A was paired with fractions D−G. The higher peptide solubility observed by pairing the fraction B or C with the neutral/basic fractions (**Table 2**) could reflect the presence of other peptides, absent in fraction A, which can interact with peptide β-LG 41−60 and lead to the prevention of its interaction with peptides β-LG 1−8 and 15−20. This hypothesis is supported by the lower amounts of peptides β-LG 1−8 and 15−20 detected in the precipitate collected in the acidic region of the IEF chamber (**Figure 4**) when peptide β-LG 41−60 is absent. Therefore, it can be concluded that the aggregating behavior of peptides β-LG 1−8 and 15−20 is enhanced by the presence of peptide β-LG 41−60 and that some specific peptides can interact with peptide β-LG 41−60 to reduce its impact on the aggregation process. Work to characterize fractions A and B is currently under way to identify the peptides that interact with peptide β-LG 41−60.

From those results, it appears that a simple acidification of a peptide mixture resulting from tryptic hydrolysis of β-LG could result in the selective separation of peptides having bioactive (β-LG 1−8 and 15−20) or emulsifying properties (β-LG 41−60). Performing a precipitation step of aggregating peptides before nanofiltration could also improve the transmission of some peptides and the separation of peptide mixtures.

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