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Fractionation of β-Lactoglobulin Tryptic Peptides by Ampholyte-Free Isoelectric Focusing

PAULE EMILIE GROLEAU,† RAFAEL JIMENEZ-FLORES,‡ SYLVIE F. GAUTHIER,† AND YVES POULIOT*,†

Centre de Recherche STELA, Pavillon Paul-Comtois, Université Laval, Québec, Canada G1K 7P4, and Dairy Products and Technology Centre, California Polytechnic State University, San Luis Obispo, California 93407

Solutions of tryptic hydrolysate of bovine β-lactoglobulin were fractionated by liquid-phase IEF in a preparative Rotofor cell at constant power for 2 h without ampholytes in order to identify interactions between peptides. The 20 peptide fractions collected were analyzed by capillary electrophoresis and SDS-PAGE under native, denaturing, and reducing conditions. The hydrolysate was shown to be composed mainly of acidic peptides (p*I* 2−5, 62%) of molecular mass below 6 kDa, and numerous disulfide bonds were detected. Purified peptides (β-LG 15−20, 71−75, 76−82, and 84−91) were also focused individually and in mixtures and matched to components of the IEF fractions obtained from the tryptic hydrolysate of β-LG. The separation of acidic (β-LG 84−91) and basic (β-LG 76−82) peptides was achieved by IEF, whereas uncharged peptides (β-LG 15−20 and 71−75) were poorly separated due to their low electrophoretic mobility. Because no peptide−peptide interaction could be identified by IEF fractionation, it is suggested that electrical fields may decrease electrostatic interactions between charged peptides.

KEYWORDS: Whey proteins; β-lactoglobulin; tryptic hydrolysate; peptides; peptide−peptide interactions; isoelectric focusing; nanofiltration

INTRODUCTION

The emerging market for nutraceuticals and functional foods has stimulated the production of enzymatic hydrolysates from whey proteins (*1*) with improved functional properties and biologically active peptides (*2*, *3*). The levels of bioactive peptides in such hydrolysates are low, however, creating a demand for techniques capable of providing rapid and efficient isolation of these molecules.

Nanofiltration (NF) membranes may be used to separate peptides according to mass and charge and have been used to separate amino acids in model systems (*4*, *5*), peptides (*6*), and enzymatic hydrolysates from whey proteins (*7*). The mechanism underlying NF separation is a molecular sieve effect or a charge effect or both, depending on membrane characteristics (*8*). Although model solutions have helped to characterize selectivity, it remains difficult to predict the permeation of peptides from a complex mixture such as a hydrolysate. The selectivity of membrane separation techniques such as nanofiltration in the fractionation of enzymatic hydrolysates of proteins is believed to be impaired by peptide−peptide interactions. Pouliot et al. (*7*) have reported that the same peptide was transferred differently depending on whether it was produced by tryptic or chymotryptic hydrolysis. Similar differences have been observed for the transfer of specific peptides when the ionic strength of the solution was increased before filtration (*9*). Selectivity thus appears to be dependent on peptide properties, on surrounding peptides, and on processing conditions. Competition between peptides at pores and peptide−peptide interactions are both believed to modify individual peptide permeation through membranes.

The hydrolysis of proteins by enzymes produces substances of lower molecular mass with increased numbers of ionizable groups and increased exposure of hydrophobic groups (*10*, *11*), creating reactive peptides that are more likely to interact. Cassaens et al. (*12*) demonstrated that peptides obtained from β-lactoglobulin (β-LG) hydrolysis with trypsin and *Staphylococcus aureus* V8 protease may associate via hydrophobic interactions and disulfide bonds but that these associations are especially prevalent among plasmin-derived peptides. Chen et al. (*13*) and Otte et al. (*14*) have also reported that peptides derived from partially hydrolyzed β-LG interact via non-covalent bonds, mainly by electrostatic and hydrophobic interactions, and form stronger gels than intact β-LG. According to Otte et al. (*15*), peptides obtained from the N-terminal region of β-LG have a greater tendency to aggregate due to the clustering of hydrophobic and hydrophilic groups. The nature of the peptides and the physicochemical characteristics of their surroundings thus both influence peptide behavior in mixtures such as protein

* Author to whom correspondence should be addressed [telephone (418) 656-5988; fax (418) 656-3353; e-mail Yves.Pouliot@aln.ulaval.ca].
† Université Laval.
‡ California Polytechnic Institute.

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Table 1. Physicochemical Characteristics of the Four Synthetic β-LG Peptides Studied

peptide	amino acid sequence	M_W[a] (Da)	charge at pH 7.0	isoelectric point[b]	$H\Phi_{av}$[c] (kcal/residue)
β-LG 15–20	VAGTWY	695.7	0	5.49	1.46
β-LG 71–75	IIAEK	572.7	−1, +1	6.00	1.63
β-LG 76–82	TKIPTVF	775.0	+1	8.41	1.76
β-LG 84–91	IDALNENK	916.0	−2, +1	4.37	0.95

[a] Molecular weight was obtained by mass spectrometry. [b] Isoelectric point was calculated using the ExPASy Molecular Biology Server. [c] Average hydrophobicity was calculated according to the method of Bigelow (*30*).

hydrolysates. The fine characterization of hydrolysates is therefore necessary to better understand their properties and to optimize their fractionation by processes such as nanofiltration.

Matrix-free isoelectric focusing (IEF) is a preparative technique that separates amphoteric molecules such as proteins and peptides according to their isoelectric point (p*I*) (*16*). Charged molecules migrate through a pH gradient in an electric field to a position at which the pH corresponds to their p*I* and hence zwitterionic state. Kim et al. (*17*) have used IEF for the comparison of milk proteins from different mammals, whereas Righetti et al. (*18*) and Castelletti et al. (*19*) used IEF to separate tryptic peptides from β-casein, during continuous production in a bioreactor. In these studies, fractionation was efficient and peptides were obtained in a highly pure state.

This work was undertaken to evaluate IEF as a means of characterizing a tryptic hydrolysate of β-LG and possibly identifying peptide−peptide interactions. The IEF technique was used as a model method to study the behavior of peptides in a charged environment such as that created in nanofiltration. Fractionation by preparative matrix-free IEF was based on the amphoteric nature of the sample components themselves rather than on the use of ampholytes to create the pH gradient, a technique known as autofocusing (*20*). With this technique, evidence of peptide−peptide interactions is obtained when peptides are found in a fraction of pH not corresponding to their p*I*. Peptide fractions obtained by IEF were characterized by capillary electrophoresis and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) under native, denaturing, and reducing conditions. Pure synthetic β-LG peptides were also focused individually and in mixtures and matched to components of the IEF fractions obtained from the tryptic hydrolysate of β-LG.

MATERIALS AND METHODS

Substrate. Bovine β-lactoglobulin (97% protein, dry basis) was obtained from Davisco Food International Inc. (Le Sueur, MN), whereas trypsin VI (porcine pancreas) was purchased from Inovatech Inc. (Abbotsford, BC). This commercial preparation of trypsin contained not only 2800 units/mg of trypsin but also 490 units/mg of chymotrypsin activity. Pure peptides β-LG 15−20, β-LG 71−75, β-LG 84−91, and β-LG 76−82 were synthesized by the Service de séquence de peptides de l'est du Québec (Sainte-Foy, PQ). All were water-soluble and >90% pure. Their physicochemical characteristics are presented in **Table 1**. All other chemicals were of analytical grade.

Preparation of Tryptic Hydrolysate. Tryptic hydrolysate was prepared according to the method of Pouliot et al. (*9*). A 10% (w/v) aqueous solution was made with 12 kg of β-LG, 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). The pH was maintained at 8.0 by manual addition of 2 N NaOH using the pH-Stat technique of Adler-Nissen (*21*). Hydrolysis was stopped when the degree of hydrolysis (DH) reached 5.6% by ultrafiltering on a 10 kDa MWCO membrane (PM10, Romicon Inc., Woburn, MA) to separate peptides from the enzyme and nonhydrolyzed proteins. Filtrations were carried out at 45 °C at a transmembrane pressure of 25 psi. The permeate was concentrated by reverse osmosis on a Lab Unit 1812 (Filtration Engineering, Champlin, MN) with a TW30-1812-50 membrane at 50 °C at a pressure of 200 psi. Concentrated hydrolysate was then freeze-dried and stored at −20 °C until further analysis. The protein content of the final hydrolysate was 97% as determined by using the Kjeldahl method (*22*).

Sample Preparation. Tryptic hydrolysate was rehydrated in water (115 mg/40 mL), giving a pH of 7.6. Two other hydrolysate solutions were prepared with the pH adjusted to 5.0 or 9.0 by 0.1 N HCl or NaOH. Solutions of synthetic peptides (10 mg/40 mL in water) were also prepared (β-LG 15−20, 71−75, 76−82, and 84−91) as well as a solution containing all four peptides (5 mg of each/40 mL in water) without pH adjustment.

IEF. The hydrolysate and pure peptide solutions were fractionated by liquid-phase IEF in a preparative Rotofor cell (Bio-Rad Laboratories, Hercules, CA) at constant power (12 W) for 2 h at 4 °C. Because of the amphoteric nature of the peptides generating the pH gradient, no ampholyte was added to the focusing chamber. 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 (the H_3PO_4 membrane chamber), whereas basic peptides migrate toward the cathode (NaOH) until they reach their zwitterionic state and stabilize at a pH corresponding to their isoelectric point (p*I*). Twenty peptide fractions were collected, and their pH was measured immediately (i.e., before dropping due to absorption of atmospheric CO_2). Initial voltage and current were in the ranges of 500−800 V and 13−20 mA, respectively, and the pH gradient was measured for each run.

Analytical Methods. Protein concentration of the fractions collected from IEF separation was determined by using the BCA method (Bio-Rad Laboratories). The IEF fractions were also analyzed by SDS-PAGE on 0.75 mm 18% polyacrylamide gels. Samples (100 μL) of peptide fractions were diluted in 50 μL of 0.06 M Tris buffer (pH 6.8) with and without denaturing (SDS, 2%) or denaturing + reducing (SDS, 2% + β-mercaptoethanol, 5%) agents, and 20 μL was loaded into the wells. Gels were run at 120 V and then fixed in an aqueous methanol/acetic acid solution (40%/10%) for 30 min, blue stained in G-250 solution (0.25% w/v in 10% acetic acid) for 1 h, and decolorized in acetic acid (10%). The molecular weight markers were triosephosphate isomerase (26.6 kDa), myoglobin (16.95 kDa), α-lactalbumin (14.43 kDa), aprotin (6.51 kDa), and insulin (3.49 kDa).

IEF fractions were also analyzed by capillary electrophoresis (CE) on a Bio-Focus 3000 system (Bio-Rad Laboratories) equipped with a UV detector adjusted to 200 nm. The analyses were performed with a silica-coated capillary Celect-P150 (59 μm i.d. × 24 cm, Supelco, Bellefonte, PA). The running buffer was 0.1 M phosphate buffer (pH 2.5), and the sample buffer was a 1:10 dilution of the running buffer. Each sample (100 μL) was prepared in the sample buffer (200 μL), filtered through a 0.45 μm membrane, and centrifuged for 3 min at 20800*g*. Constant voltage (12 kV) and temperature (25 °C) were maintained during the experiment, and a current of 30−40 mA was obtained. For analysis under reducing conditions, 70 mM dithiothreitol (DTT) was included in the sample buffer. The duration of the analysis was 20 min.

RP-HPLC analyses were performed using an 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 Millenium 2.1 chromatographic software. Peptide composition of the IEF fractions was analyzed with a Nova-Pak C_{18} column (3.9 i.d. × 150 mm, Waters) using the following conditions: flow rate, 1 mL/min; column temperature, 39 °C; solvent A, trifluoroacetic acid (TFA) 0.11% (v/v) in water; solvent B, acetonitrile/water/TFA 60%/40%/0.1% (v/v). Elution was obtained with a linear gradient of solvent B from 0 to 60% over 30 min.

For quantification of the four peptides under study, standard curves were prepared with 1.0, 0.75, 0.50, and 0.25 mg/mL of synthetic peptide. Absorbance was measured at 214 nm, and peak surface area

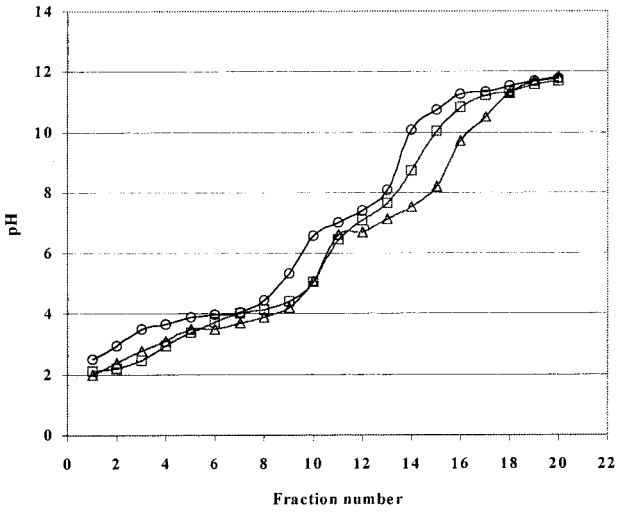

Figure 1. pH gradients generated by IEF with autofocusing of tryptic hydrolysate adjusted to initial pH 5.0 (□), 7.6 (△), and 9.0 (○).

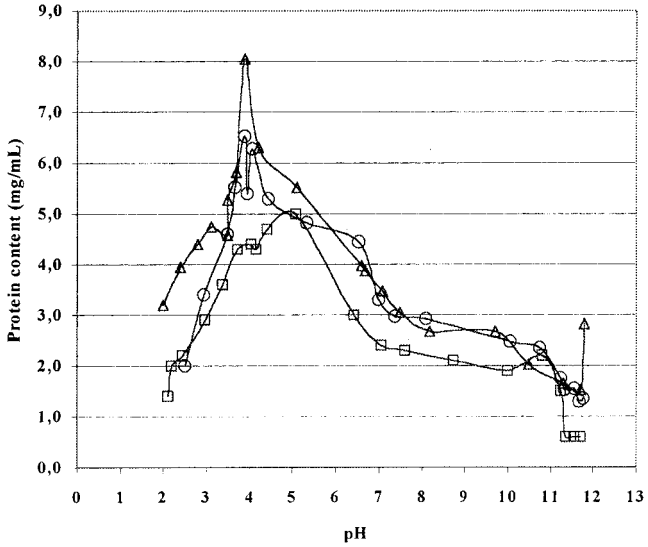

Figure 2. Protein content of tryptic hydrolysate fractions obtained by IEF with autofocusing; initial pH adjusted to 5.0 (□), 7.6 (△), and 9.0 (○).

was plotted against concentration. The linear regression equation was used to determine the concentration of these peptides in the different samples. To identify each of the peptides in the tryptic hydrolysate, a spiking technique was used and consisted of adding a synthetic peptide to the sample to observe the increase of the peak corresponding to the given peptide.

RESULTS AND DISCUSSION

IEF Fractionation of the Tryptic Hydrolysate. Figure 1 illustrates the pH gradient generated by autofocusing of the tryptic hydrolysate preadjusted to pH 5.0, 7.6, and 9.0. After 2 h of autofocusing, the pH gradient ranged from 2 to 12 and had a similar profile for all three initial pH values. Initial voltage during sample autofocusing was 500−700 V and reached 1000−1200 V at equilibrium. Yata et al. (*20*) obtained a similar pH gradient and voltage development for the autofocusing of a tryptic hydrolysate of casein. Plateaus observed on the curves reflect the presence of acidic (pH 4), neutral (pH 7), and basic (pH 11) peptides. The curve widths obtained may be the result of diffusion of peptides of similar p*I* between neighboring fractions. According to Laas (*16*), an equilibrium is reached between diffusion and electrophoretic accumulation at the p*I*. Ampholytes added to the focusing chamber generally ensure a more regular curve without plateaus, because they include isoelectric electrolytes throughout the pH gradient produced.

Figure 2 illustrates the quantitative distribution of peptides in IEF fractions throughout the pH gradient generated by autofocusing. Approximately 62% of the initial peptide material was found between pH 2 and 5 and 23% between pH 6 and 8, whereas only 14% was found between pH 8 and 12. Because trypsin cleaves proteins at the C-terminal end of Arg and Lys residues, most of the peptides have a positive charge at their C-terminal end. For them to focus in the acidic region, they must have a number of negative residues to counteract this C-terminal positive charge. In fact, β-LG contains 41 acidic residues (Asp and Glu), which make up 25% of the total amino acids (*23*). As observed for the pH gradient generated during autofocusing of the tryptic hydrolysate (**Figure 1**), peptide distribution did not vary noticeably with the different initial solution pH values (**Figure 2**).

SDS-PAGE was performed on the IEF fractions to provide a more detailed representation of peptide distribution. **Figure 3** shows the migration of peptides compared to a polypeptide molecular weight marker (M_W) in non-denaturing (**Figure 3A**),

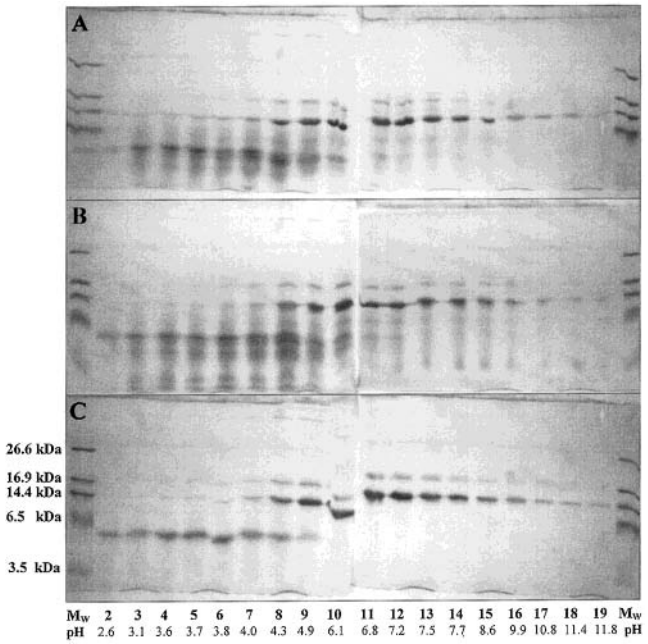

Figure 3. SDS-PAGE of IEF fractions in non-denaturing (A), denaturing (B), and denaturing + reducing (C) buffers. Lanes marked "M_W" are polypeptide molecular weight markers. pH of fractions in lanes 2–19 is indicated.

denaturing (**Figure 3B**), and denaturing + reducing (**Figure 3C**) buffers. The pH of the fractions was measured to obtain the average isoelectric point of the fractions and is indicated for each fraction. Many of the peptides were found to have a p*I* between 3 and 6 as indicated by the abundance of bands in lanes 3−10. These results are consistent with the protein contents observed throughout the pH gradient (**Figure 2**). Furthermore, molecular masses of theses peptides appear to be <6 kDa, based on the position of the nearest polypeptide marker. The hydrolysate also contains many peptides of molecular mass around 14 kDa, mostly concentrated in fractions at pH 4.9−7.7 (lanes 9−14), whereas very few peptides were found at basic pH (lanes 15−19). As expected for the small peptides, the denaturing (SDS) buffer affects separation only slightly (**Figure 3B**). In fact, SDS promotes dissociation and solubilization of proteins and has been reported to stabilize peptide secondary

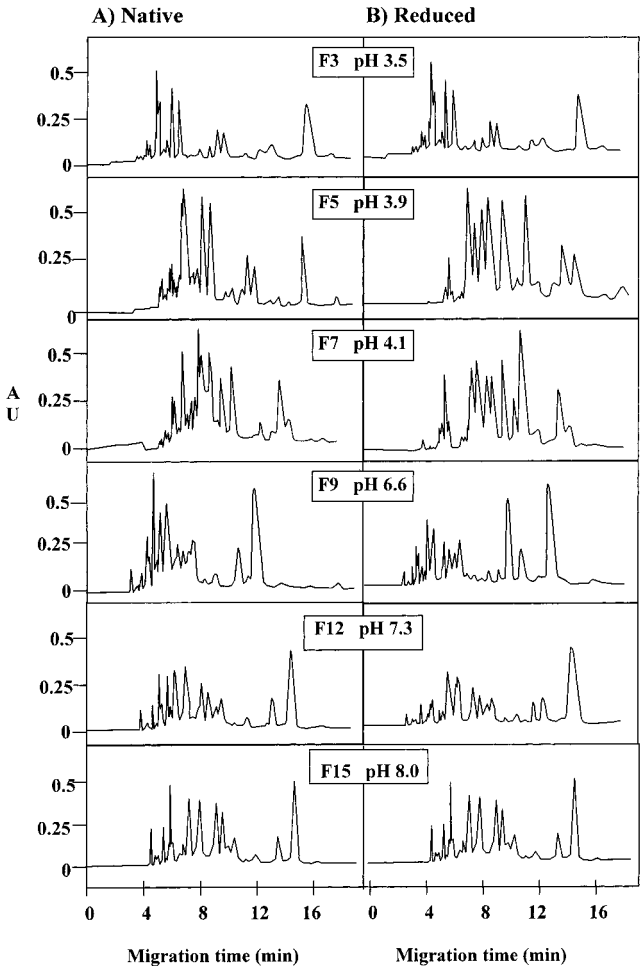

Figure 4. Capillary electrophoresis profiles of IEF fractions F3, F5, F7, F9, and F12 under native and reducing conditions. pH of the different fractions is indicated.

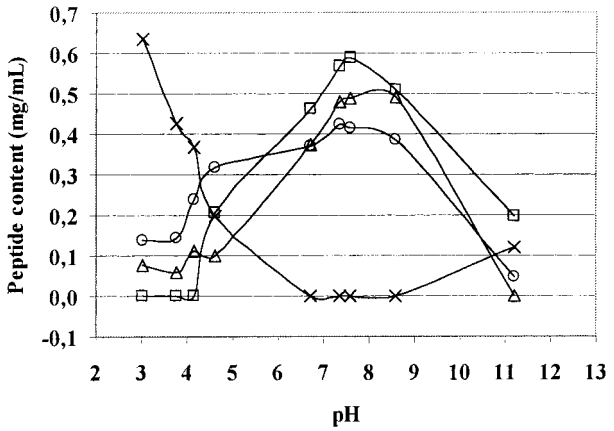

Figure 5. Peptide content (determined by RP-HPLC) of fractions obtained from tryptic hydrolysate by IEF with autofocusing versus fraction pH. Peptides are β-LG 15–20 (○), β-LG 71–75 (□), β-LG 76–82 (△), and β-LG 84–91 (×).

structure (*24*). However, the denaturing + reducing (SDS + β-mercaptoethanol) buffer did influence separation, mainly for peptides of molecular mass <6 kDa and p*I* 3.0−6.0 (lanes 3−10), indicating the presence of disulfide bonds. For β-LG hydrolysates, Caessens et al. (*25*) have observed similar results, and these authors have proposed that most of the cysteine residues are concentrated in these low-p*I*/low-mass peptides.

Fractions were also analyzed by capillary electrophoresis to confirm our gel electrophoresis observations. **Figure 4** illustrates the capillary electrophoresis profiles of IEF fractions in native and reducing (DTT) conditions. Under native conditions, profiles change noticeably as pH goes from 3.5 to 7.3, indicating a diversity of peptides. For the basic fractions, all CE profiles were similar and had fewer peaks than the acidic and neutral fractions as observed on fractions 9, 12, and 15. In fact, the same profiles are found with decreasing proportions. Under reducing conditions, CE profiles are different for fractions F5 (pH 3.9) to F12 (pH 7.3), whereas the acidic fraction at pH 3.5 (F3) and the fractions at pH >7.3 were not changed by DTT. These observations are consistent with the results obtained for SDS-PAGE analysis (**Figure 3**) and confirm the presence of disulfide bonds in the fractions containing peptides with low p*I*.

Tryptic hydrolysates of β-LG commonly contain two peptides linked by disulfide bonds: β-LG 61−69 + 149−162 and β-LG 61−70 + 149−162, which account for 7.3% of all peptides produced (*7*). These two peptides partially account for the differences observed between native and reducing condition profiles, but **Figures 3** and **4** also suggest that peptide fragments linked by new disulfide bonds may have formed. It is generally agreed that the free thiol group (Cys121) of β-LG is likely to interact with other compounds because it is often responsible for the gelling of β-LG hydrolysates (*26*). Furthermore, the peptide β-LG 102−124, which contains the free thiol group and one disulfide bond, is very likely to be reactive but has never been identified by conventional methods (*27*, *28*). One hypothesis is that this peptide forms aggregates which are removed by the prefiltration commonly done before chromatographic analysis. However, Maynard et al. (*29*) have identified the sequence β-LG 102−124 + 149−162 resulting from an intra- or intermolecular arrangement between the free thiol group of Cys 121 and the half-cysteine residue 160 located in the subsequence β-LG 149−162, suggesting that peptide β-LG 102−124 may be involved in interactions with other cysteyl-containing peptides. As reported by Caessens et al. (*25*), the free thiol group of β-LG is more exposed after hydrolysis of the protein, initiating SH−SS interchange, which could subsequently induce peptide aggregation.

IEF Fractionation of Purified Peptides. Table 1 summarizes characteristics of the pure peptides obtained by chemical synthesis selected for this study. These peptides were chosen according to their p*I* in order to study different charge distributions in the peptide sequences: neutral without any charge (β-LG 15−20), neutral with one positive charge and one negative charge (β-LG 71−75), acidic (β-LG 84−91), and basic (β-LG 76−82). All peptides are of similar molecular mass (575−800 Da) with an average hydrophobicity varying from 0.95 to 1.76 kcal/residue. Two of them (β-LG 71−75 and 84−91) are released from specific cleavage (C terminus of lysine and arginine residues), whereas β-LG 15−20 is obtained from the nonspecific cleavage of the Tyr20−Ser21 bond that has already been reported (*27*, *28*). Peptide β-LG 76−82 results from cleavage of Lys83 by the chymotrypsin present in the commercial trypsin used in this study.

Fractions 2−19 obtained from autofocusing of the tryptic hydrolysate were pooled two by two according to pH, and the nine new fractions thus obtained were freeze-dried and further analyzed by RP-HPLC to quantify the four purified peptides in each of the pooled fractions. **Figure 5** is a plot of peptide content versus fraction pH. Both charged peptides (β-LG 76−82 and 84−91) focused as expected around their respective p*I* values. Peptide β-LG 84−91, which has a p*I* of 4.37 and three charged

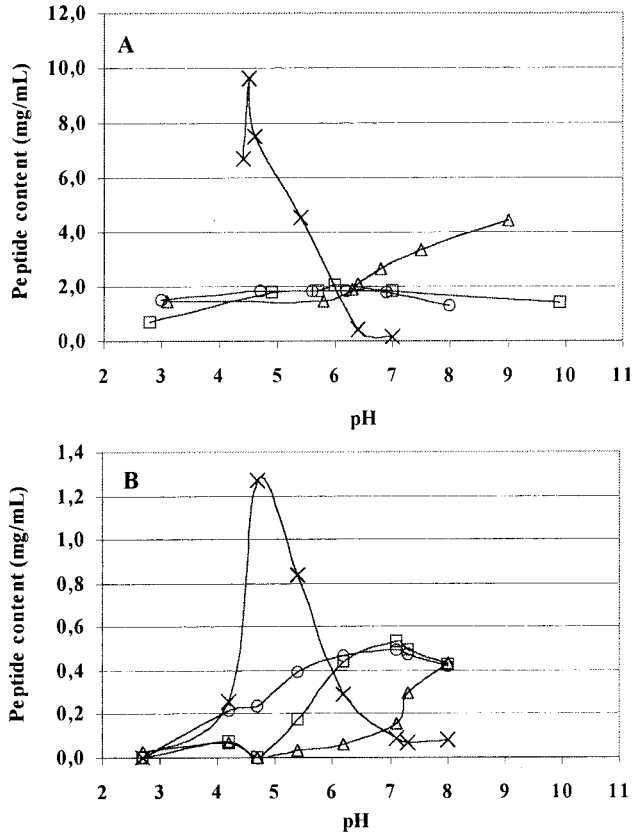

Figure 6. Peptide content (determined by RP-HPLC) of fractions obtained by IEF with autofocusing of solutions of pure peptides versus fraction pH: (A) solutions of single peptides; (B) solution of all four peptides. Peptides are β-LG 15–20 (○), β-LG 71–75 (□), β-LG 76–82 (△), and β-LG 84–91 (×).

residues (net charge −1), focused around pH 4. Peptide β-LG 76−82, which is positively charged (due to a single lysine residue) and has a p*I* of 8.4, was found in fractions from pH 7 to 9.

Both neutral peptides show a broader distribution (**Figure 5**). Peptide β-LG 71−75 has a net charge of 0 but bears a positive (Lys) and a negative (Glu) charge and has a p*I* of 6.0. It is found mostly in fractions of pH 7−9 (at pH > p*I*) but is also relatively abundant (∼0.2 mg/mL) in fractions at pH 4 and 11. Peptide β-LG 15−20 is distributed evenly from pH 4 and 10 even though its p*I* is 5.49. This neutral peptide bears no charge other than the terminal charges, and its poor focusing ability is therefore likely due to low electrophoretic mobility.

To evaluate the effect of the surrounding peptides on the electrophoretic mobility of the four pure synthetic peptides, they were focused both individually and in mixture (**Figure 6**). Running them individually confirmed that the neutral peptides (β-LG 15−20 and β-LG 71−75) did not focus, as opposed to charged peptides β-LG 76−82 and β-LG 84−91 (**Figure 6A**). As is observable in **Figure 5**, the charged peptides focused around their respective p*I* values. When the four purified peptides were focused in a mixture (**Figure 6B**), similar results were obtained for the charged peptides and the overall pattern was consistent with that observable in the tryptic hydrolysate (**Figure 5**), suggesting that surrounding peptides had little impact on their electrophoretic mobility. Both neutral peptides were partial to fractions at pH 6−8 (pH > p*I*), echoing their behavior in tryptic hydrolysate. Their complete failure to focus when analyzed individually and their broad distribution in the mixture indicate low electrophoretic mobility.

The results shown in **Figures 5** and **6** thus suggest that IEF may be useful for separating charged peptides. However, peptide−peptide interaction was not observed with this technique for the four peptides under study. It is possible that the electric field applied during IEF decreases or overrides electrostatic interactions between charged peptides, which suggests that applying an electrical field during the nanofiltration of peptide solutions may improve the separation of charged peptides.

Conclusion. Our work demonstrates that peptides from a tryptic hydrolysate of β-LG can be autofocused by IEF if enough electrophoretically mobile peptides are obtained. Most of the peptides had p*I* values between 3 and 5, at which an abundance of peptides was observed. These peptides are of low molecular mass and contain disulfide bonds. The IEF technique did not allow identification of peptide−peptide interactions. This is perhaps due to an effect of electrical field decreasing electrostatic interactions. There is a need to identify other techniques that may allow the characterization of peptide−peptide interactions in a complex solution such as enzymatic hydrolysates.

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