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Impact of Feed Solution Flow Rate on Peptide Fractionation by Electrodialysis with Ultrafiltration Membrane

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Recently, processes combining an electrical field as a driving force to porous membranes have been developed for the separation of protein or peptide mixtures to obtain more purified products with higher functionality or nutritional value. The objective of this work was to evaluate the influence of the flow rate on the productivity and selectivity as well as on the electrodialytic parameters of electrodialysis with an ultrafiltration membrane (EDUF) during the fractionation of peptides from a β-lactoglobulin tryptic hydrolysate. It appeared that the feed solution flow rate had no impact on the yield of the process but induced changes in the selectivity. In fact, increases in the flow rate decreased the migration of the peptides with limited electrophoretic mobility.

KEYWORDS: Electrodialysis; ultrafiltration; flow rate; peptide; fractionation

INTRODUCTION

Milk proteins are considered to be the most important source of food-derived bioactive peptides, and an increasing number of bioactive sequences have been identified both in fermented dairy products and protein hydrolysates (*1*). These sequences are encrypted within the primary structure of the native proteins, and enzymatic hydrolysis is the most common way to release them (*2*). Once obtained, though, hydrolysates must often be fractionated to obtain peptides of interest in a more purified form, ensuring ingredients with higher functionality or nutritional value (*3*).

Recently, Poulin et al. (*4*) demonstrated the feasibility of fractionating peptides from a β-lactoglobulin (β-lg) tryptic hydrolysate using ultrafiltration membranes stacked in a conventional electrodialysis cell (EDUF). With a feed solution adjusted to pH 5.0, 10.75% of the ACE-inhibitory sequence β-lg 142–148 was recovered in the permeate solution. Other electromembrane processes, that is, processes using an electrical field as a driving force combined with porous membranes, have also been developed recently to achieve biomolecule separation. Those works showed the influence of the different process parameters on both the product yield and purity of the fraction collected. The flow rate of the solutions is a parameter taking extremely different values according to the apparatus used. In the specially designed electromembrane filtration module, Bargeman et al. (*5*) used flow rates of 2866 mL/min; in the electrophoretic membrane contactor, Galier and Roux-de Balmann (*6–8*) report values of around 1.5 mL/min; in the membrane electrophoresis module, Van Nunen (*9*) used flow rates of 550−1100 mL/min; and in flow electrophoresis, these values oscillate between 0.3 and 2.4 mL/min (*10*). Moreover, the authors reported different impacts of this parameter, sometimes influencing the process (*9*) and sometimes not (*10*).

Thus, the aim of the present work was to evaluate for the first time, in an electrodialysis with ultrafiltration membrane system, the impact of the flow rate of the hydrolysate solution and, hence, the residence time per pass in the electrical field on the yield and the selectivity of the process. The objectives were (1) to evaluate if the variations in the flow rate of the feed solution could influence the quantity of peptides recovered in the permeate as well as its composition and (2) to determine the impact of different flow rates on the evolution over time of the electrodialytic parameters (electrical conductivity and pH).

MATERIALS AND METHODS

Materials. NaCl and KCl were obtained from Laboratoire MAT (Québec, QC, Canada). HCl and NaOH 1.0 M solutions were obtained from Fisher Scientific (Montréal, QC, Canada). A bovine β-lg tryptic hydrolysate, prepared as previously described by Groleau et al. (*11*), was obtained from Advitech Solutions (Québec, QC, Canada). The chromatographic profile of the hydrolysate solution adjusted to pH 5.0 is presented in **Figure 1**, in which 23 peptides are identified. The hydrolysate was characterized by Lapointe et al. (*12*), and according to this work, 10 peptides have a p*I* under 5.0, 4 have a p*I* over 8.0, and the rest have p*I* values between 5.0 and 8.0.

Electrodialysis Cell and Configuration. The electrodialysis cell was a MicroFlow type cell (effective area of 10 cm^2) (ElectroCell AB, Täby, Sweden) with one Neosepta CMX-S cationic membrane (Tokuyama Soda Ltd., Tokyo, Japan), one Neosepta AMX-SB anionic membrane (Tokuyama Soda Ltd.), and one cellulose ester ultrafiltration membrane with a molecular weight cutoff (MWCO) of 20 kDa (Spectrum Laboratories Inc., Rancho Dominguez, CA). The cell

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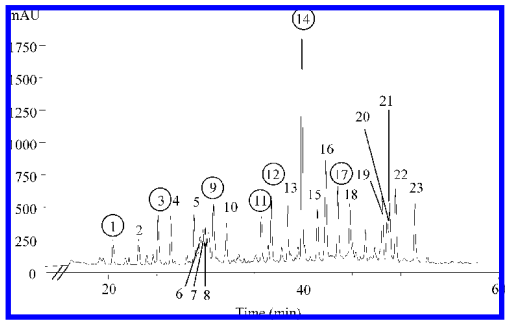

Figure 1. Chromatographic profile (RP-HPLC) of the 0.25% (w/v) β-lg tryptic hydrolysate solution adjusted to pH 5.0. Migrating peptides are circled.

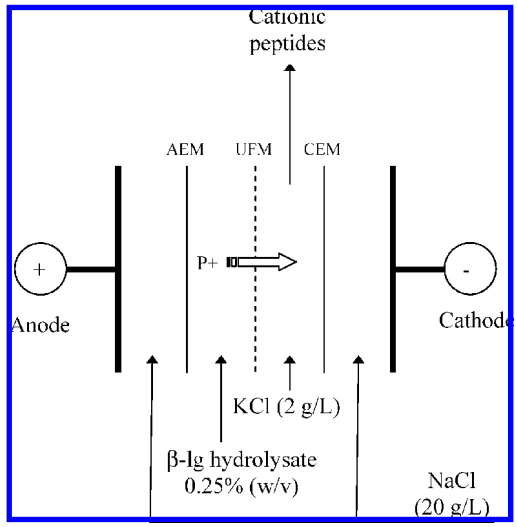

Figure 2. Configuration of the electrodialysis module for the separation of cationic peptides from a β-lg hydrolysate. AEM, anion-exchange membrane; UFM, ultrafiltration membrane; CEM, cation-exchange membrane; P^+, cationic peptides.

configuration, presented in **Figure 2**, and the ED system were the same as the ones used in our previous work (*4*). The system was not equipped to maintain the temperature of the solutions constant.

Protocol. The experiment was conducted to demonstrate the impact of the feed solution flow rate on the separation of peptides from a β-lg hydrolysate. Electroseparation was performed in batch process using a constant voltage difference of 5.5 V. The duration of the treatment was 180 min. The electrode, permeate, and feed compartments contained a 20 g/L NaCl aqueous solution (250 mL), a 2 g/L KCl aqueous solution (250 mL), and a 0.25% (w/v) β-lg tryptic hydrolysate aqueous solution (200 mL), respectively (**Figure 2**). The permeate solution flow rate was 200 mL/min, whereas the flow rate of the electrode solution was 300 mL/min, and the feed solution flow rate was fixed to 100, 150, 200, or 250 mL/min. The hydrolysate solution pH was adjusted to 5.0 before each run with 0.1 M HCl and was not controlled afterward. Three replicates of each condition were performed. Samples of 1.5 mL of the hydrolysate and KCl solutions were taken before voltage was applied and every 30 min during the treatment. The peptide content of the permeate samples was determined with a Micro-BCA protein assay kit. The molecular profiles of the hydrolysate and permeate solutions samples were determined by RP-HPLC. Conductivity and pH of the permeate and feed solutions were recorded throughout the process. After each treatment, the UF membrane electrical conductivity and thickness were measured to evaluate its potential fouling.

Analysis Methods. *pH.* A pH-meter model SP20 (Thermo Orion, West Chester, PA) was used with a VWR Symphony epoxy gel combination pH electrode (Montréal, Canada).

Conductivity. A YSI conductivity meter, model 3100, was used with a YSI immersion probe model 3252, cell constant $K = 1\ cm^{-1}$ (Yellow Springs Instrument Co., Yellow Springs, OH).

Total Peptide Determination. The peptide concentration was determined using Micro-BCA protein assay reagents (Pierce, Rockford, IL). Because the amino acid composition, sequence, and molecular mass of β-lg hydrolysate and albumin proteins such as BSA vary considerably (*13*), BSA may not be an appropriate reference standard for protein measurements of hydrolysate samples (*14*). To overcome these limitations of the BCA protein assay, the same β-lg hydrolysate as in the EDUF runs was used as standard instead of BSA. Assays were conducted on microplates by mixing 125 μL of the sample with 125 μL of the working reagent and incubation at 37 °C for 2 h. The microplate was then cooled to room temperature for 15 min, and the absorbance was read at 562 nm on a microplate reader. Concentration was determined with a standard curve in a range of 2−40 μg/mL.

Molecular Profiles. The peptide composition of the permeate and hydrolysate solutions was determined by RP-HPLC according to the method of Groleau et al. (*15*). The system used was an Agilent 1100 series (Agilent Technologies, Palo Alto, CA) consisting of an autosampler (G1329A), two pumps (bin G1323A), and a diode array detector (DAD G1315A). Peptides were analyzed with a Luna 5 μm C_{18} column (2 i.d. × 250 mm, Phenomenex, Torrance, CA). Solvent A, TFA 0.11% (v/v) in water, and solvent B, acetonitrile/water/TFA (90%/10%/0.1% v/v), were used for elution at 0.2 mL/min. A linear gradient of solvent B, from 1 to 50% in 60 min, was used. The detection wavelength was 214 nm.

Membrane Electrical Conductivity. The membrane electrical conductivity was measured according to the method of Bazinet and Araya-Farias (*16*), using a specially designed clip from the Laboratoire des Matériaux Échangeurs d'Ions (Créteil, France).

Membrane Thickness. The thickness of the membrane was measured using a Mitutoyo Corp. digimatic indicator (model ID-110 ME) and a digimatic mini-processor (model DP-1HS), especially devised for plastic film thickness measurement. The resolution was of 1 μm and the range of 10 mm.

Statistical Analysis. The peptide migration data as a function of time for the four flow rates were subjected to analysis of variance ($P < 0.05$) using SAS software version 9.1 (SAS Institute Inc.).

RESULTS AND DISCUSSION

Electrodialysis Parameters: Electrical Conductivity and pH. *Electrical Conductivity.* Conductivity of the permeate and feed solutions was monitored throughout the process, as presented in the panels **a** and **b**, respectively, of **Figure 3**. The conductivity of the KCl solution decreased in a linear fashion whatever the flow rate of the feed solution, starting from an average value of 3081 ± 32 μS/cm and ending at values between 2663 and 2581 μS/cm, with an average of 2626 ± 35 μS/cm. This change in conductivity corresponds to a 15% demineralization. For the hydrolysate solution, the conductivity decreased when the flow rate was fixed at 150, 200, or 250 mL/min, starting from an average value of 302 ± 9 μS/cm and ending at an average value of 244 ± 22 μS/cm. This decrease corresponds to a 19% demineralization. However, when the feed solution flow rate was 100 mL/min, the conductivity did not follow any trend, starting at 320 ± 12 μS/cm and ending at 363 ± 217 μS/cm. Those results were in accordance with the configuration of the ED cell: K^+ and Cl^- ions migrated from the KCl solution to the electrolyte and feed solutions, respectively, resulting in a decrease of the conductivity. As electroneutrality has to be maintained in the compartment (*17*), Cl^- or intrinsic anions from the hydrolysate solution migrated to the electrolyte solution. The difference observed for the 100 mL/min condition would be explained by the counterpressure caused by a flow rate twice as important for the KCl solution (200 mL/min). This pressure gradient would force both the K^+ and Cl^- ions to cross the UF membrane from the KCl to the feed solution, creating an imbalance in their electric-driven migration and in the hydrolysate solution conductivity. Conse-

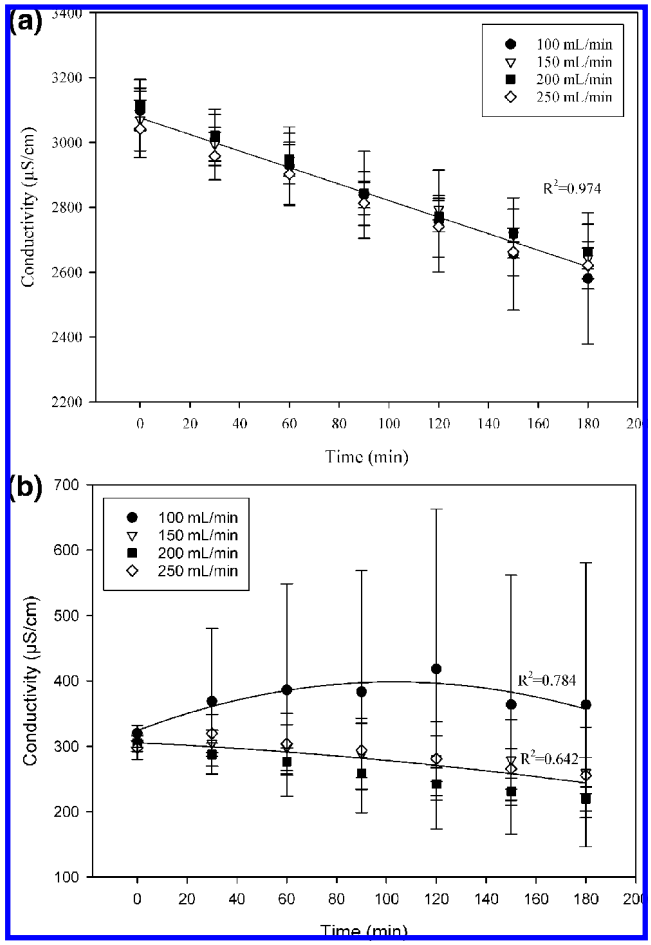

Figure 3. Evolution of the electrical conductivity of (**a**) the KCl solution and (**b**) the hydrolysate solution as a function of time during electrodialysis with ultrafiltration membrane for four different flow rates.

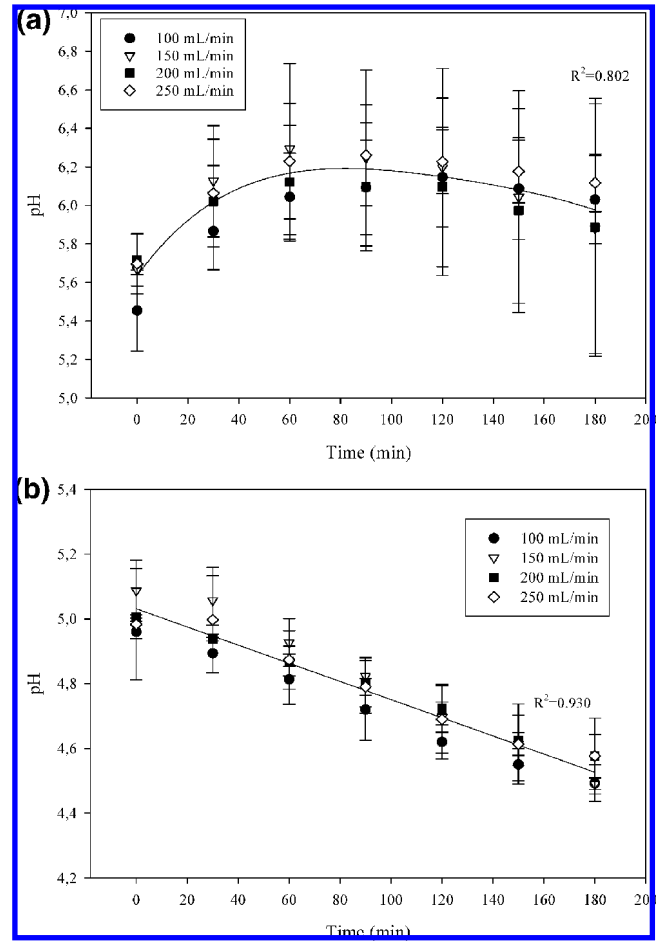

Figure 4. Evolution of the pH of (**a**) the KCl solution and (**b**) the hydrolysate solution as a function of time during electrodialysis with ultrafiltration membrane for four different flow rates.

quently, the behavior of the conductivity in that condition could not be directly related to the configuration of the cell and to the normal migration of the ionic species under the influence of the electrical field.

pH. As presented in **Figure 4b**, the pH of the hydrolysate solution changed in the same way whether the flow rate was adjusted to 100, 150, 200, or 250 mL/min. It started at an average value of 5.01 ± 0.06 and decreased until it reached an average value of 4.54 ± 0.05. This variation can be explained by water dissociation at the anion-exchange membrane, resulting in H_3O^+ production at the interface with the hydrolysate solution and leading to its low acidification. Water splitting occurred as the conductivity of the feed solution is low; not many intrinsic ions can carry the current, and the limiting current density is quickly reached. The pH of the permeate solution, presented in **Figure 4a**, slightly changed over the 180 min of EDUF for the four flow rates tested, increasing from an average value of 5.64 ± 0.12 to an average value of 5.98 ± 0.11. A decrease in the pH could have been expected as positively charged peptides migrate in this solution and could release protons and acidify the solution. The opposite variation observed here would be associated either with the dissociation of water molecules at the cation-exchange membrane and production of OH^- ions in the KCl solution or by leakage of hydroxyde ions through the cation-exchange membrane from the electrode compartment. Similar results were obtained in our previous works (*4*).

Peptide Migration. *Total Peptide Determination.* Peptide concentration in the KCl solution evolved in the same way whatever the flow rate of the feed solution was, as presented in

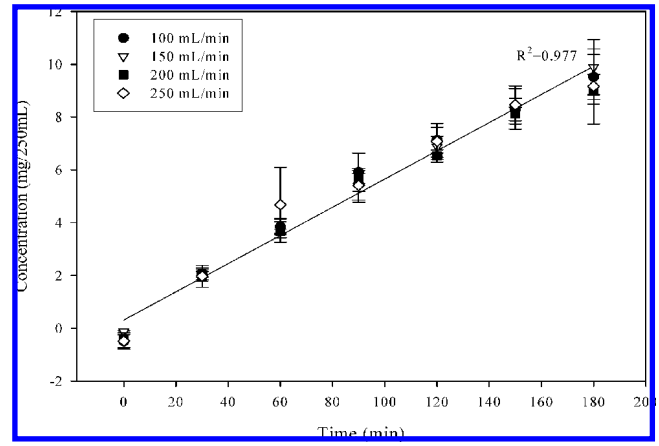

Figure 5. Evolution of the peptide concentration in the KCl compartment as a function of time during electrodialysis with ultrafiltration membrane of a 0.25% β-lg hydrolysate solution adjusted to pH 5.0 for four different flow rates.

Figure 5. The peptide concentration in the KCl solution or permeate increased in a linear fashion as the process was performed, from an initial average value of 0 mg/mL to a final one of 0.038 mg/mL. With a specially designed large-scale membrane-electrophoresis module, Van Nunen (*9*) observed that changes in the flow rate of the feed solution (from 10 to 50 L/h) hardly influenced the amino acid (methionine) flux through a 350 Da MWCO membrane, differences being rather explained by a modification of the limiting current value. Along the same

Table 1. Evolution of the Relative Migration (Percent) in the KCl Solution of the Seven Peptides of Interest Associated with Their Peak Number for Four Different Flow Rates; Statistics Are Given under Each Column Referring to the Significance of Difference between the Four Treatments

flow rate (mL/min)	time (min)	peak 1 424.5 Da p*I* 9.76	peak 3 572.4 Da p*I* 6.00	peak 9 672.4 Da p*I* 5.84	peak 11 932.5 Da p*I* 8.75	peak 12 836.5 Da p*I* 9.80	peak 14 695.3 Da p*I* 5.49	peak 17 774.5 Da p*I* 8.41
100	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	60	6.23 ± 0.41	0.99 ± 0.23	2.88 ± 0.85	1.60 ± 0.18	2.63 ± 0.95	0.32 ± 0.04	2.11 ± 0.25
	120	12.59 ± 0.93	1.92 ± 0.28	5.92 ± 2.10	2.87 ± 0.80	5.60 ± 1.83	0.73 ± 0.08	4.38 ± 0.60
	180	18.83 ± 1.02	3.27 ± 1.07	8.61 ± 2.09	4.48 ± 0.64	8.34 ± 3.03	1.21 ± 0.22	6.57 ± 0.89
150	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	60	6.18 ± 1.32	0.85 ± 0.17	2.45 ± 0.82	1.70 ± 0.06	3.54 ± 0.39	0.34 ± 0.08	1.89 ± 0.31
	120	12.58 ± 2.09	1.78 ± 0.25	5.55 ± 1.79	3.08 ± 0.28	7.16 ± 1.00	0.74 ± 0.15	4.09 ± 0.58
	180	19.98 ± 2.88	3.27 ± 0.56	7.83 ± 2.51	4.96 ± 0.12	11.33 ± 1.13	1.18 ± 0.21	6.27 ± 1.00
200	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	60	6.05 ± 0.92	0.87 ± 0.16	2.63 ± 0.35	1.59 ± 0.13	3.46 ± 0.33	0.34 ± 0.06	1.86 ± 0.18
	120	12.45 ± 1.12	1.64 ± 0.30	5.43 ± 1.82	3.01 ± 0.23	6.93 ± 1.22	0.74 ± 0.10	3.86 ± 0.51
	180	18.52 ± 1.45	2.80 ± 0.56	7.72 ± 2.97	4.69 ± 0.55	10.79 ± 1.70	1.18 ± 0.10	5.94 ± 0.54
250	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	60	7.07 ± 1.67	1.58 ± 0.44	2.60 ± 0.73	1.41 ± 0.46	3.40 ± 0.80	0.64 ± 0.39	2.45 ± 0.61
	120	13.81 ± 4.77	1.95 ± 0.73	4.44 ± 1.21	2.34 ± 0.83	7.39 ± 2.42	0.87 ± 0.22	4.53 ± 1.37
	180	18.93 ± 5.84	3.20 ± 0.95	6.60 ± 0.87	3.30 ± 0.60	9.16 ± 3.70	1.31 ± 0.29	6.72 ± 1.62
P		<0.9186	<0.8882	<0.0621	<0.0217	<0.5352	<0.9665	<0.8971

line, Liu et al. (*10*) reported that the flow rate at which the sample was fed in a multichannel flow electrophoresis did not have an effect on the protein migration speed. The transport rate, estimated to be 3.1 g/m^2·h, is 2.5 times lower than in previous work done by Poulin et al. (*4*), who calculated a transport rate of 7.8 g/m^2·h in the same process conditions but for a hydrolysate concentration 4 times higher (1% instead of 0.25%). It would seem that the increase in the transport rate was not correlated linearly with the feed solution concentration, a result that is opposite to what was obtained by Bargeman et al. (*18, 19*). In fact, in the selective isolation of cationic peptides from α_{s2}-casein by electromembrane filtration, they showed that a 2.5-fold increase in the hydrolysate concentration resulted in a 2.1-fold increase of the target peptide. The difference in our case could be explained by the fact that bringing the solution to an acidic pH induced the precipitation of some peptides and the formation of aggregates (*15*). Therefore, at higher hydrolysate concentrations, more aggregates are formed, causing more membrane fouling and interfering with the peptide migration.

Molecular Profiles. The results for the individual peptide migration presented in **Table 1** confirmed the trend that migration evolves linearly with time, as revealed by total peptide determination. Moreover, it appeared from these results that independent of the flow rate value, only 7 of 23 peptides identified in the raw hydrolysate had migrated in the KCl solution. The same peptides were identified by Poulin et al. (*4*) in the cathode side permeate for an ED process with two UF membranes and a β-lg hydrolysate solution adjusted to pH 5.0. Although the same peptides were found regardless of the feed flow rate, their relative migration was influenced by this parameter. In fact, when the hydrolysate solution circulated at 250 mL/min, statistical analysis revealed that peptide 11 ($P < 0.0217$) and, to a larger extent, peptide 9 ($P < 0.0621$) had a lower transmission than at the three other lower flow rates. Migration of the former was 18% lower and that of the latter, 29% lower. This behavior could be explained by relating molecular characteristics of these peptides, namely, their mass and isoelectric point, to electrophoretic mobility. Adamson and Reynolds (*20*) stated that electrophoretic mobility of a peptide is proportional to its actual charge and inversely proportional to its molecular size, related to its mass. Peak 9, associated with

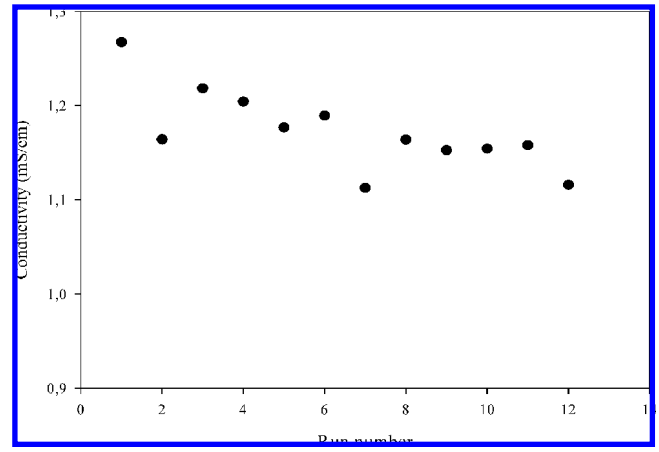

Figure 6. Evolution of the electrical resistance of the ultrafiltration membrane through the 12 electrodialysis runs set of experiments.

sequence β-lg 9–14, has a p*I* of 5.84, giving it a weak positive charge at pH 5.0, reducing its mobility. Peak 11 (sequence β-lg 1–8), even though its p*I* is 8.75, has a lower electrophoretic mobility, having the highest molecular weight (932.5 g/mol) of the migrating peptides. Hence, a faster flow rate resulted in a shorter residence time per pass in the electrical field and a lower transport rate of those two peptides with limited electrophoretic mobility. Van Nunen (*9*) also showed that increasing the flow rate could increase the separation factor and, therefore, the selectivity, when separating lipase from lyzozyme. The other five peptides had average migrations of 18.83, 3.14, 9.91, 4.36, and 6.38% for peaks 1, 3, 12, 14, and 17, respectively, after 180 min of EDUF. The peptide associated with peak 12 was identified as the ACE-inhibitory sequence β-lg 142–148 and is also the one with the second highest transmission. The total migration of those five peptides was superior to what was obtained by Poulin et al. (*4*) after 180 min of processing; they worked at a higher voltage difference of 6.0 V in a MP type ED cell with two UF membranes. For instance, an increase of 14% was observed for peptide 12, β-lg 142–148. This discrepancy is explained by the difference in the electrical field strength of the two systems, caused by the cell thickness. In the present paper, the distance between the electrodes was 2.0 cm, whereas

in the previous works of Poulin et al. (*4*) with two UF membranes it was 4.5 cm. This means that in the first case the electrical field was 2.75 V/cm, whereas in the second case it was 1.33 V/cm, a decrease of 48%. This showed that the electrical field strength plays an important role in the output of the process, as already stated in the literature (*5, 6*).

Membrane Fouling Evaluation. Membrane fouling is generally associated with an increase of the membrane electrical resistance or a decrease of its electrical conductivity. The UF membrane electrical conductivity was measured after each treatment to evaluate its fouling and is presented in **Figure 6**. The initial value was 1.267 mS/cm and then varied between 1.117 and 1.190 mS/cm between the 12 electroseparation runs. The difference between the 1st and the 12th utilizations of the UF membrane was then only 6%. As this was not considered to be a significant decrease in the electrical conductivity, the fouling of the membrane was also said to be nonsignificant. Membrane thickness was also measured, but, again, no significant changes were observed (0.301 ± 0.003 mm). This confirmed the previous results of Poulin et al. (*4*), who demonstrated that EDUF could minimize membrane fouling.

Conclusion. The aim of this work was to study the impact of modifying the flow rate of the feed solution on the yield, selectivity, and electrodialytic parameters of an EDUF process for the fractionation of peptides from a β-lg tryptic hydrolysate. The results showed that increasing or decreasing the flow rate of the hydrolysate solution had no effect on the total peptide migration in the permeate solution. However, at the highest value tested, the selectivity of the process was influenced: migration of peptides 9 and 11 was lower at 250 mL/min. This behavior could be explained by the limited electrophorectic mobility of those peptides. For the ED parameters, no major differences were observed with the changes in the flow rate. At all tested flow rates, the pH of the feed solution decreased and the pH of the permeate solution slightly increased, whereas the conductivity had a different behavior only for the hydrolysate solution when the flow rate was 100 mL/min.

Future experiments with faster flow rates should be done to determine if selectivity can be improved more. Other experiments on the electrical field strength and the feed solution concentration will also be pursued.

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