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# Use of an electrodialytic reactor for the simultaneous β-lactoglobulin enzymatic hydrolysis and fractionation of generated bioactive peptides

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ABSTRACT

The enzymatic hydrolysis of β-lactoglobulin and the fractionation of peptides were performed in one step in an electrodialysis cell with ultrafiltration membranes stacked. After 240 min of treatment, 15 anionic and 4 cationic peptides were detected in the anionic and cationic peptide recovery compartments. Amongst these 15 anionic peptides, 2 hypocholesterolemic, 3 antihypertensive and 1 antibacterial peptides were recovered and concentrated with migration rates ranging from 5.5% and 81.7%. Amongst the 4 cationic peptides, the peptide sequence ALPMHIR, identified as lactokinin and known to exert an important antihypertensive effect, was recovered with an estimated 66% migration rate. To our knowledge, it was the first attempt to perform hydrolysis under an electric field and to simultaneously separate anionic and cationic peptides produced.



## 1. Introduction

Enzymatic hydrolysis of various animal and vegetable proteins can generate a large variety of bioactive peptides that exhibit several biological activities such as cardiovascular protection (Erdmann, Cheung, & Schröder, 2008), antibacterial (Pompilio et al., 2011), antihypertensive (Hernández-Ledesma, del Mar Contreras, & Recio, 2011; Ruiz-Giménez et al., 2012) and antioxidant capabilities (Hipkiss, 1998; Husson et al., 2011). For the food industry, these bioactive peptides have the potential to produce high value-added nutraceutical products.

Bioactive peptides derived from milk protein hydrolysis have been extensively studied in recent years (Clare & Swaisgood, 2000; Hannu, 2009; Pihlanto, 2011; Pritchard, Phillips, & Kailasapathy, 2010). β-Lactoglobulin (β-LG) is one of the major milk proteins used for the generation of bioactive peptides by microbial fermentation or enzymatic hydrolysis (Korhonen & Pihlanto, 2006; Silva & Malcata 2005). β-LG is a water soluble protein which normally exists as a dimer with a subunit molecular weight of 18,350 Da. Each monomer contains 162 amino acids, with one free cysteine and two disulphide bridges (Brownlow et al., 1997). Its hydrolysis is generally catalysed by the serine protease trypsin (EC 3.4.21.4). Indeed, the tryptic hydrolysis of β-LG allowed the recovery of various peptidic fragments including bioactive peptides (Cheison, Lai, Leeb, & Kulozik, 2011; Cheison, Schmitt, Leeb, Letzel, & Kulozik, 2010; Gauthier & Pouliot, 2003). For instance, the tryptic fragment f142–148 from bovine β-lactoglobulin has been identified as an efficient inhibitor of angiotensin-I-converting enzyme (Mullally, Meisel, & FitzGerald, 1997). In several studies, other tryptic fragments have been identified as hypocholesterolemic (Nagaoka et al., 2001) or antibacterial peptides (Pellegrini, Dettling, Thomas, & Hunziker, 2001). Nowadays the experimental conditions to produce peptides by enzymatic hydrolysis of β-LG proteins are well-understood (Cheison et al., 2011; Otte, Zakora, Qvist, Olsen, & Barkholt, 1997), however the main technological limitation is the isolation of individual peptides generated from the hydrolysate which may contain a complex mixture of peptides of similar size (Poulin, Amiot, & Bazinet, 2006). Although original strategies of chromatography (Kuo-Chiang, 2010) or pressure-driven filtration processes (Butylina, Luque, & Nyström, 2006) have been developed, several drawbacks limit their applications at an industrial scale (Bazinet & Firdaous, 2009). Moreover, concerning pressure-driven filtration technologies, membrane fouling may occur limiting and changing the selectivity during the separation (Chabeaud, Vandanjon, Bourseau, Jaouen, & Guérard, 2009). Recently, electrodialysis with ultrafiltration membranes (EDUF) has been successfully used for the

*Abbreviations:* $A^-_{RC}$, anionic peptide recovery compartment; AEM, anion exchange membrane; β-LG, β-lactoglobulin; BCA, bicinchoninic acid; BSA, bovine serum albumin; $C^+_{RC}$, cationic peptide recovery compartment; CA, cellulose acetate; CEM, cation exchange membrane; EDUF, electrodialysis with ultrafiltration membrane; IEM, ion-exchange membrane; MS, mass spectrometry; MW, molecular weight; MWCO, molecular weight cut-off; TFA, trifluoroacetic acid; UFM, ultrafiltration membrane.

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separation of peptides from various hydrolysates of bovine haemoglobin (Vanhoute et al., 2011), alfalfa white protein (Firdaous et al., 2009; Firdaous et al., 2010), snow crab by-products (Doyen, Beaulieu, Saucier, Pouliot, & Bazinet, 2011a) and β-LG (Poulin et al., 2006). In all cases, the hydrolysates were prepared prior to the separation by EDUF treatment.

The purpose of this present work is to investigate the feasibility of combining enzymatic hydrolysis of β-LG with the separation of released peptides in a single-step EDUF process. The electromigration rates of the released peptides which migrated through ultrafiltration membranes (UFMs) were studied. Finally, the sequences and biological activities of the main recovered peptides were discussed based on data available in the literature.

## 2. Materials and methods

### 2.1. Materials

Bovine pancreatic trypsin, with an activity ⩾7500 BAEE units/mg and a protein purity of 90–100%, was purchased from Sigma–Aldrich (St. Louis, MO, USA). One BAEE unit will produce a $\Delta A_{253}$ of 0.001 per min at pH 7.6 at 25 °C using α-*N*-benzoyl-L-arginine ethyl ester as substrate. HCl and NaOH solutions were obtained from Fisher Scientific (Montreal, QC, Canada). NaCl and KCl were purchased from ACP Inc. (Montréal, QC, Canada). The commercial bovine β-LG used in this study was graciously provided by Davisco Foods International Inc. (BioPURE β-LG, Le Sueur, Minnesota, USA).

### 2.2. EDUF cell configuration

The electrodialysis cell used for this experiment was an MP type cell (100 $cm^2$ of effective surface area) manufactured by ElectroCell Systems AB Company (Täby, Sweden). The configuration was the same as the one used by Doyen et al. (2011a) and Poulin et al. (2006) (Fig. 1). Briefly, EDUF configuration consisted of one Neosepta CMX-SB cationic membrane (Tokoyuma Soda Ltd, Tokyo Japon), one Neosepta AMX-SB anionic membrane (Tokoyuma Soda Ltd, Tokyo, Japan) and two cellulose acetate (CA) UFMs with a molecular weight cut-off (MWCO) of 50 kDa (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). The configuration was formed of 4 compartments. Two of them, containing 2 L of aqueous KCl (2 g/L) were used for recovery and concentration of peptides (anionic ($A^-_{RC}$) and cationic ($C^+_{RC}$) peptide recovery compartments): they were located near the anode and the cathode respectively. The third compartment contained the electrode NaCl rinsing solution (3 L, 20 g/L), and, the last compartment contained the feed solution (β-LG, 2 L) at 12.5 g/L. The solutions were circulated using four centrifugal pumps and the flow rates were controlled using flowmeters (Fig. 1). Permeate and feed solution flow rates were 1.5 L/min while the flow rate of the electrode solution was 2 L/min.

### 2.3. Protocols

#### 2.3.1. Hydrolysis of β-lactoglobulin and fractionation protocol

Trypsin-catalysed hydrolysis of β-LG was carried-out in the EDUF cell. For the hydrolysis reaction, 25 g of β-LG were suspended in 2 L of distilled water (1.25% β-LG protein, w/v) and the pH adjusted to 7.8. Enzymatic hydrolysis was started by the addition of 10 mL trypsin solution (125 mg/L of trypsin (w/v)) and a constant electric field of 14 V/cm was applied between EDUF electrodes. The hydrolysis and fractionation procedures were performed during 240 min in order to obtain a large electrodialytic migration and sufficient recovery of peptides. The system was started initially at room temperature and the temperature in the β-LG compartment was recorded every 30 min during the 240 min experiment. During hydrolysis, the reaction was maintained at pH 7.8, corresponding to the optimum pH value of trypsin, with 0.5 M NaOH using a Thermo Scientific Orion 9206BN probe (VWR International Inc., Mississauga, Ontario, Canada). Ten-millilitre samples of hydrolysate and solutions from each recovery compartment were collected before applying voltage and every 30 min during the treatment. In the hydrolysate samples, trypsin was immediately inactivated by adding 0.5 M HCl until pH 3.0 was reached. Following each 240 min of treatment, the final volumes of β-LG hydrolysate as well as $A^-_{RC}$ and $C^+_{RC}$ compartments were recovered and the enzyme contained in the hydrolysate compartment was inactivated as described previously. Afterwards, a clean-in-place procedure for the EDUF cell was performed to ensure the recovery of the UFMs and IEMs performances (Doyen et al., 2011a).

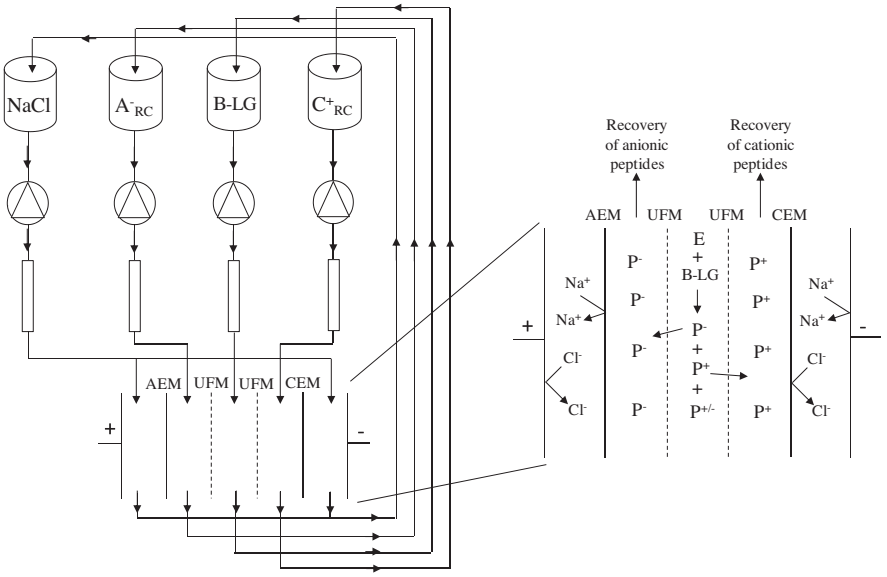


**Fig. 1.** EDUF cell configuration for the simultaneous hydrolysis of β-LG protein and recovery of anionic and cationic peptides. AEM: anion-exchange membrane, CEM: cation-exchange membrane, UFM: ultrafiltration membrane, $A^-_{RC}$: anionic peptide recovery compartment, $C^+_{RC}$: cationic peptide recovery compartment, β-LG: β-lactoglobulin protein, E: trypsin enzyme, $P^+$: cationic peptide, $P^-$: anionic peptide.

*2.3.2. Controls*

Three different controls were produced to assess the feasibility of the simultaneous hydrolysis and fractionation treatment in the EDUF cell. The first control procedure was prepared in a beaker in which a conventional β-LG hydrolysis was carried-out to compare the reaction kinetics for conventional peptide production with the EDUF technology. Thus, 2 L of a 1.25% β-LG solution, was hydrolysed by trypsin at 37 °C and pH 7.8 under continuous stirring (magnetic stirrer) for a 240 min time period. The pH was maintained constant using a Thermo Scientific Orion 9206BN probe (VWR International Inc., Mississauga, Ontario, Canada). Following hydrolysis, trypsin was inactivated at pH 3.0 by adding HCl and the hydrolysate was freeze-dried for further analysis. The second control consisted in performing EDUF treatment of β-LG protein without adding trypsin. This control was used to evaluate a potential migration of β-LG through ultrafiltration membranes. In the third control, trypsin alone was treated by EDUF; it allowed the evaluation of potential migration of trypsin through ultrafiltration membranes. The second and third controls were performed with the same electrodialytic and pH parameters used for hydrolysis of β-lactoglobulin and fractionation protocol (Section 2.3.1).

*2.4. Analyses*

*2.4.1. Solution conductivities*

Conductivities of $A^-_{RC}$, $C^+_{RC}$ and β-LG compartments were measured every 30 min during the 240 min of separation with a YSI conductivity meter (model 3100) equipped with a YSI immersion probe (model 3252, cell constant $K = 1\ cm^{-1}$, yellow Springs Instrument Co., Yellowsprings, OH, USA). The conductivities were measured in order to evaluate the mineralisation or demineralisation of the solutions during the process.

*2.4.2. Total peptide determination in liquid samples*

Total peptide migration in the $A^-_{RC}$, $C^+_{RC}$ was monitored at 30 min over a period of 240 min using the BCA protein assay (Pierce, Rockford, IL, USA). BCA protein assays were also performed to assess the migration of β-LG and trypsin during the EDUF treatments corresponding to the second and third controls. The microplate was then cooled to room temperature and the absorbance was read at 562 nm on a microplate reader (THERMOmax, Molecular devices, Sunnyvale, CA). Concentration was determined with a standard curve in a range of 25–2000 μg/mL of bovine serum albumin (BSA) (Doyen et al., 2011a).

*2.4.3. Total peptide content in freeze-dried samples*

The $A^-_{RC}$, $C^+_{RC}$ and hydrolysate final solutions were freeze-dried to concentrate solids and to allow the injection of uniform amount of peptide during the subsequent MS analysis (50 μg) for MS analysis. The initial protein concentration of β-LG and the final peptide concentration in the $A^-_{RC}$, $C^+_{RC}$ and β-LG hydrolysate fractions, recovered after 240 min of EDUF treatment, were obtained by the total nitrogen determination. Total nitrogen was analysed by combustion of 100 mg sample using a LECO-FP528 carbon and nitrogen analyser (LECO, St. Joseph, Michigan). Nitrogen concentrations in the samples were converted into peptide percentages by multiplying the nitrogen result by a conversion factor of 6.38, the value commonly used for milk proteins (Lilbaek, Fatum, Ipsen, & Sorensen, 2007).

*2.4.4. Protein and peptide profiles*

The peptide composition of the $A^-_{RC}$, $C^+_{RC}$ and hydrolysate solutions was determined by RP-HPLC according to the method of Firdaous et al. (2009) adapted to the specific conditions of the β-LG and peptides generated during hydrolysis. The system used was an Agilent 1100 series. Peptides were analysed with a Luna 5 μm $C_{18}$ column (2 i.d. × 250 mm, Phenomenex, Torrance, CA, USA). Solvent A, TFA 0.11% (v/v) in water, and solvent B, acetonitrile/water/trifluoroacetic acid (TFA) (90%/10%/0.1% v/v), were used for elution at a flow rate of 0.2 mL/min. A linear gradient of solvent B, from 3% to 97% in 85 min, was used. The detection wavelength was 214 nm which is typically used to monitor peptide bonds (Firdaous et al., 2009; Stachelhaus, Mootz, Bergendahl, & Marahiel, 1998).

*2.4.5. Protein and peptide molecular mass determination*

The molecular weight (MW) of proteins and peptides recovered in the $A^-_{RC}$, $C^+_{RC}$ and hydrolysate compartments was determined by liquid chromatography–mass spectrometry (LC-MS) analyses according to Firdaous et al. (2009). The system used was an Agilent 1100 series (Agilent Technologies, Palo Alto, CA, USA). Peptides were analysed with the same method and the same column used for RP-HPLC analyses. To reduce the effect of TFA, mass-spectrometry was performed after infusing (50 μL/min) a mixture of 50% propionic acid and 50% isopropanol to the existing flow before the MS interface. Signals were recorded in positive mode using a 90-V fragmentation with a scan range of 300–3000 $m/z$ (Firdaous et al., 2009).

*2.5. Peptide migration rates and bioactivities*

Peptide electromigration rates, expressed in percent, were determined from the HPLC results. The area under the curve of each peak detected on HPLC chromatograms for one permeate solution was compared to the area of the corresponding peak on the HPLC chromatogram of β-LG hydrolysate obtained in the beaker after 240 min of trypsin hydrolysis. Indeed, since no treatment of electroseparation was applied to this sample, it is assumed in this experiment that the hydrolysis would be the same as in the beaker. Consequently, β-LG control performed in beaker which reflected the total amount of peptide obtained and recovered after β-LG hydrolysate was used as a reference for peptide migration rate estimation. The following equation was used to calculate the peptide migration rate:

$$\mathrm{Tr}\ (\%) = (A_{\mathrm{permeate}}/A_{\mathrm{hydrolysate}}) \times 100$$

where Tr represents peptide migration in percent, $A_{\mathrm{permeate}}$ the area of a given peak in the permeate and $A_{\mathrm{hydrolysate}}$ the area of the corresponding peak in the feed solution at $t = 240$ min.

*2.6. Statistical analyses*

The changes in conductivity and peptide concentration as a function of time were subjected to a repeated measures analysis of variance ($P < 0.05$ as probability level for acceptance) using SAS software version 4.2 (SAS Institute Inc., Cary, NC, USA). Test of comparison of two means ($t$-test) ($P < 0.05$ as probability level for acceptance) were used to compare final values of conductivity and peptide concentration between $A^-_{CR}$ and $C^+_{CR}$.

## 3. Results and discussion

*3.1. Evolution of heat in the β-LG compartment*

Fig. 2 shows that temperature in the β-LG compartment increased rapidly from 25.2 °C (room temperature) to 35.7 °C after the first 60 min of protein digestion and fractionation by EDUF. Afterwards, until the end of fractionation, the temperature remained stable at a value of 35.7 °C. The increase in temperature of the β-lactoglobulin compartment was explained by the well known Joule effect (heating effect) (Bazinet & Firdaous, 2009),

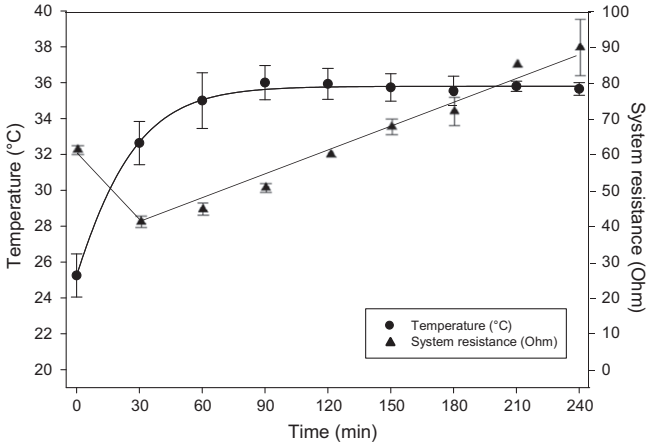


**Fig. 2.** Evolution of heat in the β-lactoglobulin (β-LG) compartment and apparent system resistance during 240 min of EDUF process.

due to the increase in apparent system resistance (Fig. 2) (except between $t = 0$ min and $t = 30$ min due to the EDUF equilibrium in terms of hydrodynamic parameters and ion concentration), and mainly pump operations. As β-LG compartment temperature was naturally maintained at 36 °C after 90 min of EDUF process (Fig. 2), the use of an automatic system for the control of temperature at 37 °C (optimum operating temperature of trypsin) was not necessary.

### 3.2. Solution conductivities

Fig. 3 represents the changes in solution conductivity in the $A^-_{RC}$, $C^+_{RC}$ and β-LG compartments during the 240 min of EDUF treatment. The duration of the process significantly affected the conductivity into both $A^-_{RC}$ and $C^+_{RC}$ compartments ($P < 0.0001$). The conductivities of $A^-_{RC}$ and $C^+_{RC}$ as a function of time decreased continuously ($P \geqslant 0.860$) to reach values of 0.78 ± 0.1 and 0.79 ± 0.01 mS/cm which corresponded to similar demineralisation rates of 75.4% and 71.0%, respectively. Unlike $A^-_{RC}$ and $C^+_{RC}$, the conductivity of β-LG solution increased dramatically during the first 60 min of fractionation with a mineralisation rate close to 300%. Afterwards, the compartment mineralisation increased more slowly during the last 180 min. These tendencies were observed and explained by Doyen et al. (2011a) and Poulin et al. (2006) for the same EDUF configuration. The decrease of $A^-_{RC}$ and $C^+_{RC}$ conductivities was explained by the fact that $K^+$ and $Cl^-$ ions from $A^-_{RC}$ and $C^+_{RC}$ solutions migrated in the rinsing electrode solution towards the cathode and the anode, respectively. With respect to the β-LG compartment, the mineralisation was explained by the presence of $K^+$ ions migrating from the $A^-_{RC}$ and $Cl^-$ ions migrating from the cationic compartment $C^+_{RC}$.

### 3.3. Total peptide migration in anionic and cationic compartments

Process duration ($P < 0.0001$) and the type of recovery compartment ($A^-_{RC}$ or $C^+_{RC}$, $P = 0.0048$) significantly affected the progress of peptide migration. Indeed, peptide migration in the $A^-_{RC}$ increased continuously during the first 210 min of the process and, afterwards seemed to reach a plateau (Fig. 4). However, in $C^+_{RC}$, peptide migration increased for the first 90 min of treatment and then reached a plateau (Fig. 4). Consequently, after 240 min of EDUF treatment, and taking into account the volume change in the $A^-_{RC}$ (0.2 L/h) due to electro-osmosis phenomenon, it was observed that peptide migration in the $A^-_{RC}$ was approximately one and a half time larger than in $C^+_{RC}$ with values of 191.1 ± 20.7 μg/mL and 138.7 ± 48.8 μg/mL, respectively which corresponded to peptide migration rates of 9.8 ± 0.1 and 6.9 ± 2.4 g/m$^2$ h. This difference is explained by the fact that peptides derived from tryptic hydrolysis of β-LG are mostly anionic at pH 7.8 (Groleau, Gauthier, & Pouliot, 2003; Groleau, Morin, Gauthier, & Pouliot, 2003) and consequently were recovered more rapidly in the anionic compartment.

The peptide migration rate was different compared to the results obtained by Poulin et al. (2006). These authors observed that the migration rate was higher in the $C^+_{RC}$ than in the $A^-_{RC}$ with values of 1.7 and 3.4 g/m$^2$ h at pH 7.0, respectively. This opposite trend could be explained by the difference of electrodialytic parameters used. Although the cell configuration was the same for both studies, Poulin et al. (2006) applied a voltage difference of 6.0 V and used an ultrafiltration membrane with a 20 kDa MWCO while a voltage difference of 60.0 V and a membrane pore size of 50 kDa were used in the present study. Consequently, the migration of highly charged peptides with high MW, mostly negatively charged peptides, with the parameters used in this work was increased and may possibly explain the difference in migration rates observed.

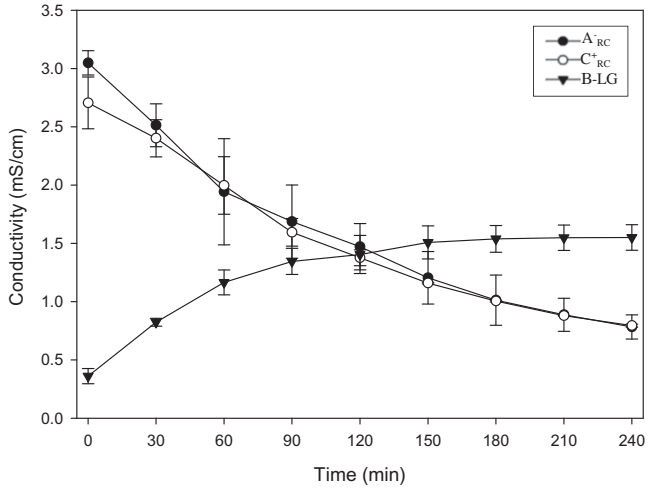


**Fig. 3.** Changes in conductivity of $A^-_{CR}$, $C^+_{CR}$ and β-lactoglobulin (β-LG) solutions at pH 7.8 during 240 min of EDUF process. $A^-_{RC}$: anionic peptide recovery compartment, $C^+_{RC}$: cationic peptide recovery compartment.

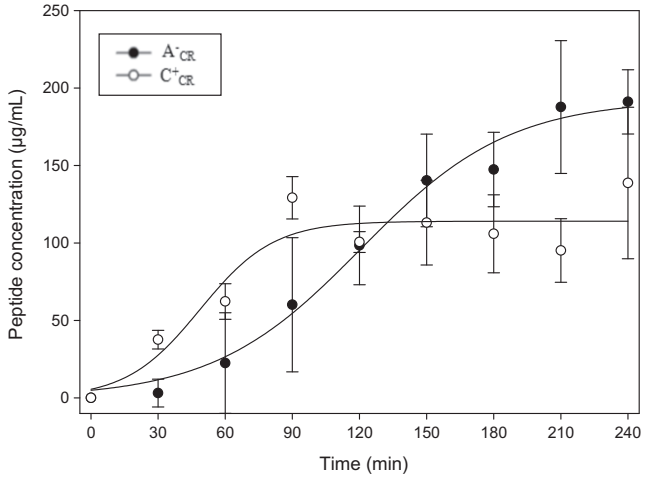


**Fig. 4.** Changes in peptide concentration in the $A^-_{CR}$ and $C^+_{CR}$ during 240 min of EDUF treatments at pH 7.8. $A^-_{RC}$: anionic peptide recovery compartment, $C^+_{RC}$: cationic peptide recovery compartment.

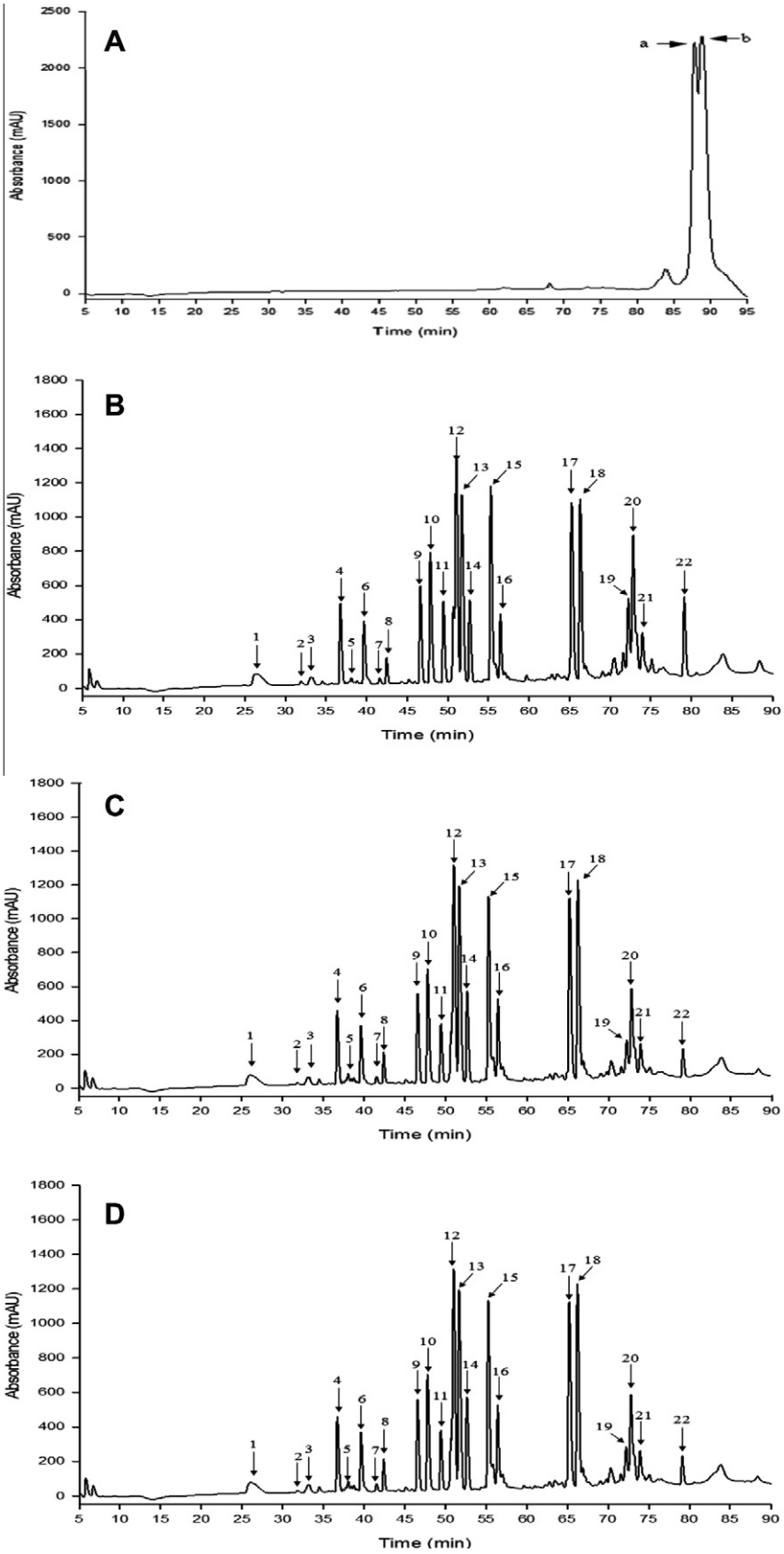


**Fig. 5.** Chromatograms of initial β-LG protein (A) and β-LG hydrolysates after 60 (B), 120 (C) and 240 min (D) of EDUF treatment.

### 3.4. Evaluation of β-LG protein and trypsin potential migration during EDUF treatment

Results obtained by the BCA assay after 240 min of EDUF treatment performed without trypsin, demonstrated the absence of β-LG (negatively charged at pH 7.8 (Bromley, Krebs, & Donald, 2005; Das & Kinsella, 1989; Sawyer & Kontopidis, 2000)) in the $A^-_{RC}$. Bovine trypsin was not detected in the $C^+_{CR}$ although it is positively charged at 7.8 since its pI value is about 10.1–10.5 (Tietze, 1953; Walsh, Perlmann, & Lorand, 1970). These results confirmed that the EDUF treatment restricted the migration of β-LG and trypsin during the EDUF treatment even though their molecular weights were lower than the 50 kDa MWCO value associated with the membrane. Consequently, during the entire EDUF treatment no loss of β-LG protein as well as trypsin occurred in the feed compartment which meant that hydrolysis reaction was not affected.

### 3.5. Peptide profiles

#### 3.5.1. Peptide profiles and molecular weights of β-LG hydrolysate

Fig. 5A represents chromatograms of initial β-LG. The two major peaks *a* and *b* had retention times of 87.76 and 88.76 min, respectively and were composed of proteins with molecular weights of 18,362.86 and 18,276.73 Da determined by MS. Peak *a* was identified as β-LG A and peak *b* as β-LG B, the two major variants of β-LG protein in milk (Brownlow et al., 1997). Moreover, the MW difference calculated to be 86.13 Da corresponds to the MW difference between β-LG A and B due to the substitution of Asp and Val in β-LG A by Gly and Ala in β-LG B (MW difference of 86.45 Da) (Brignon & Dumas, 1973). The peak detected at 83.9 min, probably corresponded to another milk protein, such as α-lactalbumin or bovine serum albumin (BSA) since the purity of the β-LG protein preparation was 92% according to the manufacturer.

Fig. 5B–D represents typical chromatograms of β-LG hydrolysate obtained after 60, 120 and 240 min of EDUF treatment, respectively. β-LG was totally hydrolysed after 60 min (Fig. 5B) since no trace of native protein was detected by MS analysis. Regardless of the treatment duration, HPLC analysis showed that hydrolysates were composed of 22 major peaks which were quantified on the basis of peak heights. However, as the hydrolysis progressed, it appeared that the relative proportions of the peptides varied. Indeed, heights of peaks numbered 4, 6, 9, 10, 15, 19, 20, 21 and 22 decreased between 60 and 240 min (Fig. 5B–D) while heights of peaks numbered 5, 7, 8, 13, 16, 17 and 18, increased. Finally, heights of peaks numbered 1, 2, 3, 12 and 14 remained stable regardless of the duration of treatment. The decrease in peptide quantity (peak numbers 4, 6, 9, 10, 15, 19, 20, 21 and 22) between 120 and 240 min could be explained by the fact that some peptides migrated in $A^-_{RC}$ and $C^+_{CR}$ during electroseparation. Another hypothesis to explain some peptide peak height decreases could be their hydrolysis by trypsin which resulted in an increase in lower MW generated peptides (peaks numbered 5, 7, 8, 13, 16, 17 and 18).

The characterisation of the 22 peaks containing peptides was performed in terms of MW, potential sequence, location on β-LG sequence, net charge and isoelectric point (pI) (Table 1). Potential

**Table 1**
Characterization of peptides obtained after 240 min of EDUF treatment.

| Peak# | $R_t$ (min)[a] | Obs MW[b] | Calc MW[c] | Potential sequence | Location[d] | Net charge[e] | *p*I |
|---|---|---|---|---|---|---|---|
| 1 | 26.08 | 572.37 | 573.36 | IIAEK | f71–75 | – | 6.0 |
| | | 346.22 | nd | nd | nd | nd | nd |
| 2 | 31.82 | 1191.91 | 1192.54 | WENGECAQKK | f61–70 | – | 6.1 |
| 3 | 33.25 | 700.64 | 701.45 | KIIAEK | f70–75 | + | 8.6 |
| | | 1063.65 | 1064.44 | WENGECAQK | f61–69 | – | 4.5 |
| | | 1249.87 | 1249.62 | FKDLGEEHFK[g] | f35–44 | – | 5.4 |
| 4 | 36.72 | 915.51 | 916.47 | IDALNENK | f84–91 | – | 4.4 |
| **5** | 38.26 | 2440.30 | 2441.1 | VHKECCHGDLLECADDRADLAK[g] | f524–544 | – | 4.9 |
| 6 | 39.62 | 672.38 | 673.38 | GLDIQK | f9–14 | – | 5.9 |
| 7 | 41.53 | 948.75 | nd | nd[f] | nd | nd | nd |
| 8 | 42.41 | 1244.87 | 1245.58 | TPEVDDEALEK | f125–135 | – | 3.8 |
| 9 | 46.58 | 932.43 | 933.44 | LIVTQTMK | f1–8 | + | 8.8 |
| 10 | 47.81 | 836.71 | 837.48 | ALPMHIR | f142–148 | + | 9.8 |
| | | 652.47 | nd | nd | nd | nd | nd |
| 11 | 49.43 | 1193.04 | 1193.68 | VLVLDTDYKK | f92–101 | – | 5.9 |
| | | | 1192.54 | WENGECAQKK | f61–70 | – | 6.1 |
| 12 | 51.01 | 902.68 | 903.57 | TKIPAVFK | f76–83 | + | 10.0 |
| | | 673.43 | 673.39 | GLDIQK | f9–14 | – | 5.9 |
| 13 | 51.58 | 1635.40 | 1635.77 | TPEVDDEALEKFDK | f125–138 | – | 4.0 |
| 14 | 52.65 | 1064.80 | 1064.45 | WENGECAQK | f61–69 | – | 4.5 |
| 15 | 55.27 | 2848.71 | 2847.59 | TKIPAVFKIDALNENKVLVL DTDYK | f76–100 | – | 5.8 |
| | | 2906.74 | 2906.3 | ATEEQLKTVMENFVAFVDKCCAADDK[g] | f562–587 | – | 4.3 |
| 16 | 56.44 | 2778.53 | nd | nd | nd | nd | nd |
| | | 2720.52 | nd | nd | nd | nd | nd |
| 17 | 66.21 | 2929.90 | nd | nd | nd | nd | nd |
| 18 | 66.23 | 2313.27 | 2313.25 | VYVEELKPTPEGDLEILLQK | f41–60 | – | 4.3 |
| 19 | 72.22 | 5144.39 | nd | nd | nd | nd | nd |
| | | 3487.58 | 3486.78 | VYVEELKPTPEGDLEILLQKWENGECAQKK | f41–70 | – | 4.6 |
| 20 | 72.81 | 5202.42 | 5203.3 | TCVADESHAGCEKSLHTLFGDELCKVASLRETYGDMADCCEKQEPER[g] | f76–122 | – | 4.6 |
| 21 | 73.91 | 5074.23 | 5073.4 | HPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETMREK[g] | f169–211 | – | 5.7 |
| 22 | 79.13 | 2707.64 | 2707.37 | VAGTWYSLAMAASDISLLDAQSAPLR | f15–40 | – | 4.2 |

[a] Retention time (Figs. 5 and 6).
[b] Observed MW.
[c] Calculated MW.
[d] Peptide location on the mature β-lactoglobulin protein.
[e] Calculated at pH 7.8.
[f] Not determined: not corresponding to a peptide sequence derived from the enzymatic hydrolysis of β-LG by trypsin.
[g] Peptides generated by hydrolysis of bovine serum albumin.

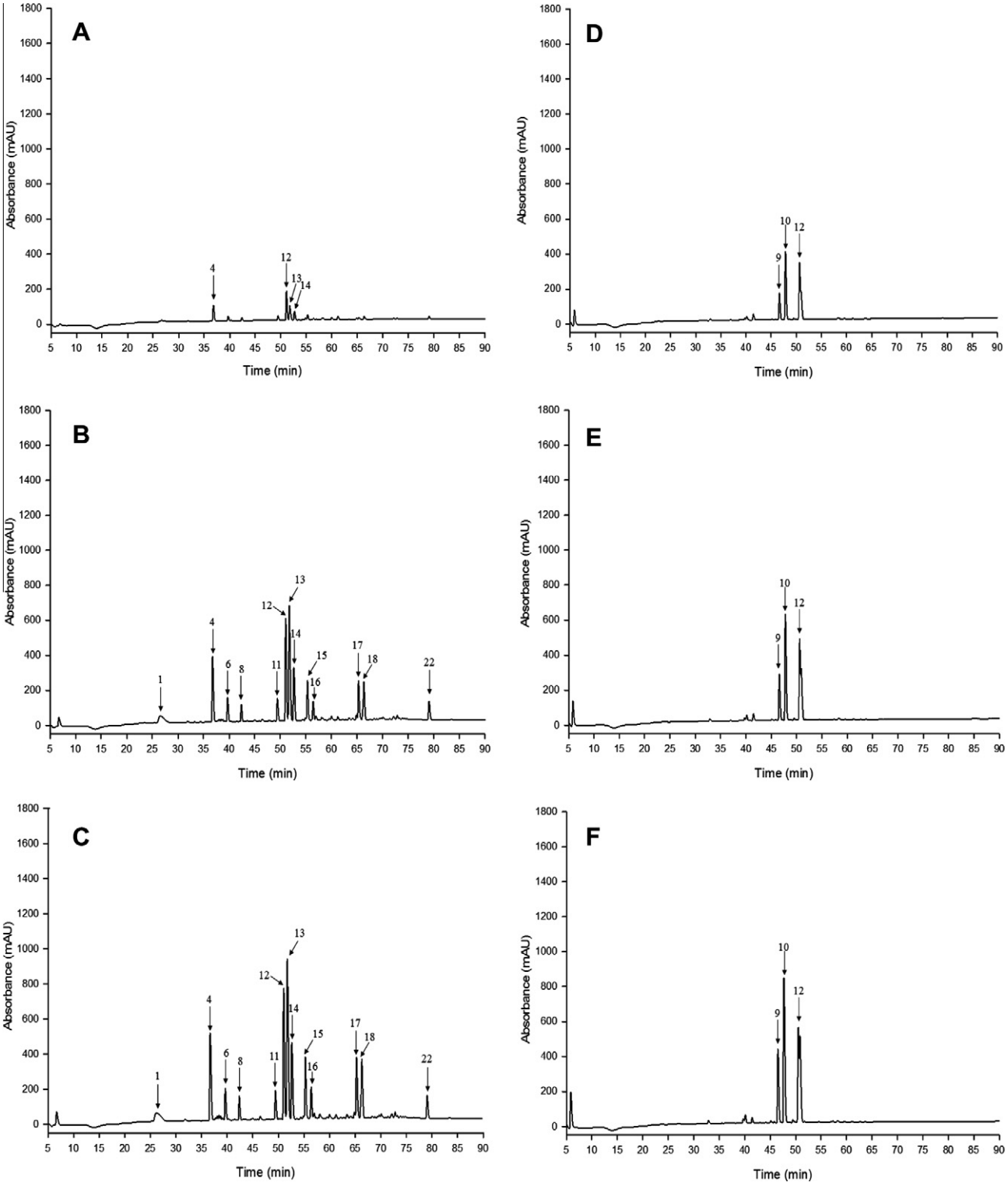


**Fig. 6.** Chromatograms of $A^-_{RC}$ and $C^+_{RC}$ solutions after respectively, 60 (A, D), 120 (B, E) and 240 min (C, F) of EDUF treatment, respectively. $A^-_{RC}$: anionic peptide recovery compartment, $C^+_{RC}$: cationic peptide recovery compartment.

sequences, peptide locations, net charge and pI were obtained by using tools available on the ExPASy Bioinformatics Resource Portal (Swiss Institute of Bioinformatics) and sequence of bovin (*Bos taurus*) β-LG (UniProtKD/TrEMBL # P02754). Amongst 31 peptides obtained from β-LG digestion, 19 peptides with MW ranging from 572.37 to 2848.71 Da were derived directly from tryptic hydrolysis of β-LG protein. Moreover, the potential sequences of 6 peptides appeared to be generated from tryptic hydrolysis of BSA (UniProtKD/TrEMBL # P02769) (Table 1). Six peptide sequences marked as "nd" in Table 1 were apparently not generated from tryptic digestion of bovine α-lactalbumin (UniProtKD/TrEMBL # P00711). Consequently, it was proposed that the 6 peptides were

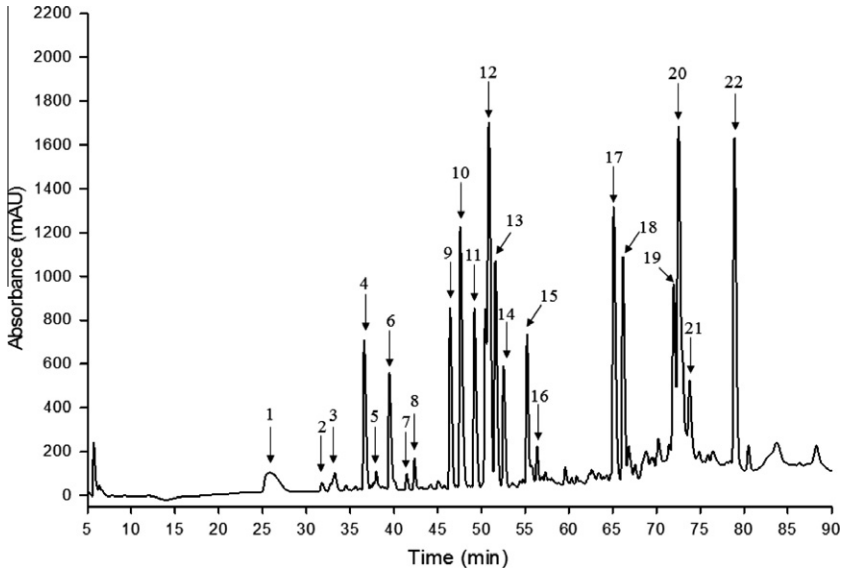


**Fig. 7.** Chromatograms of β-LG hydrolysate control obtained after 240 min of hydrolysis.

**Table 2**
Peptide migration rates in $A^-_{RC}$ and $C^+_{RC}$ compartments of recovery.

| Peak# | Migration rate (%) in $A^-_{RC}$ | | | Migration rate (%) in $C^+_{RC}$ | | |
|---|---|---|---|---|---|---|
| | *t* = 60 min | *t* = 120 min | *t* = 240 min | *t* = 60 min | *t* = 120 min | *t* = 240 min |
| 1 | | 31.2 | 42.4 | | | |
| 4 | 10.2 | 47.1 | 66.0 | | | |
| 6 | | 19.1 | 26.4 | | | |
| 8 | | 55.2 | 77.7 | | | |
| 9 | | | | 14.6 | 26.3 | 43.9 |
| 10 | | | | 24.1 | 39.3 | 57.1 |
| 11 | | 13.0 | 17.0 | | | |
| 12 | 4.3 | 17.3 | 22.7 | 14.4 | 23.1 | 32.3 |
| 13 | 6.9 | 57.5 | 81.7 | | | |
| 14 | | 45.9 | 68.8 | | | |
| 15 | | 27.8 | 43.3 | | | |
| 16 | | 44.7 | 76.7 | | | |
| 17 | | 12.4 | 19.6 | | | |
| 18 | | 19.4 | 31.1 | | | |
| 22 | | 4.4 | 5.5 | | | |

$A^-_{RC}$: anionic peptide recovery compartment.
$C^+_{RC}$: cationic peptide recovery compartment.

obtained after peptide–peptide interactions as already observed by Doyen, Beaulieu, Saucier, Pouliot, and Bazinet (2011b) after EDUF treatment.

*3.5.2. Profiles and molecular weights of peptides recovered in anionic and cationic compartments of recovery*

Amongst the $A^-_{RC}$ 22 peaks detected in β-LG hydrolysate, only 4 peaks (4, 12, 13 and 14) were recovered after 60 min of peptide hydrolysis and migration (Fig. 6A). Table 1 lists the peptide sequences identified as IDALNENK (peak 4), GLDIQK (peak 12), TPEVDDEALEKFDK (peak 13) and WENGECAQK (peak 14) with MWs ranging from 673.39 Da to 1635.40 Da. After 120 min of EDUF treatment, peptide migration to the $A^-_{RC}$ increased drastically with the recovery of 9 additional peaks (1, 6, 8, 11, 15, 16, 17, 18 and 22) (Fig. 6B). At the end of the experiment (Fig. 6C), the same 13 peaks were also recovered; however, peak heights increased between 120 and 240 min which meant that peptide quantities increased too. Only 3 cationic peaks which corresponded to 4 peptides with MW ranging from 652.47 and 933.44 Da were recovered regardless of process duration (Fig. 6D–F). The potential sequences for 3 of the cationic peptides were LIVTQTMK (peak 9), ALPMHIR (peak 10) and TKIPAVFK (peak 12) originating from β-LG. The last peptide sequence (peak 10, 652.47 Da MW peptide) was not determined and could have come from the hydrolysis of unknown contaminating proteins observed in the β-LG chromatogram (Fig. 5A). As observed for $A^-_{RC}$, the heights of the 3 peaks increased continually as a function of digestion time. The difference in peptide migration between $A^-_{RC}$ and $C^+_{RC}$ confirmed the results obtained with the BCA protein assay which demonstrated that peptide migration in $A^-_{RC}$ was higher than in $C^+_{RC}$ since the majority of peptides obtained after trypsic β-LG hydrolysis are anionic at pH 7.8.

The anionic peptide sequences IDALNENK, GLDIQK, TPEVDDEALEKFDK and WENGECAQK were only detected after 60 min of frac-

**Table 3**
Recovery of bioactive peptides from β-LG hydrolysis.

| Peak# | Peptide location | Recovery compartment | Bioactivity | Reference |
|---|---|---|---|---|
| 1 | f71–75 | $A^-_{RC}$ | Hypocholesterolemic | Nagaoka et al. (2001) |
| 4 | f84–91 | $A^-_{RC}$ | Antihypertensive | Chobert et al. (2005) |
| 6, 12 | f9–14 | $A^-_{RC}$ | Hypocholesterolemic | Nagaoka et al. (2001) |
| | | | Antihypertensive | Pihlanto-Leppala et al. (1998) |
| 10 | f142–148 | $C^+_{RC}$ | Antihypertensive | Mullally et al. (1997) |
| 11 | f92–101 | $A^-_{RC}$ | Antibacterial | Pellegrini et al. (2001) |
| 22 | f15–40 | $A^-_{RC}$ | Antihypertensive | Chobert et al. (2005) |

$A^-_{RC}$: anionic peptide recovery compartment.
$C^+_{RC}$: cationic peptide recovery compartment.

tionation. The relatively rapid migration of the GLDIQK peptide is probably explained by its low MW (673.39 Da). The lower migration rates of the 3 other peptides could be explained by the fact that they had low pI values and consequently strong negative charges. Peaks 1, 6, 11 and 15 (after 120 min of treatment) had pI values of about 6 and therefore migrated slowly at pH 7.8 in the EDUF apparatus. However, in spite of very low pI values for peptides detected in peaks 18 and 22, their migration occurred only after 120 min of operation due to their high MWs (2313.25 and 2707.37 Da, respectively). Peak 8 was detected only after 120 min of EDUF treatment despite its low pI value (3.8) and relatively small size (1245.58 Da). This may be explained by the fact that this peptide (TPEVDDEALEK peak 8) is a fragment of peak 13 (TPEVDDEALEKFDK) which migrated after 60 min of EDUF treatment. Consequently, TPEVDDEALEK peptide migration occurred only after 120 min due to the time required for trypsin to hydrolyse peptide in peak 13. This possibly explains the hypothesis presented to explain the peptide peak height decreases.

Peaks 10 and 12 exhibited pI values of 9.8 and 10.0, respectively. At the pH used for the experiments, these highly positively charged peptides migrated from 60 min of EDUF separation towards $C^+_{RC}$ compartment. Furthermore, the peptide corresponding to peak 9 exhibits a pI of 8.8. Thus, this peptide was slightly positively charged at the pH used for experiments (7.8). For that, its rapid migration in $C^+_{RC}$ compartment could be rather eased by its low MW (932.43 Da).

### 3.6. Peptide migration rates and bioactivities

Chromatograms of the control β-LG hydrolysate obtained after 240 min of trypsin hydrolysis (Fig. 7) were used to estimate peptide migration rates $A^-_{RC}$ and $C^+_{CR}$. According to Table 2, peptide migration rate increased continuously as a function of time for both recovery compartments. As shown in Table 3, amongst the 13 peaks detected in the $A^-_{RC}$, peptide sequences IDALNENK (peak 4, f84–91) and VAGTWYSLAMAASDISLLDAQSAPLR (peak 22, f15–40) were previously identified as antihypertensive (Chobert et al., 2005). Peptide sequence GLDIQK (peak 6 and 12, f9–14) was identified as hypocholesterolemic by Nagaoka et al. (2001) and antihypertensive by Chobert et al. (2005) and Pihlanto-Leppala, Rokka, and Korhonen (1998). However, as this peptide was detected at $t$ = 39.62 (peak 6) and 51.01 min (peak 12), its migration rate could not to be accurately calculated. Peptide sequence IIAEK (f71–75) was identified as a hypocholesterolemic peptide by Nagaoka et al. (2001). However, as peak 1 was composed of two different peptides (Table 1), the hypocholesterolemic peptide migration rate could not be accurately calculated. Peak 11 (Table 1) with sequence VLVLDTDYKK (f92–101) was previously identified as an antimicrobial peptide (Pellegrini et al., 2001) with a migration rate of 17%.

Amongst the 4 cationic peptides recovered (Table 1), the sequence ALPMHIR corresponded to lactokinin (f142–148) known to exert antihypertensive activity (Mullally et al., 1997). According to MS analysis, peak 10 collected in the $C^+_{CR}$ was composed of 2 different peptides; lactokinin and a peptide with a MW of 652.47 Da. Consequently, lactokinin peptide migration rate could not be calculated precisely. However an estimate could be given, according to peptide abundance values obtained after MS analysis. Indeed, lactokinin abundance was higher than the 652.47 Da MW peptide since it value was 100% whereas abundance was calculated as 66% for peptide with a MW of 652.47 Da.

## 4. Conclusion

EDUF appeared to be an innovative process for the simultaneous production and fractionation of bioactive peptides from β-LG by enzymatic hydrolysis in one step. Indeed, EDUF allowed a specific separation and concentration of anionic and cationic peptides after 240 min of treatment. Moreover, some bioactive peptides generated from trypsic hydrolysis of β-LG protein were specifically recovered in the anionic and cationic compartments of recovery such as hypocholesterolemic, antihypertensive and antibacterial peptides with migration rate ranging from 5.5% to 66%.

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