Chapitre 3. Hypothèse de recherche et objectifs

3.1. Hypothèse de recherche

« La modification du ratio peptides hydrophiles/hydrophobes engendrée lors de l'hydrolyse trypsique de la β-LG, prétraitée sous HPH, affecte la transmission et la sélectivité membranaire lors du fractionnement par UF suite à la formation d'une couche de colmatage ».

Afin de vérifier cette hypothèse de recherche, plusieurs objectifs spécifiques, listés ci-dessous, ont été formulés.

3.2. Objectifs spécifiques

1. Caractériser l'ensemble des espèces peptidiques composant les hydrolysats trypsiques de β-LG générés après pré-pressurisation de la protéine.
2. Évaluer les performances du procédé d'UF lors du fractionnement des hydrolysats trypsiques de β-LG générés suite à un prétraitement par HPH de la protéine native.
3. Identifier les peptides bioactifs et leurs abondances relatives dans les perméats d'UF.
4. Caractériser les espèces peptidiques majoritairement responsables du colmatage membranaire.

Chapitre 4. Impact d'un prétraitement de la β-LG sous hautes pressions hydrostatiques sur les performances du système d'ultrafiltration lors du fractionnement de ses hydrolysats trypsiques et la récupération des peptides bioactifs

Résumé

Le traitement de la β-LG par hautes pressions hydrostatiques (HHP) a amélioré efficacement le processus d'hydrolyse enzymatique, cependant, une modification des profils peptidiques générés risque d'altérer les performances du procédé d'ultrafiltration (UF) utilisé pour le fractionnement des hydrolysats obtenus. Dans cet ordre d'idées, l'objectif principal de ce travail était d'évaluer l'impact de la filtration d'hydrolysats trypsique de la β-LG prétraitée à 0,1 (témoin), 400 et 600 MPa, sur les performances du procédé baromembranaire. En effet, l'analyse des profils peptidiques générés a montré qu'une augmentation de l'abondance relative de certains peptides, dont ceux bioactifs, était plus marquée après un traitement à 400 MPa, comparé aux autres conditions. Cependant, les flux de perméation des hydrolysats à 400 MPa étaient plus faibles comparés aux autres conditions, en mode recirculation totale ainsi qu'en mode concentration. Durant la désorption membranaire des peptides, l'ALPMHIR, un peptide antihypertensif, a été identifié comme l'espèce peptidique colmatante majeure. D'autres peptides chargés négativement ont été désorbés de la surface membranaire avec une abondance relative plus élevée à 400 MPa comparativement à 0.1 et 600 MPa. La détection de peptides chargés négativement à la surface d'une membrane ayant la même charge est expliquée par des interactions hydrophobes entre les espèces peptidiques et le matériau membranaire ainsi que la différence de taille entre ces espèces et le seuil de coupure. Ainsi, malgré l'efficacité de la technique de pressurisation de la β-LG sous HPH en termes de production en peptides bioactifs, elle est à l'origine d'un colmatage membranaire accrue. Par conséquent, il est nécessaire d'optimiser les conditions de filtration afin de minimiser l'altération des performances du système

Mots clés : Hautes pressions hydrostatiques, β-lactoglobuline, hydrolyse trypsique, colmatage des membranes de filtration, peptides bioactifs.

Abstract

High hydrostatic pressure-assisted enzymatic digestion of β-LG accelerated protein digestion but drastically modified the resulting peptide profile, which may affect the performance of ultrafiltration (UF) which is used to fractionate the hydrolysates. Consequently, the aim of this work was to evaluate the change in UF performance of tryptic hydrolysates generated after β-LG pre-pressurization at 0.1 (control), 400 and 600 MPa. Compared to the other conditions, high peptide relative abundance, including abundance of several bioactive peptides, was observed for the 400 MPa hydrolysate. During total recirculation and concentration mode, the permeate flux of the 400 MPa hydrolysate was lower than for other conditions. After peptide desorption from the membrane, ALPHMIR, an antihypertensive peptide, was identified as the main fouling material. For the 400 MPa condition, a larger number of peptides, mainly negatively charged and with higher relative abundance in the hydrolysate, were identified on the membrane surface compared to other conditions. While the repulsion phenomenon should occur between these peptides and the membrane material, both negatively charged, their detection at membrane surface is due to a size effect and hydrophobic interaction rather than a charge mechanism. Consequently, even if pressure treatment of β-LG improved the production of bioactive peptides, it is necessary to optimize hydrodynamic conditions or membrane material during filtration to minimize loss of UF performance.

KEYWORDS

High hydrostatic pressure, β-lactoglobulin, Tryptic hydrolysis, Ultrafiltration membrane fouling, Bioactive Peptides

4.1. Introduction

High hydrostatic pressure (HHP) is an emerging non-thermal process in which an applied isostatic pressure (typically 100 to 1000 MPa) is instantaneously and uniformly transmitted to liquid or solid-based products [93]. This process is of particular interest in the food industry since its application extends product shelf life with minimal effects on product nutritional values [94] ; [95]. However, HHP provokes protein unfolding since secondary, tertiary, and quaternary structures of proteins are particularly susceptible to modification under pressurization due to the disruption of noncovalent bonds (hydrogen, hydrophobic and ionic bonds) [96]. Nevertheless, the unfolding of proteins under HHP has generated useful and innovative research applications in the field of food sciences. Among these, HHP has been used in combination with various proteolytic enzymes to increase protein susceptibility to hydrolysis due to the exposure of new cleavage sites in the unfolded pressure-treated proteins [97] ; [13] ; [98] ; [36]. Consequently, the rate of reaction and peptide yield improved while reaction time and production costs decreased, depending on the pressurization parameters (level of pressure, time and temperature), the type and concentration of protein, and the physicochemical parameters (pH, ionic strength) of the solution [9] ; [67].

Several studies have compared the peptide patterns obtained from native and pressure-treated proteins. Indeed, Maynard *et al.* [1] found a lower concentration of intermediate hydrolysis peptides generated after tryptic hydrolysis of under pressurization while Knudsen *et al.* [11] indicated that a higher amount of hydrophobic and high molecular weight peptides was generated after tryptic hydrolysis of pressure-treated β-LG, specifically at the beginning of hydrolysis step [11]. Similar results were also obtained with chymotrypsin hydrolysis of in β-LG combination with HHP [11].

Pressure-driven membrane processes such as ultrafiltration (UF) have proven to be useful for generating bioactive peptide-enriched fractions with the desired molecular weight ranges from a wide range of protein hydrolysates [99] ; [100] ; [5] ; [101]. However, during filtration of protein hydrolysates, a decrease in permeate flux as a function of time is generally observed due to concentration polarization and fouling [102]. These drastic modifications of performance are dependent on the initial protein hydrolysate properties (peptide molecular weight and concentration, hydrophobicity and charge of peptide species, pH and ionic strength) as well as the membrane material (molecular weight cut-off, charge and hydrophobicity), and hydrodynamic conditions (transmembrane pressure and cross-flow velocity) used [75] ; [91] ; [88] ; [103]. The fractionation of tryptic whey protein hydrolysate by UF (1 and 5 kDa) showed that peptide transmission was mainly based on charge effects [104]. Using a tryptic casein hydrolysate, it was demonstrated that UF by polysulfone (PS) and polyethersulfone (PES) materials induced adsorption of casein peptides through hydrophobic interactions [103]. During UF using a 10 kDa molecular weight cut-off (MWCO), Nau *et al.* [105]

showed that transmission of peptides generated after tryptic hydrolysis of β-casein was mainly governed by electrostatic interactions between the peptides and membrane material, specifically for low molecular weight peptides. Bouhallab and Henry [106] observed a drastic rejection of peptides after chymotryptic hydrolysis of β-casein, induced by hydrophobic interactions between the β-casein peptide (f193-209) and membrane material [106].

Consequently, while enzymatic hydrolysis assisted by HHP applied to a hydrolysate improves peptide yield and hydrolysate bioactivities, the resulting changes to the peptide profile may negatively affect the UF process used to concentrate bioactive peptides. Thus, the objectives of this study were to: 1) evaluate the performance of the UF process during fractionation of tryptic β-LG hydrolysates obtained after pressurization of the native protein; 2) determine the impact of pressurization and UF on the recovery of bioactive peptides; and 3) characterize peptides potentially involved in membrane fouling.

4.2. Materials and methods

4.2.1 Materials

Bovine whey protein β-LG was kindly provided by Davisco Foods International Inc. (Le Sueur, MN, USA). Trypsin VI (lot No: PS9371), a pancreatic enzyme preparation was obtained from Neova (Abbotsford, BC, Canada). Sodium hydroxide (NaOH) was purchased from Fisher Scientific (Ottawa, Toronto, Canada) while hydrochloric acid (HCl) and nitric acid (HNO_3) were both provided by Anachemia Canada Co. (Montréal, QC, Canada). Calcium chloride dehydrate ($CaCl_2.2H_2O$) was purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA) and sodium dodecylbenzene sulfonate (SDoS) was obtained from Sigma-Aldrich Co. (St-Louis, MO, USA).

4.2.2 Preparation of β-LG solution and High Hydrostatic Pressure Treatment

Three liters of β-LG solution were prepared in triplicate for each condition (control, 400 and 600 MPa) at a concentration of 1.5% (w/v) in distilled water. Solutions were stirred for 60 min at room temperature and stored for 12 h at 4°C before pressurization and analysis. Before HHP treatment, β-LG solutions were transferred into flexible plastic bags. The solutions were pressurized at 400 and 600 MPa for 10 min at room temperature in a discontinuous hydrostatic pressurization unit (Hiperbaric 135, Hiperbaric, Burgos, Spain), as described previously [107]. Non-pressurized β-LG solutions (0.1 MPa) were used as controls.

4.2.3 Tryptic hydrolysis of β-lactoglobulin

Tryptic hydrolysis (E/S ratio of 1:100 (w/w)) of pressure-treated and control β-LG solutions were performed

at 37°C, pH 8 for 100 min. During hydrolysis, the pH was maintained by adding 5 N NaOH. After 100 min of enzymatic digestion, the hydrolysis was stopped by adding 10 M HCl to reduce the pH to 1.5 and inactivate the enzyme. Afterwards, pH of hydrolysates was increased to 8.0 by adding 5 N NaOH and stored at 4°C before ultrafiltration experiments.

4.2.4 Ultrafiltration System

Pretreated and control β-LG tryptic hydrolysates were ultrafiltered using a crossflow filtration unit (model SEPA-CF, Sterlitech, Kent, WA, USA) described previously [107]. The UF unit was equipped with a PES flat-sheet membrane (Synder Filtration, Vacaville, CA, USA) with MWCO of 1 kDa. The filtration surface area was 0.014 m^2. Before UF experiments, the membrane was conditioned [107] and the pure water flux was measured in triplicate. Membrane coupons with a similar pure water flux value (±10%) were kept for further UF experiments. A spacer and a shim with respective thicknesses of 7.1 x 10^{-5} and 4.1 x 10^{-5} m were used on the feed side to ensure a transitional flow regime as a function of an intermediate Reynolds number.

4.2.5 Operational Modes

4.2.5.1 Total Recycle Mode.

In total recirculation mode, both permeate and retentate were recycled into the feed tank to ensure a constant volume. From the three liters of pressure-treated and control β-LG tryptic hydrolysates solutions, one liter was recirculated to determine the optimal filtration parameters to use for the concentration mode. The permeate flux was measured at different transmembrane pressures (TMPs) from 170 to 570 kPa at a constant retentate recirculation rate of 89.6 x 10^{-3} $m.s^{-1}$. Each pressure level was held for 10 min until a stable permeate flux value was reached. As described by Leu *et al.* [107], critical (J_{crit}) and limiting (J_{lim}) fluxes were calculated at the end of UF for non-pressurized and pressure-treated β-LG tryptic hydrolysates at 400 and 600 MPa [107]. Total recycle mode was performed in triplicate, and for each replicate, a new membrane was used.

4.2.5.2 Concentration Mode

The remaining two liters of the β-LG tryptic hydrolysates were used for UF experiments in concentration mode, for which only retentate was recycled into the feed tank while permeate was removed and collected separately. The same recirculation flow rate and crossflow velocity values obtained for total recycle mode were used and kept constant during concentration mode. As a function of the J_{crit} obtained during total recycle mode, a constant TMP of 310 kPa was chosen during UF concentration experiments to avoid early

fouling on UF membranes. During the experiment, permeation fluxes of pressure-treated and control hydrolysates were measured every 10 min until reaching a 4X volume concentration factor. At the end of the experiment, permeates and retentates of control and pressure-treated β-LG hydrolysates were immediately freeze-dried and stored at -30°C for further analysis. Concentration mode was performed in triplicate and a new membrane was used for each replicate.

4.2.6 Membrane Fouling Evaluation

After control and pressure-treated hydrolysate concentrations by UF, a rinsing step with water was performed and the UF system was dismantled to recover UF membranes to evaluate peptide fouling. First, flat-sheet membranes were soaked in 100 mL of 0.01M HNO_3 for 24h at 4°C with stirring to desorb hydrophilic peptides [108]. The HNO_3 solution was recovered and stored at -20°C. The UF membranes recovered after desorption by HNO_3 were then soaked in 100 mL of 0.5% (w/v) SDoS solution for 24h at 4°C to recover hydrophobic peptides [75]. The SDoS desorption solutions were stored at -20°C. Both desorption solutions were finally freeze-dried and stored at -30°C until analysis.

4.2.7 Analysis

4.2.7.1 Molecular weight profiles of control and pressure-treated β-LG solutions

Aggregation profiles of control and pressure-treated β-LG solutions were determined after polyacrylamide gel electrophoresis (PAGE) under native and denaturing conditions using 4-20% acrylamide Bio-Rad Mini-Protean TGX precast gels (Bio-Rad Laboratories, Hercules, CA, USA). For native conditions, 10 µL of β-LG solution was diluted to 1:10 with distilled water. Twenty microliters of each diluted β-LG solution were mixed with the same volume of native sample buffer and 10 µL of each sample was loaded into the wells. The molecular weight marker used was Precision Plus Protein All Blue Standards (Bio-Rad Laboratories, Hercules, CA, USA). The running buffer was prepared by adding 0.1 L of Tris/Glycine (10x) buffer (Bio-Rad Laboratories, Hercules, CA, USA) to 0.7 L of distilled water and 0.2 L of methanol to fill the chamber. Migration was performed at 15 mA for approximately 30 min. The gel was stained for 60 min with a solution of 1% (w/v) Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA), diluted in a mixture of 10% acetic acid, 40% ethanol and 50% distilled water. Finally, the gel was destained overnight in a mixture of 10% methanol, 10% acetic acid and 80% deionized water. For denaturing conditions, the protocol was similar except that 50 µL of β-mercaptoethanol was added to 950 µL of the Laemmli sample buffer (2x) (Bio-Rad Laboratories, Hercules, CA, USA) and the mixture was heated at 100°C for 5 min. Moreover, the migration buffer consisted of a mixture of 0.1 L Tris/Glycine/Sodium dodecyl sulfate (SDS)

(10x) buffer (Bio-Rad Laboratories, Hercules, CA, USA) to 0.9 L of distilled water.

4.2.7.2 Characterization of peptide fractions

Control and β-LG hydrolysates generated after pressurization of β-LG protein, permeates recovered after 4X UF concentration, as well as freeze-dried desorption solutions (HNO_3 and SDoS), were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC). All samples were filtered through a 0.45 µm polyvinylidene fluoride (PVDF) membrane and stored at -20°C prior to RP-HPLC-mass spectrometry (MS) analysis. SDoS solutions were washed using a Pierce Detergent Removal Resin (Thermo Scientific, Rockford, IL, USA) to remove SDoS from the foulant peptide species to prevent any detergent-MS interference and to optimize MS signals. The samples were injected into an Agilent 1100 series system (Santa Clara, CA, USA) equipped with degasser, pump, auto-sampler, and UV detector (set at 214 nm). Peptide profiles were obtained using a Luna 5 µm C18 column (2 mm i.d. × 250 mm, Phenomenex, Torrance, CA, USA). Solvents A and B were 0.1% v/v trifluoroacetic acid (TFA) in water and acetonitrile/water/TFA (90/10/0.1% v/v), respectively, and were used for elution at a flow rate of 0.2 $mL.min^{-1}$ at 40°C. A linear gradient of solvent B was used from 3 to 55% in 85 min, and from 55 to 100% in 105 min. Peptide analysis was performed with LC/MSD ChemStation software Rev. A. 10.02 (Agilent, Santa Clara, CA, USA). Mass spectrometry analyses were performed to determine the molecular weight (MW) of peptides generated after enzymatic hydrolysis of β-LG as well as peptides recovered in permeates and desorption solutions. The MS system was the same as the one used previously [109]. From the MW obtained after MS analysis, potential peptide sequences, locations in the protein sequence, net charge and pI were obtained using tools available on the ExPASy Bioinformatics Resource Portal (Swiss Institute of Bioinformatics) and the bovine (*Bos taurus*) β-LG sequence (UniProtKD/TrEMBL # P02754).

4.2.8 Statistical analysis

All pressurization, UF experiments and analyses were carried out in triplicate. The statistical analysis of the data (permeate flux and peptide relative abundance) included an analysis of variance (ANOVA) for repeated measures with mixed procedure at a 95% confidence level ($p<0.05$). All statistical analyses were carried out with SAS 9.4 software (SAS Institute Inc., Cary, NC, USA).

4.3. RESULTS

4.3.1 Impact of high hydrostatic pressure on β-lactoglobulin

Native PAGE of control and pressure-treated β-LG is presented in Figure 15. Protein aggregation occurred under all pressurization conditions (0.1, 400 and 600 MPa), with three specific aggregate populations (X1-

X3). However, the intensity of bands corresponding to these protein aggregates (X1-X3) was higher at 600 MPa than at 0.1 and 400 MPa. Moreover, large aggregates were observed in the loading well (X4) only for the 600 MPa condition.

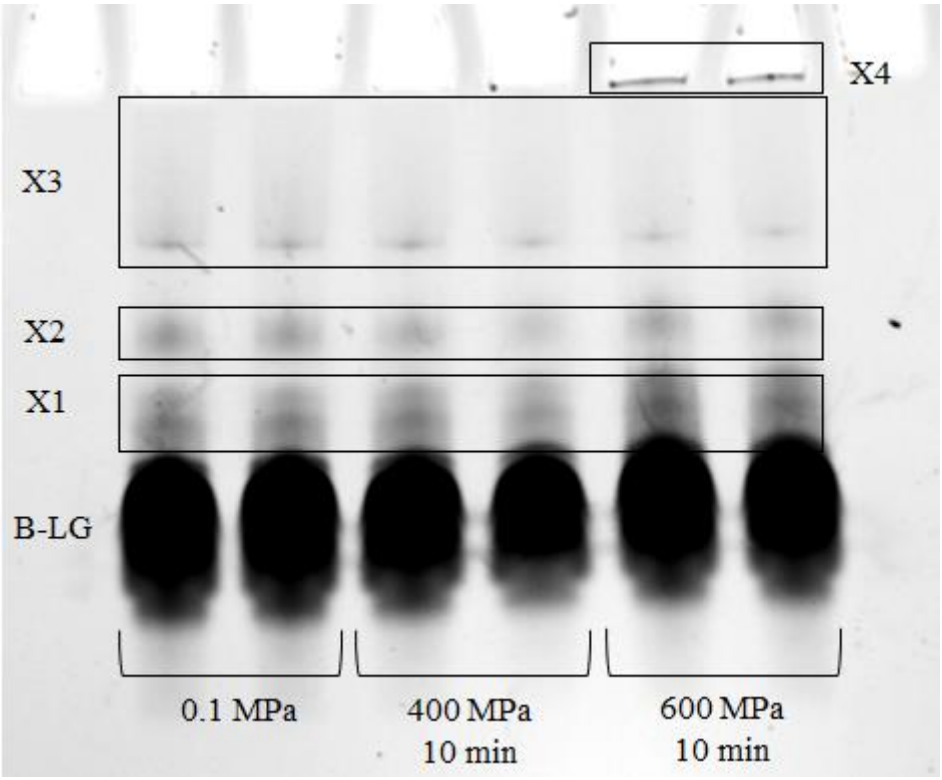

Figure 15: Native PAGE gels of control (0.1 MPa) and pressure-treated (400 and 600 MPa for 10 min) β-LG solutions.

4.3.2 Profiles and molecular weights of tryptic β-lactoglobulin peptides

Figure 16 shows the RP-HPLC peptide profiles of the initial β-LG tryptic hydrolysates obtained after pre-treatment of β-LG at 400 MPa (**Figure 16B**) and 600 MPa (**Figure 16C**) for 10 min and prior to the UF process. The control condition (0.1 MPa) hydrolysate is also shown (**Figure 16A**). In addition, Table 3 presents the MWs of peptides recovered from the different hydrolysates after MS analysis as well as their amino acid sequences, obtained by comparing theoretical MW from ExPASy to the MW determined by MS. The isoelectric point, net charge and abundance in each of the different fractions were also presented. In total, 20 peaks corresponding to 21 different potential peptides (since peak #17 could correspond to YANKY or QEPER due to similar MWs) were identified in the 0.1, 400 and 600 MPa β-LG hydrolysates. Of the 21 peptides, 13 derived from tryptic hydrolysis of β-LG and 7 were identified as potential peptide

species generated after bovine serum albumin (BSA) tryptic hydrolysis (Table 3). Since the purity of β-LG was 93%, according to the manufacturers, it is not surprising that peptides originating from other whey proteins were identified. Differences in peptide profile and composition were detected as a function of the pressurization treatment applied to native β-LG protein. The compositions of control and test hydrolysates obtained after pressurization of β-LG at 600 MPa were comparable since the same 17 peptides were commonly detected (**Figure 16A**, **16C**). However, the relative abundance of the peptides in the 0.1 MPa hydrolysate was higher, overall, than that obtained after β-LG pressurization at 600 MPa, specifically for peaks #5, 7, 9, 13 and 15. Compared to the 0.1 and 600 MPa hydrolysates, differences in peptide profile and composition were observed for hydrolysates obtained after pressurization of β-LG at 400 MPa. Indeed, 20 peptides (peaks #18 and 19 were newly generated peptides) were detected while only 18 and 17 peptides were characterized at 0.1 and 600 MPa, respectively. Moreover, the relative abundance of peptides at 400 MPa hydrolysate was higher than other two conditions, specifically for those corresponding to the peaks #2, 3, 5, 8, 9, 11, 12, 13, 16, 17 and 20. However, the relative abundance of peak #7 was drastically reduced.

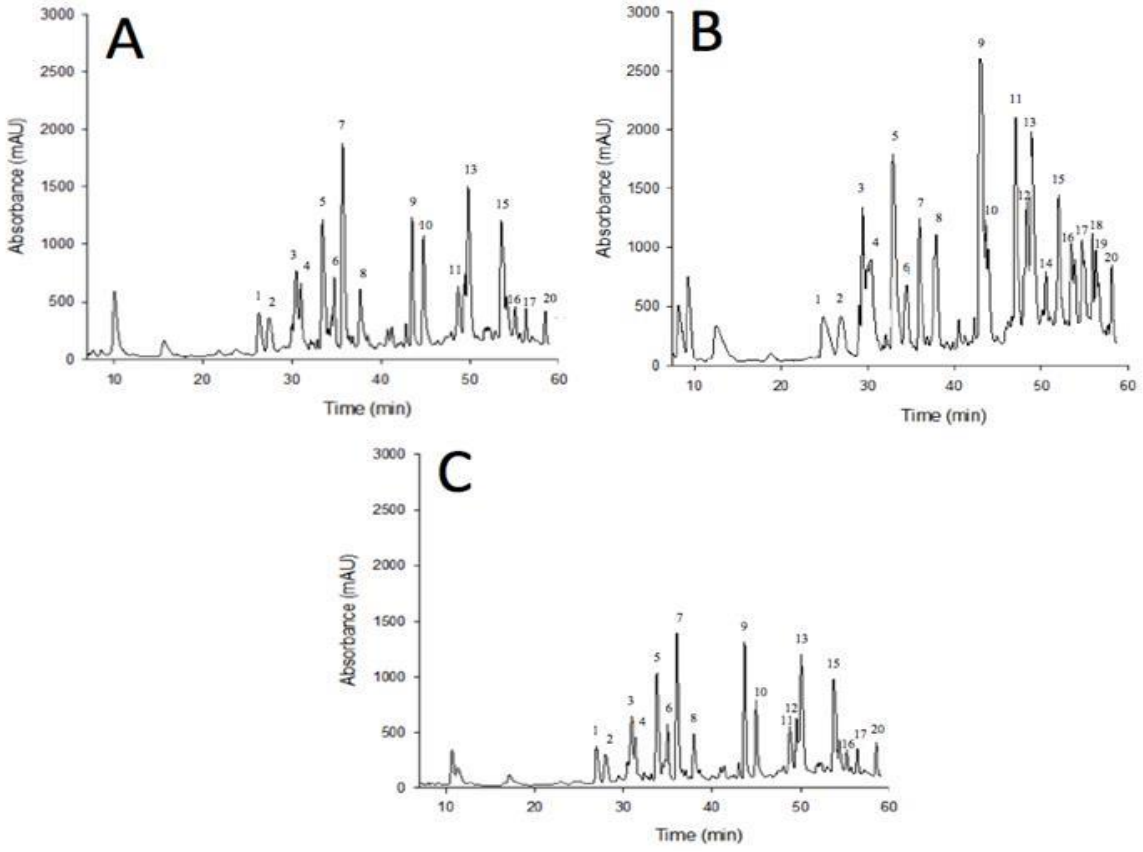

Figure 16. Peptide profiles of β-LG hydrolysates after HHP pre-treatments at 0.1 MPa (A), 400 MPa (B) and 600 MPa (C).

Table 3. Molecular weight identification and sequences of peptides recovered from tryptic hydrolysates of β-LG pretreated with high hydrostatic pressure (400 MPa, 600 MPa) or the control (0.1 MPa).

Protein[1]	Peak	Rt (min)[2]	Amino Acid Seq[3]	Location Seq[4]	Calc MW[5]	Obs MW[6]	pI[7]	Net Charge[8]	Detection[9]		
									Control	400 MPa	600 MPa
β-LG	1	25.1	IDALNENK	f84-91	916.47	915.7	4.3	-	✔	✔	✔
β-LG	2	27.2	GLDIQK	f9-14	673.39	672.9	5.9	-	✔	✔	✔
β-LG	3	29.7	IIAEK	f71-75	573.36	572.9	6.0	-	✔	✔	✔
β-LG	4	30.5	TPEVDDEALEK	f125-135	1245.59	1244.5	3.83	-	✔	✔	✔
β-LG	5	33.0	LIVTQTMK	f1-8	933.54	932.8	8.8	+	✔	✔	✔
BSA	6	34.2	NECF	107-110	512.18	512.9	3.85	-	✔	✔	✔
β-LG	7	36.1	FDKALK	f136-141	721.42	721.9	9.5	+	✔	✔	✔
β-LG	8	38.0	ALPMHIR	f142-148	837.48	837.8	9.8	+	✔	✔	✔
β-LG	9	43.0	VAGTWY	f15-20	696.34	695.8	5.4	-	✔	✔	✔
β-LG	10	44.0	WENGECAQK	f61-69	1064.44	1064.7	4.5	-	✔	✔	✔
β-LG	11	47.2	VLVLDTDY	f92-99	937.49	936.8	3.4	-	✔	✔	✔
β-LG	12	48.5	IPAVF	f78-82	546.33	548.9	5.5	-	x	✔	✔
BSA	13	49.5	AEFVEVTK	f233-240	922.49	922.4	4.2	-	✔	✔	✔
BSA	14	50.8	NECFLSHKDDSPDLPK	f107-122	1844.85	1844.4	4.49	-	✔	✔	✔
BSA	15	52.5	GDELCK	f79-84	664.30	664.9	4.1	-	✔	✔	✔
BSA	16	53.7	YLY	f145-147	458.23	458.9	6.02	-	✔	✔	✔
BSA	17	54.9	YANKY/QEPER	f164-168/f102-106	658.32	659.0	9.1/4.26	+/-	✔	✔	✔
BSA	18	56.1	QEAKDAFLGSF	f331-337	1212.59	1212.6	4.19	-	x	✔	x
β-LG	19	57.8	WENGECAQKK	f61-70	1192.54	1190.6	6.1/6.23	-/-	x	✔	x
β-LG	20	58.2	VAGTWYSLAMAASDISLLDA QSAPLRVY	f15-42	2969.51	2968.3	4.11	-	✔	✔	✔

[1] Native protein originating peptides: β-LG (beta-lactoglobulin), BSA (bovine serum albumin) and α-LAC (alpha-lactalbumin)
[2] Retention time
[3] Amino acid sequence
[4] Peptide location in the primary sequence of the milk protein identified in the first column
[5] Calculated molecular weight.
[6] Observed molecular weight
[7] Isoelectric point
[8] Calculated at pH 8.0.
[9] Sample in which peptide was detected (C: control; P400: 400 MPa hydrolysate and P600: 600 MPa hydrolysate)
[3, 4, 6 7, 8] Data obtained using tools available on the ExPASy Bioinformatics Resource Portal (*Bos taurus* β-LG, UniProtKD/TrEMBL # P02754).

4.3.3 Separation of tryptic β-LG peptides by UF membranes

4.3.3.1 Total Recycle Mode

Figure 17 presents the evolution of permeate flux as a function of TMP from 170 to 570 kPa at a constant retentate recirculation rate of 89.6 $m.s^{-1}$ for control and pre-treated β-LG tryptic hydrolysates. The pure water flux increased linearly from 147 to 450 $kg.m^{-2}.h^{-1}$ from initial to final TMP (data not shown). For the control and 600 MPa hydrolysates, a total and similar ($p>0.05$) increase in permeate fluxes of 37% was calculated from 170 to 570 kPa with initial and final values of 107.9±6.07 to 148.4±14.7 $kg.m^{-2}.h^{-1}$, respectively. The 44% increase in permeate flux (74.7±12.2 to 108.5±9.5 $kg.m^{-2}.h^{-1}$) for tryptic hydrolysates generated after pressurization of β-LG at 400 MPa was higher than for the two other conditions. However, permeate flux values were significantly lower ($p<0.05$), specifically from 300 to 570 kPa.

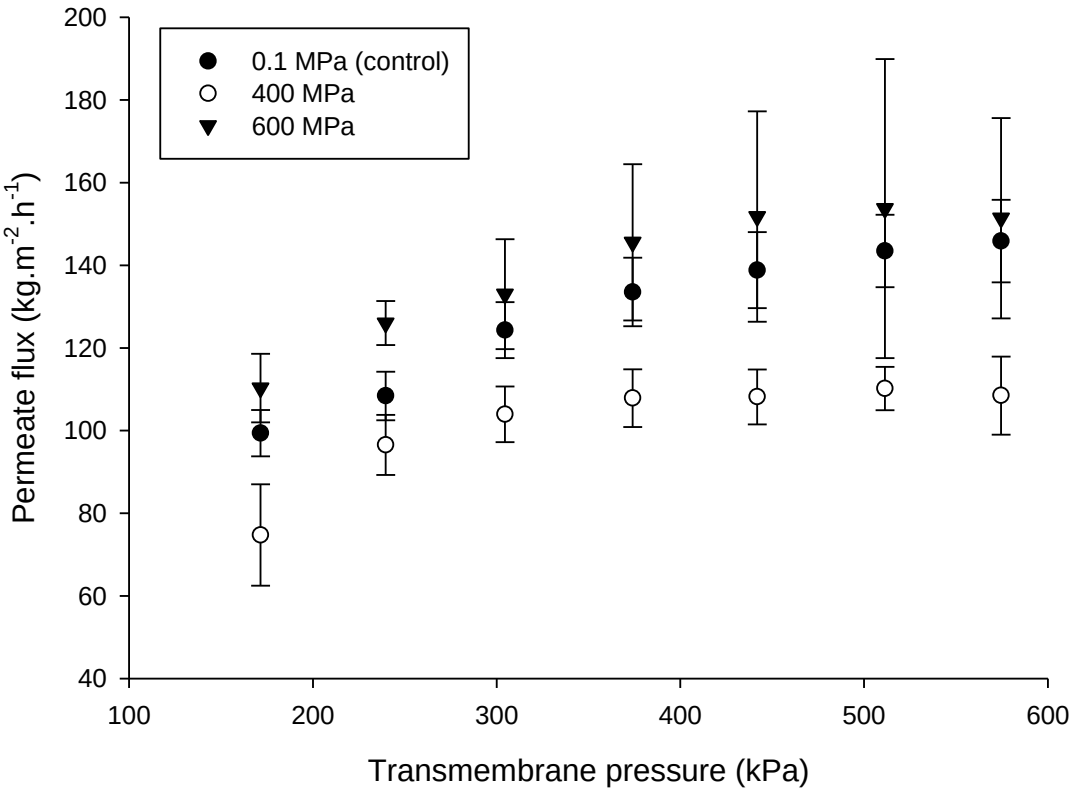

Figure 17. Permeate flux-TMP relationship for control β-LG tryptic hydrolysates (0.1 MPa) and hydrolysates generated after pressurization of native β-LG at 400 and 600 MPa. Data are means of triplicate experiments ± standard deviation.

The permeate flux-TMP relationship (**Figure. 17**) allowed us to determine J_{crit} (obtained when a deviation from linearity was observed in the TMP-permeate flux relationship) and J_{lim} (highest flux

obtained as a function of TMP applied) for control and pressurized samples [107]. Both J_{crit} and J_{lim} were similar for the 0.1 and 600 MPa conditions at 141.2±10.9 and 148.9±4.17 kg.m^{-2}.h^{-1}, respectively, and the critical TMP (TMP_c) was 372 kPa. The J_{crit} and J_{lim} values were significantly lower ($p<0.05$) for hydrolysates generated after protein pre-treatment at 400 MPa than for the 0.1 and 600 MPa conditions at 105.2±3.7 and 107.9±7.00 kg.m^{-2}.h^{-1}, respectively. Critical TMP was lower for the 400 MPa condition compared to the other conditions, with values of 323 (400 MPa) and 372 kPa (0.1 and 600 MPa). Consequently, the TMP used for the concentration experiments was fixed to 310 kPa, a pressure lower than TMP_c for all conditions while allowing appropriate permeate flux.

4.3.3.2 Concentration mode

The UF concentration experiments were performed up to a volume concentration factor (VCF) of 4.04±0.17 X. Figure 18A shows that the decrease in permeate flux was similar for the 0.1 and 600 MPa conditions ($p>0.05$), with a total flux decline of 43.1% (112.6±2.47 to 65.14±1.27 kg.m^{-2}.h^{-1}). However, significantly lower permeation flux values were obtained at 400 MPa ($p<0.05$) and the total flux decline reached 55% (112.0±3.97 to 50.37±4.77 kg.m^{-2}.h^{-1}). Moreover, the 400 MPa condition needed about 10% more UF time than the other conditions to reach a VCF of 4.0X. These results were confirmed by those presented in Figure 18B, which demonstrates that permeate flux values were considerably lower at 400 MPa than at 0.1 and 600 MPa ($p<0.05$) at the final VCF of 4.0X.

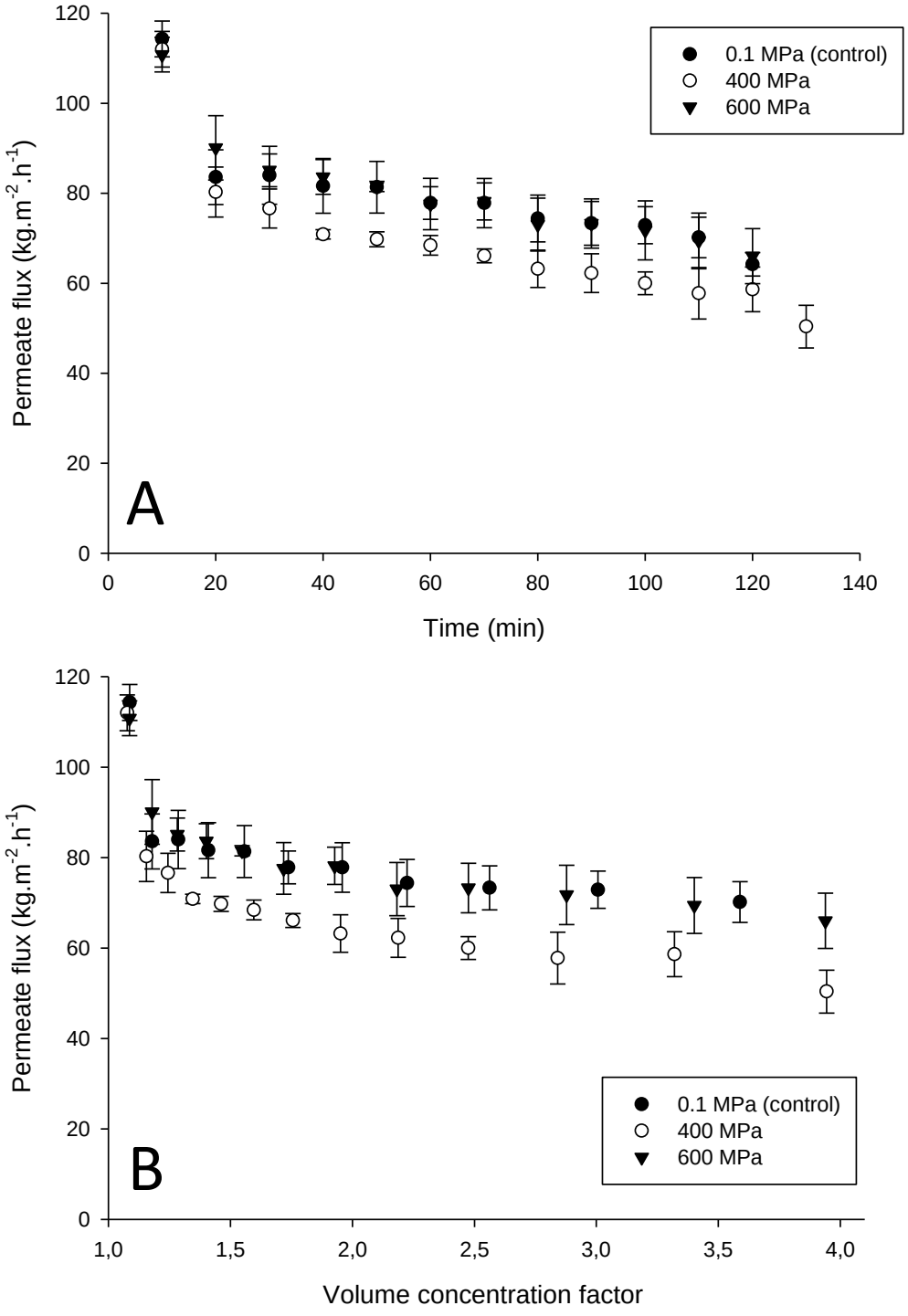

Figure 18. Permeate flux as a function of time (A) and volume concentration factor (B) for control (0.1 MPa) (●) and hydrolysates from β-LG pre-treated by HHP at 400 MPa (○) and 600 MPa (▼). Data are means of triplicate experiments ± standard deviation.

4.3.4 Characterization of permeate after UF concentration

Figure 19 shows the peptide profiles of permeates obtained from β-LG tryptic hydrolysates (0.1, 400 and 600 MPa) after UF concentration, up to a VCF of 4.10±0.15X. Only one replicate is presented since similar profiles were obtained for the three replicates. Differences were observed in the relative abundance of some peptides as a function of the treatment applied. The relative abundance of peaks #1, 2, 5, 6, 8, 13, 16, 17 and 20 were similar for all treatment conditions ($p>0.05$). However, transmission of peaks #3 and 4 were higher at 400 and 600 MPa compared to the control ($p<0.05$). The relative abundance of peak #7 was similar for the 0.1 and 600 MPa treatments but substantially lower for the 400 MPa ($p<0.05$) treatment. Peak #11 was more intense at 0.1 and 400 MPa than at 600 MPa ($p<0.05$) while the relative abundance of peak #10 was lower at 400 MPa ($p<0.05$). Peak #15 had higher relative abundance at 0.1 MPa than at the two other conditions while peaks #14 and 18 were only detected at similar relative abundance for 0.1 and 400 MPa. Finally, peak #19 was higher in concentration at 0.1 and 600 MPa compared to 400 MPa.

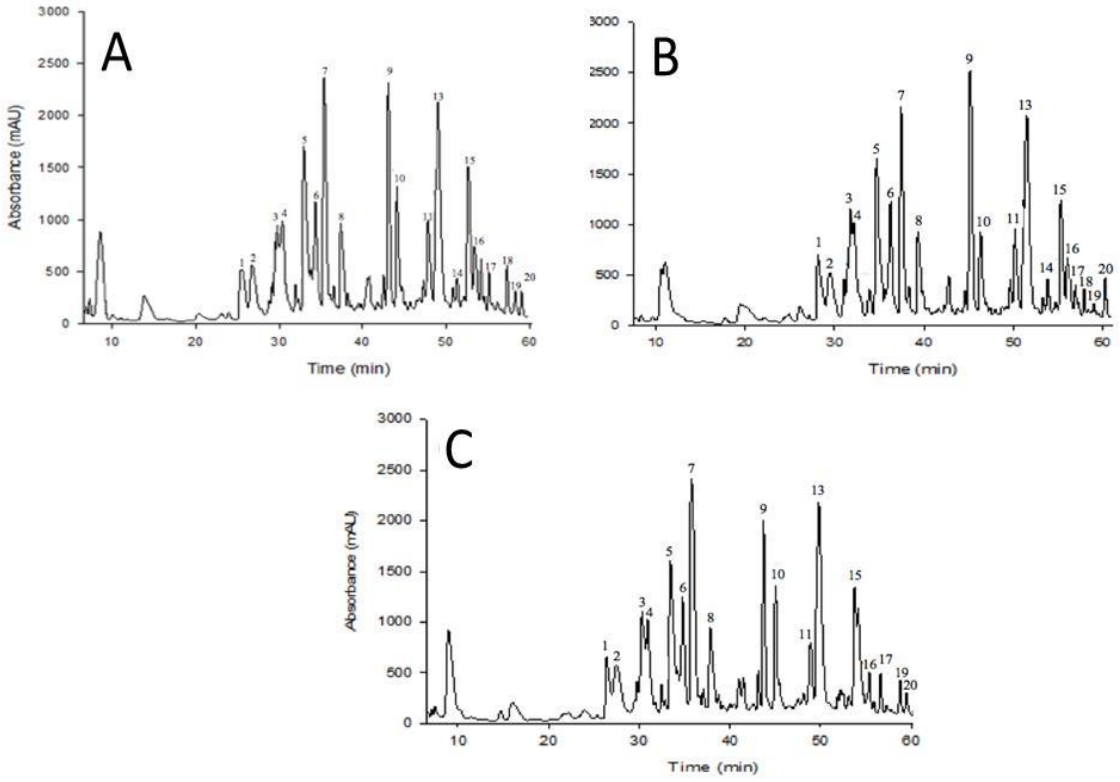

Figure 19. Peptide profiles of permeates from control (A) and hydrolysates recovered after β-LG pressurization at 400 MPa (B) and 600 MPa (C) after UF concentration to a VCF of 4X.

4.3.5 Relative proportions of the bioactive peptide fraction in hydrolysates and permeates

Seven of the 20 peptides detected in hydrolysates and permeates, IDALNENK (peak #1), GLDIQK (peak #2), IIAEK (peak #3), TPEVDDEALEK (peak #4), ALPMHIR (peak #8) and VAGTWY (peak #9), IPAVF (peak #12, only observed in hydrolysates), were identified as bioactive peptides by several authors [15, 28-31]. The relative abundance of this multi-bioactive peptide fraction in hydrolysates and permeates was calculated by dividing the total area under the curve for all bioactive peptide peaks with the total area of all peptide peaks for the same fraction (hydrolysate or permeate) and pressure condition using Equation 1:

$$\mathrm{R} = 100 * \frac{\mathrm{A}bio}{\mathrm{A}tot} \quad (1)$$

A_{bio} and A_{tot} represent, respectively, the areas under the curve of bioactive peptides and all detected peptides for the same hydrolysate or permeate condition.

Interestingly, the relative proportion of bioactive peptides in the hydrolysate solutions reached 38.64% for the 400 MPa condition, higher than for the 0.1 and 600 MPa conditions at 26.7% and 20.5%, respectively. The same pattern occurred with the permeates fractions (VCF of 4.0X) since the relative proportion of bioactive peptides was 31.15% for the 400 MPa condition compared to 24.8 and 24.23% for the 0.1 MPa and 600 MPa conditions.

4.3.6 Characterization of fouling peptides

Figure 20 shows the profiles of peptides desorbed from UF membranes by HNO_3 (**Figure 20A**, **20C** and **20E**) or SDoS (**Figure 20B**, **20D** and **20F**). A total of seven peptides, mainly generated from β-LG tryptic hydrolysis, were characterized. Three peptide sequences, (LIVTQTMK (peak #5), AEFVEVTK (peak #13) and YANKY and/or QEPER (peak #17)), were detected in the HNO_3 desorption solutions of the 0.1 and 600 MPa hydrolysates (**Figure 20A** and **20E**, respectively) and with higher relative abundance ($p<0.05$) in the 0.1 MPa hydrolysate.

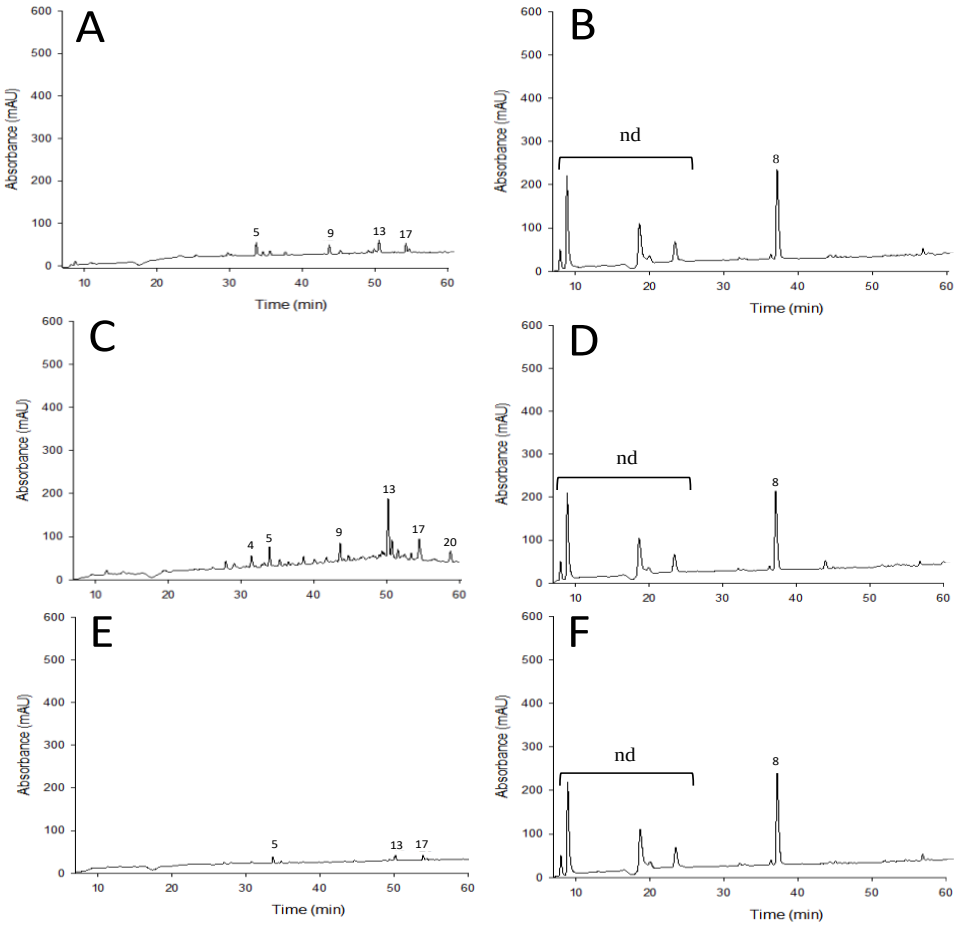

Figure 20. Peptide profiles of desorption solutions (HNO_3 (A, C and E) and SDoS (B, D and F)) of control hydrolysate (A, B) as well as hydrolysates generated after pressurization of β-LG at 400 MPa (C, D) and 600 MPa (E, F). The four peaks identified as "nd" (not determined) in B, C and F did not represent peptide (no sequence detected by mass spectrometry) and could be residual SDoS.

The peptide sequence VAGTWY (peak #9) was only detected in the control hydrolysates. For tryptic hydrolysates generated after pressurization of β-LG at 400 MPa, these four peaks (#5, 9, 13 and 17) were also detected but at higher relative abundance ($p<0.05$). Two other peptide sequences, identified as TPEVDDEALEK (peak #4) and VAGTWYSLAMAASDISLLDAQSAPLRVY (peak #20) (Table 2), fouled the UF membrane after reaching a VCF of 4.0X. When using the SDoS desorption solution, only the antihypertensive ALPMHIR [32] (peak #8) (Table 4) was desorbed from the UF membrane surface and at similar relative abundance for all experimental conditions (0.1, 400 and 600 MPa; **Figure 20B**, **20D** and **20F**).

Table 4. Characterization of peptides recovered from membranes after desorption by HNO_3 (0.01 M) or SDoS (0.5%)

Protein[1]	Peak#	Rt (min)[2]	Amino Acid Seq[3]	Hydrophobic amino acid (%)[4]	Solution recovery	Detection[5]		
						Control	400 MPa	600 MPa
β-LG	4	30.5	TPEVDDEALEK	36.4	HNO_3	**x**	✔	**x**
β-LG	5	33.0	LIVTQTMK	50.0	HNO_3	✔	✔	✔
β-LG	8	38.0	ALPMHIR	71.4	SDoS	✔	✔	✔
β-LG	9	43.0	VAGTWY	50.0	HNO_3	✔	✔	**x**
BSA	13	49.5	AEFVEVTK	50.0	HNO_3	✔	✔	✔
BSA	17	54.9	YANKY/QEPER	20.0	HNO_3	✔	✔	✔
β-LG	20	58.2	VAGTWYSLAMAASDISLLDA QSAPLRVY	57.1	HNO_3	**x**	✔	**x**

[1] Native protein originating peptides: β-LG (beta-lactoglobulin) and BSA (bovine serum albumin)
[2] Retention time
[3] Amino acid sequence, data obtained using tools available on the ExPASy Bioinformatics Resource Portal (*Bos taurus* β-LG, UniProtKD/TrEMBL # P02754).
[4] Proportion of hydrophobic amino acids in the peptide sequence
[5] Sample in which peptide was detected (C: control; P400: 400 MPa hydrolysate and P600: 600 MPa hydrolysate)

4.4. DISCUSSION

This study was designed to evaluate the effects on UF performance of modifying peptide profiles by pre-pressurization of β-LG, followed by trypsin hydrolysis at atmospheric pressure. The results showed that pre-pressurization of β-LG at 400 MPa for 10 min before hydrolysis modified the peptide pattern and improved the production of bioactive peptides but decreased UF performance.

4.4.1 Impact of HHP on native β-LG

β-lactoglobulin is a globular protein composed of two intramolecular disulfide bridges and a free thiol group (Cys-121) buried in its hydrophobic core [1]. Numerous studies have demonstrated that HHP treatment induces the unfolding of β-LG, which exposes the free thiol group and induces formation of internal disulfide bonds through exchange reactions that generate soluble β-LG aggregates. Consequently, the number of dimers in the solution decreases while higher molecular weight aggregates are produced [66] ; [57]. Indeed, Chicón *et al.* [66] demonstrated that dimers to tetramers were generated after pre-pressurization of β-LG variant A at 200 to 400 MPa [66]. Our results were consistent with these observations since increased protein aggregation was observed at 600 MP (**Figure 15**) compared to the other conditions. Moreover, the presence of high molecular weight β-LG aggregates trapped in the loading gel (**Figure 15**) was related to the generation of β-LG oligomers after pressurization, as observed by Dumay *et al.* [34], who demonstrated that β-LG aggregate molecular weights ranged from 500 to 10^3 kDa after pressurization of a 2.5 or 5.0 % protein solution at 450 MPa for 15 min [57]. The presence of aggregates in the 0.1 MPa condition was not surprising since the powder form of pure β-LG used is known to induce protein aggregation when heat is applied [110].

4.4.2 Impact of HHP on tryptic hydrolysis of β-LG

Our results showed that tryptic hydrolysis of pre-pressurized β-LG at 400 MPa for 10 min generated two new peptides (QEAKDAFLGSF (peak 18) from BSA and WENGECAQKK (peak 19) from β-LG) and increased the relative abundance of many peptide species. This clearly demonstrated that pre-pressurization of β-LG increased the proteolytic rate and improved the generation of specific peptides. These results were not in line with those obtained by Maynard *et al.* [1] who found that pressurization of β-LG up to 800 MPa before hydrolysis at atmospheric pressure had no effect on the peptide profile or on the hydrolysis of large hydrophobic intermediate tryptic peptides [1]. However, our observations agreed with those of Chicón *et al.* [66] who compared peptide profiles after tryptic hydrolysis at atmospheric pressure (5, 30 and 60 min) of untreated and pressure-treated β-LG variant A (100-400

MPa for 20 min) [66]. These authors demonstrated that proteolytic activity was enhanced by pressure since no intact protein was detected after enzymatic digestion [66]. Similar observations were made by Knudsen *et al.* [11] after tryptic hydrolysis of pre-pressurized β-LG A variant (150-450 MPa for 15 min) since pre-pressurization before hydrolysis increased the number of the binding sites for the enzyme [11]. Moreover, conformational changes under pressure increased exposure of hydrophobic regions, which increased the reactivity of the free thiol group [25]. Thus, and as mentioned by several authors, the presence of hydrophobic and disulfide-linked peptides was higher in tryptic hydrolysates generated after pre-pressurization of β-LG compared to control samples. More specifically, Knudsen *et al.* [11] observed that β-LG peptides f76-100 and f15-40 were present in higher relative abundance in tryptic hydrolysates generated after pre-pressurization of β-LG at 300 MPa for 15 min [11]. Chicón *et al.* [66] demonstrated that, in particular, β-LG tryptic fragments f15-40, (f41-70)S-S(f149-162) and (f41-70)S-S(f149-162) were generated in tryptic hydrolysates obtained after pre-pressurization of β-LG at 400 MPa [66]. These sequences were not detected in our hydrolysates since these peptide fragments were generated at the beginning of tryptic hydrolysis [66]. Thereafter, these intermediate products were cleaved into peptide fragments f21-40, f41-60, (f61-69)S-S(f149-162), (f61-70)S-S(f149-162) and f15-20 [66]. Surprisingly, only the low-molecular weight intermediate tryptic peptide f15-20 (peak #9) was detected in our 400 MPa samples. However, the absence of other sequences may be explained by the difference in enzymatic hydrolysis parameters and pressurization times used in this study, compared to others, in terms of protein concentration, enzyme/substrate ratio and hydrolysis time [11] ; [66]. Indeed, Maynard *et al*. [1] observed that intermediate peptide products were extensively hydrolyzed for pressure-treated β-LG over 100 min, the same hydrolysis time as used here [1].

Compared to the 400 MPa hydrolysate sample, the 600 MPa hydrolysate had a lower relative abundance of peptides. Consequently, the pre-pressurization of β-LG at 600 MPa was less efficient than un-pressurized protein (0.1 MPa) in terms of peptide yield. Recent work by Leeb *et al*. [38]. demonstrated that extensive denaturation of β-LG at 80°C and pH 5.1 decreased the relative abundance of several peptides, including the peptide sequences f(9–14), f(142–148), f(71–75), f(78–83) and f(92–100), due to reduced accessibility of trypsin to possible cleavage sites. Interestingly, the relative abundance of these peptide sequences was also decreased in the 0.1 and 600 MPa hydrolysates compared to the 400 MPa sample. In this respect, a parallel may be drawn between thermal and high hydrostatic pressure treatments since larger β-LG aggregates were generated as a function of pressure. Consequently, the tryptic hydrolysis may have been less efficient when β-LG