



Membrane fractionation of a β -lactoglobulin tryptic digest: Effect of the pH

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ABSTRACT

The tryptic digestion of β -lactoglobulin leads to the release of a large range of biologically active peptides. Ultrafiltration/nanofiltration technology can be used to fractionate protein hydrolysates in order to obtain permeate products with increased functionality and free from intact proteins and enzymes. The influence of the pH in the fractionation of the hydrolysate through a polyethersulfone membrane (MWCO 5 kDa) was investigated in this work. In this case peptide transmission was mainly governed by charge mechanisms and reached its maximum value when the pH value is close to the peptide isoelectric point. Almost complete rejection of acidic peptides was achieved at basic pH values due to electrostatic repulsive forces with the negatively charged membrane and, in agreement with Donnan theory, positively charged peptides shown lower transmission than the neutral species. The highest peptide recovery and the best separation factor between bioactive and non-bioactive peptides were obtained following nanofiltration at pH 8.0.

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1. Introduction

Milk proteins, both casein and whey proteins, contain a large range of biologically active peptides which may confer important biological properties *in vivo* (Madureira et al., 2010). Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions which may ultimately influence health (Kitts and Weiler, 2003). These peptides are inactive within the sequence of the parent protein and can be released by enzyme digestion of during microbial fermentation.

It has been shown that whey protein hydrolysates and in particular β -lactoglobulin (β -lg) digestions, have potential health benefits involving digestive, antimicrobial, immunomodulatory, antithrombotic, hypocholesterolemic and antihypertensive activities (Korhonen and Pihlanto, 2006; Mullally et al., 1997; Pellegrini et al., 2001; Nagaoka et al., 2001). Given the above and the fact that β -lg is easily isolated from sweet whey (Fernández et al., 2011, 2012), this makes β -lg an interesting substrate for the production of preparations enriched in bioactive peptides.

Separation technologies which discriminate small differences in charge, size and hydrophobicity can be employed to fractionate protein hydrolysates. Membrane separation techniques seem to be well suited for this purpose (Korhonen and Pihlanto, 2003).

Membrane separation using tight ultrafiltration (UF) and nanofiltration (NF) approaches have been used to fractionate protein hydrolysates (Garem et al., 1998; Pouliot et al., 1999; Groleau et al., 2004; Lapointe et al., 2005). Publications on this area highlight the important role of membrane-peptide electrostatic interactions in the differential separation of peptides with similar molecular weight (MW) but with different net charge.

In the case of peptides with zero net charge, their transmission (Tr) is mainly dependent on the size exclusion effect as described by Ferry (1936) and corrected by Zeman and Wales (1981). However, it has been shown that other physicochemical properties such as hydrophobicity may play a role when uncharged organic solutes are filtrated (Verliefde et al., 2008). In the case of charged peptides both size and electrical repulsion-attraction effects are involved in their Tr. These charge interactions can be described for single solutions according to the Donnan theory (Garem et al., 1997). Unfortunately, the transposition of this theory to complex amino acid and peptide mixtures is not easy.

The objective of the present work was therefore to evaluate the influence of pH on the fractionation of a tryptic hydrolysate of bovine β -lg through a polyethersulfone (PES) membrane having a molecular weight cut-off (MWCO) of 5 kDa and the description of optimal pH conditions in order to obtain a permeate product enriched in bioactive peptides and free from the native protein and the enzyme. Finally, this study tried to clarify if peptide hydrophobicity influences its transmission when charged mechanisms are minimized, i.e., when the pH of the mixture is close to the isoelectric point (pI) of the peptide.

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2. Material and methods

2.1. Preparation of a tryptic hydrolysate of β -lg

A 1.5 L solution containing 3 g/L of bovine β -lg (Davisco Food Co., MN, USA) was used for hydrolysate preparation. The protein content of the commercial substrate was 97.9% (w/w) and β -lg represented 91.5% of the total protein. The substrate solution was equilibrated at 37 °C and the hydrolysis reaction was initiated by adding TPCK-Trypsin (T1426-5G, Sigma, MO, USA) at a final enzyme:substrate ratio (E:S) of 1:170 (w:w). The pH of the reaction mixture was maintained at pH 8.0 using a pH-stat model 842 Titrand (Metrohm Ion Analysis, Herisau, Switzerland). The base used for maintaining constant pH was 1.0 N NaOH. The degree of hydrolysis (DH) was determined from the quantity of base consumed according to the pH-stat method (Adler-Nissen, 1986). Membrane permeation experiments were initiated when the DH of the hydrolysis reaction reached 9% (the theoretical maximum DH during tryptic digestion of β -lg is 10.6%).

2.2. Permeation experiments

The tryptic hydrolysate was filtered using a Pellicon 2 mini holder (Millipore, USA) and a GJ Series 120 pumping system (Micropump Inc., Vancouver, Canada) through a 5 kDa PES tangential flow membrane (Biomax 5, Millipore, USA). The effective membrane area was 0.1 m². The transmembrane pressure and temperature were set to 7.5×10^4 Pa and 37 °C, respectively. The experiment was carried out at four pH values (pH 4.0, 6.0, 8.0 and pH 10.0) with the aim of studying the role of pH dependent changes in physicochemical properties on the selective permeation of hydrolysate peptides.

Permeate and retentate streams were recycled into the feed tank, where the temperature and pH were controlled, to maintain constant concentration of the feed. The membrane system was run for 15 min during which time the pH and temperature of the feed solution as well as transmembrane pressure were carefully controlled and corrected if necessary. At this point, measurements of permeate flux (in triplicate) and sampling of permeate and retentate (in triplicate) were performed.

The membrane cassette was flushed with distilled water prior to and after filtering process solutions as well as prior to cleaning. The membrane was cleaned after each experiment following manufacturer recommendations. Cleaning procedure of the membrane consisted on the recirculation of a 0.1 N NaOH solution during 30 min at 40 °C. Cleaning efficiency was calculated by the measurement of water permeability. After the cleaning process the PES membrane was stored in 0.1 N NaOH solution at 4 °C.

The Tr of each peptide through the membrane was calculated according to Eq. (1):

$$\text{Tr}(\%) = \frac{A_{\text{Pi}}}{A_{\text{Ri}}} \times 100 \quad (1)$$

where A_{Pi} and A_{Ri} are the i peptide corresponding peak area obtained from the UPLC profiles of permeate and retentate, respectively.

The theoretical size transmission was calculated according to Zeman and Wales (1981)

$$\text{Tr} = (1 - (\lambda \cdot (\lambda - 2))^2) \cdot \exp(-0.7146\lambda^2) \quad (2)$$

with

$$\lambda = \left(\frac{\text{MW}}{\text{MWCO}} \right)^{0.4}$$

In this sieving model, the NF membrane is assumed to be a network of perfectly cylindrical and parallel pores in which solvent velocity follows Poiseuille's law with a parabolic profile and solutes are assimilated to hard spheres.

The separation factor ($S_{x/y}$) was calculated as the ratio of mean Tr values (%) between two peptides or group of peptides according to Eq. (3):

$$S_{x/y} = \frac{\sum_{i=1}^n T_{ri}/n}{\sum_{j=1}^m T_{rj}/m} \quad (3)$$

where n and m are the number of peptides included in groups x and y respectively. This value was used to evaluate the ability of the membrane to fractionate specific peptides.

2.3. Analytical methods

Peptides contained in the β -lg hydrolysate were isolated using an ultra high pressure chromatography (UPLC) system (Waters Acquity UPLC, Dublin Ireland) fitted with a BEH C18 column (1.7 μ m, 2.1×50 mm, Waters, Dublin Ireland). Peptides were separated by gradient elution at a flow rate of 0.2 μ L/min. Mobile phase A consisted of water, 0.05% (v/v) formic acid and 0.05% (v/v) trifluoroacetic acid (TFA) while mobile phase B was acetonitrile containing 0.05% (v/v) formic acid and 0.05% (v/v) TFA. Peptide separation was performed with the following programme: isocratic elution at 1% B for 1 min followed by a linear gradient from 1% to 13.5% B in 26 min, then a linear gradient to 21.5% B over 28 min, to 35% B over 25 min, to 80% B over 25 min, isocratic elution at 80% B for 5 min and return to starting conditions in 0.1 min followed by isocratic elution over 13 min. The injection volume was 10 μ L, total analysis time per sample was 90 min and detector response was monitored at 214 nm.

MS analysis was performed using a microTOF Q-II ESI MS system (Bruker Daltonics, Sunnyvale, USA) as previously described by Zhu and FitzGerald (2010). Eluant from the UPLC column was directly injected into the ESI sprayer. The MS and tandem MS analysis were controlled by MicroTOF software (version 2.3, Bruker Daltonics). Full scans were performed between an m/z range of 50–3000. Tandem MS determination was carried out with five automatically selected precursor ions present in the MS scan using CID. Electrospray conditions were as follows: capillary temperature, 200 °C; capillary voltage, 4500 V; dry gas (N_2) flow, 6.0 L min⁻¹.

MS/MS spectra were analysed using Data Analysis software (version 4.0, Bruker Daltonics) and BioTools (version 3.2, Bruker Daltonics). Data were analysed on the MASCOT (Matrix Science, v2.3.0) search engine (Perkins, Pappin, Creasy, & Cottrell, 1999) against NCBI nr 201011 with the following parameters: enzyme semi-trypsin; MS tolerance 20 ppm; MS/MS tolerance 0.05 Da; variable modifications oxidation (Met) and phosphorylation (Ser and Thr); maximum missed cleavages 4; taxonomy other mammalia. Unidentified m/z data were analysed in Sequence Editor (Bruker Daltonics) with the following parameters: sequence gi520 from NCBI database (β -lg variant A) and β -lg variant B, which was modified from sequence gi520 (D 64 to G 64 and V 64 to A 64); enzyme trypsin; MS tolerance 20 ppm; MS/MS tolerance 0.05 Da; disulfide bond cross-linking between C 66 and C 160; maximum missed cleavages 4.

2.4. Statistical analysis

All experiments were performed in triplicate. Data obtained were reported as mean and standard deviation and the difference between treatments was analyzed using one-way ANOVA with

Microsoft Office Excel 2007 (Microsoft Co., USA). Significant differences were at $p < 0.05$. Data points plotted in the Figures represent means $\pm$ deviation values for the triplicate analysis.

3. Results and discussion

3.1. Characterization of the hydrolysate

The peptides identified following UPLC separation and ESI-QTOF analysis are shown in Table 1. This method allowed 85.8% coverage of the amino acid sequence of β -lg. The only region which was not identified corresponds to f(102–124). A similar observation was reported when using HPLC separation coupled with ion-trap mass analysis (Lapointe et al., 2005). This region of β -lg contains a free-cysteine residue which does not participate in disulfide linkage in the native protein. As a consequence, the formation of aggregates during hydrolysis at basic pH is a possibility which may contribute to the difficulty in identifying this peptide. The presence of peptides such as KIIAEK (f(70–75)) and TKIPAVFK (f(76–80)), containing missed specific tryptic cleavages, indicate that hydrolysis of the protein was incomplete.

Two genetic variants, A and B, are commonly found in bovine β -lg. The primary sequences differ at position 64 (D in variant A and G in variant B) and at position 118 (V in variant A and A in the case of variant B). As expected, peptides corresponding to these variants were identified: sequences WENDECAQK (f(61–69)), WENDECAQKK (f(61–70)) correspond to β -lg A while peptides WENGCAQK (f(61–69)), WENGCAQKK (f(61–70)) correspond to β -lg B (Table 1).

Some of the peptides identified were linked by disulfide bridges (peptides with retention times of 50 and 71 min) as no reducing agent was used during the hydrolysis and subsequent analysis.

3.2. Influence of pH in permeate flux

Permeate flux as a function of pH through the 5 kDa PES membrane is summarized in Fig. 1. A 42% decrease in permeate flux (from 17.99 to 10.16 L h⁻¹ m⁻²) was observed when the pH was decreased from pH 10.0 to 6.0. Under the experimental conditions, similar flux values were observed at pH 4.0 and 6.0. Interestingly, it was observed that hydrolysate turbidity changed from being completely transparent at pH 10.0 to being opaque at pH 4.0. Groleau et al. (2003) reported the formation of pH dependant interactions in a tryptic digest of β -lg. It is evident from Table 1 that the majority of the peptides in the hydrolysate have pI values close to pH 4.0. Therefore, when the pH is set to this value, repulsive interactions between peptides are minimized potentially favouring hydrophobic interactions. The contribution of membrane charge, which is negative over the pH range tested (Susanto and Ulbricht, 2005), may also contribute to the observed differences in permeate flux. At basic pH values the tendency for membrane fouling may be reduced due to electrostatic repulsion between peptides (which have low pI values) and the membrane, as both have negative charge under these conditions.

3.3. Influence of pH on individual peptide Tr

The Tr values of the peptides which were identified in the UPLC profile was calculated. The results obtained for peptides SLAMAASDISLLDAQSAPLR (f(21–40)), VYVEELKPTPEGDLLEILLQK (f(41–60)) and VAGTWYSLAMAASDISLLDAQSAPLR (f(14–60)) are not discussed here as large deviation in permeation values were observed during triplicate experiments. In the case of the intact β -lg, as expected, total retention of the protein was achieved at all pH values tested.

Table 1
Characterization of the hydrolysate by ESI-QTOF and main physicochemical characteristics of the peptides.

AA sequence	MW (Da)	pI ^a	A/B/N ^b	H ₂ O _{av} (kcal/res) ^c	Charge ^d				Bioactivity references	
					pH 4.0	pH 6.0	pH 8.0	pH 10.0		
ALK f(139–141)	330.43	8.80	B	1.55	1	1	1	0 (–)		
KIIAEK f(71–75)	572.70	6.00	N	1.63	1	0	0 (–)	0 (–)		[5]
KIIAEK f(70–75)	700.88	8.59	B	1.61	2	1	1	0 (–)		
IDALNENK f(84–91)	916.0	4.37	A	0.95	0 (+)	–1	–1	–2		
GLDIQK f(9–14)	672.78	5.84	N	1.14	0 (+)	0 (–)	0 (–)	–1		[24]
TPEVDDEALEK f(125–135)	1245.31	3.83	A	0.85	–1	–4	–4	–5		
LIVTQTMK f(1–8)	933.17	8.75	B	1.34	1	1	1	0 (–)		
ALPMHIR f(142–148)	837.05	9.80	B	1.54	2	2	1	0 (–)		[6]
VLVIDTDYKK f(92–101)	1193.41	5.93	N	1.45	0 (+)	0	0 (–)	–1		
TKIPAVFK f(76–82)	903.13	10.00	B	1.76	2	2	2	0		
VLVIDTDYK f(92–100)	1065.23	4.21	A	1.44	–1	–1	–1	–2		[4]
TPEVDDEALEKFDK f(125–138)	1635.74	4.02	A	0.97	–1	–4	–4	–5		
WENG(D)ECAQK + LSFNPTQLEEQCHI (Ag.) f(61–70)+f(149–162)	2833.14 (2891.18)	4.91 (4.57)	A	0.85	+2 (+1)	–2 (–3)	–3 (–4)	–4 (–5)		
WENG(D)ECAQK + LSFNPTQLEEQCHI (Ag.) f(61–69)+f(149–162)	2704.97 (2763.01)	4.48 (4.25)	A	0.82	+3 (+2)	–1 (–2)	–2 (–3)	–3 (–4)		
SLAMAASDISLLDAQSAPLR f(21–40)	2117.40	4.21	A	1.01	–1	–1	–1	–2		
VYVEELKPTPEGDLLEILLQK f(41–60)	2313.67	4.25	A	1.37	1	–3	–3	–4		
VAGTWYSLAMAASDISLLDAQSAPLR f(14–60)	2708.08	4.21	A	1.11	–1	–1	–1	–2		

^a Isoelectric point were calculated using the ExPASy Molecular Biology Server.

^b Classified as A: acidic (pI < 5), B: basic (pI > 7) and N: neutral (5 < pI < 7).

^c Calculated according to Bigelow (1967).

^d Charge of the most abundant ion at the indicated pH.

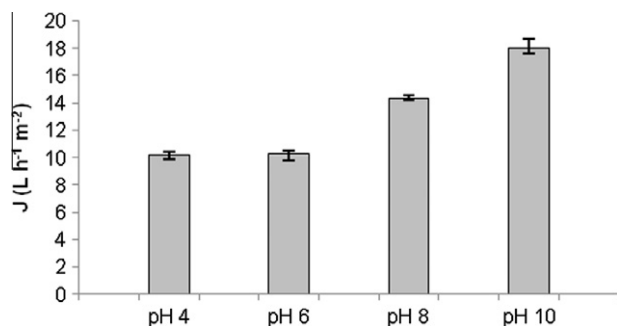


Fig. 1. Permeate flux ($\text{L h}^{-1} \text{m}^{-2}$) through the 5 kDa PES membrane at different pH values.

Peptide pI and ultimately charge is a central parameter influencing Tr behaviour under the different pH conditions. Therefore, peptides were grouped into acidic ($\text{pI} < 5$), neutral ($5 < \text{pI} < 7$) and basic ($\text{pI} > 7$) peptides. The differential pH dependent Tr of these peptide groupings was determined.

3.3.1. Acidic peptides

Peptides within this group include IDALNENK (f(84–91)), VLVLDTDYK (f(92–101)), TPEVDDEALEK (f(125–135)) and TPEVDDEALEKFDK (f(125–138)). This group also contains the disulfide linked peptides WENG(D)ECAQK-LSFNTQLEEQCHI (f(61–69) and f(149–162)) and WENG(D)ECAQKK-LSFNTQLEEQCHI (f(61–70) and f(149–162)), since in their reduced state they are acidic peptides.

Fig. 2 summarizes the Tr values obtained with the 5 kDa PES membrane for the acidic peptides at different pH values. The results demonstrate that Tr is decreased as the pH increased. This is clearly evident in the case of the lowest molecular mass peptide IDALNENK (f(84–91)). Some Tr of all the acidic peptides was observed at pH 4.0. However, Tr values were $< 50\%$ when peptide molecular mass was > 1.5 kDa. Furthermore, complete retention of peptides greater than 5 kDa was observed at pH 10.0.

The pH dependant Tr behaviour of acidic peptides can be readily explained according to charge mechanisms. As already outlined, PES membranes are negatively charged at pH values higher than 4.0. Acidic peptides ($\text{pI} < 5$) are negatively charged at pH values higher than 5.0. Therefore, repulsive forces between the peptides and the membrane may explain the decrease in peptide Tr as the pH was increased.

Of the peptides in the acidic grouping, only the fragment VLVLDTDYK (f(92–101)) has biofunctional properties as it is reported to exert antimicrobial activity when released from β -lg (Pellegrini et al., 2001). Therefore, the results herein would indicate that an appropriate membrane processing strategy may be to use operating conditions where this group of peptides are maximally retained by the 5 kDa PES membrane.

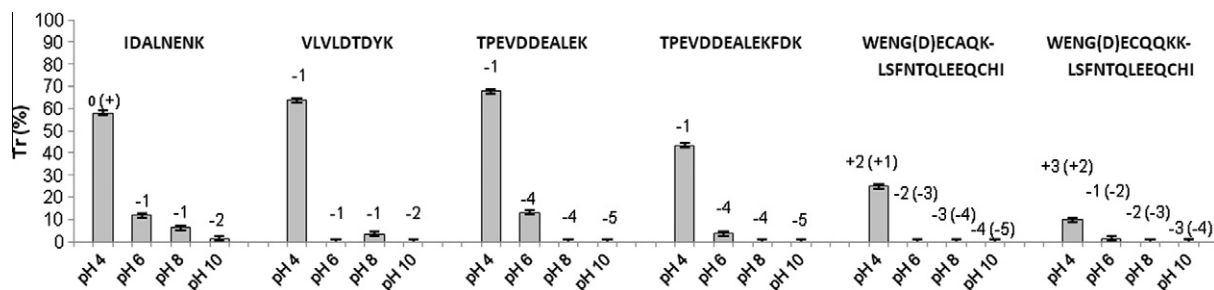


Fig. 2. Transmission coefficient of Acidic Peptides through the 5 kDa PES membrane as a function of pH. Acidic peptides are classified from the smallest to the biggest in X-axis and each bar represents the Tr calculated for a given pH, the charge of the peptide at each pH value being indicated over this information.

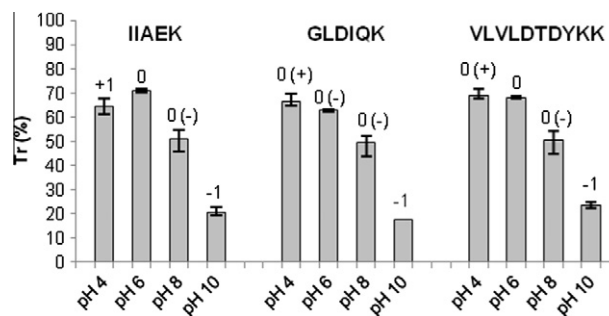


Fig. 3. Transmission coefficient of neutral peptides through the 5 kDa PES membrane as a function of pH. Neutral peptides are classified from the smallest to the biggest in X-axis and each bar represents the Tr calculated for a given pH, the charge of the peptide at each pH value being indicated over this information.

3.3.2. Neutral peptides

Peptides within this grouping include: IIAEK (f(71–75)), GLDIQK (f(9–14)) and VLVLDTDYKK (f(92–101)). Fig. 3 summarises the Tr values obtained with the 5 kDa PES membrane for these neutral peptides at different pH values. As was the case with acidic peptides, when the pH of the hydrolysate was higher than the pI of the peptides Tr is decreased due to repulsive forces between the peptides and the membrane. In the case of neutral peptides this takes place at pH values higher than 6.0. However, when pH is changed from pH 4.0 to pH 6.0 Tr remains constant. Good Tr through the 5 kDa PES membrane was observed at pH 4.0 and 6.0. This is as expected since neutral peptides have low net negative charge at these pH values.

No relationship between molecular mass and the Tr of neutral peptides was observed herein. This is not surprising since the three neutral peptides studied all have molecular masses significantly lower than 5 kDa.

Nagaoka et al. (2001) have reported that peptide IIAEK (f(71–75)), or Lactostatin, have an hypocholesterolemic effect while Pihlanto-Leppälä et al. (2000) have shown the ACE-inhibitory activity of peptide GLDIQK(f(9–14)). Furthermore, VLVLDTDYKK (f(92–101)) may be a precursor of VLVLDTDYK (f(92–100)), an acidic peptide that is reported to exhibit antibacterial properties (Pellegrini et al., 2001). Therefore, a pH value between 4.0 and 6.0 could be used to obtain a permeate preparation enriched in neutral bioactive peptides. Furthermore, membrane fractionation at pH 6.0 would be more favourable in order to decrease co-permeation of acidic peptides.

3.3.3. Basic peptides

Peptides within this grouping include ALK (f(139–141)), KIAEK (f(70–75)), ALPMHIR (f(142–148)), TKIPAVFK (f(76–82)) and LIV-TQTMK (f(1–8)). Table 1 shows that all these peptides have molecular masses lower than 1 kDa. This group of peptides mostly have lower molecular masses than peptides in the acidic group and this

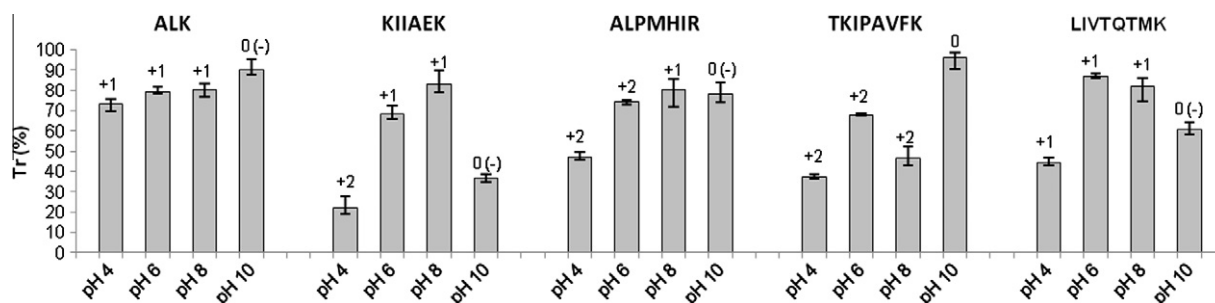


Fig. 4. Transmission coefficient of basic peptides through the 5 kDa PES membrane as a function of pH. Basic peptides are classified from the smallest to the biggest in X-axis and each bar represents the Tr calculated for a given pH, the charge of the peptide at each pH value being indicated over this information.

could influence, to some extent, the Tr differences observed between both groups

Fig. 4 summarizes the Tr values obtained with the 5 kDa PES membrane for the basic peptides at different pH values. In line with results obtained with the acidic and neutral peptides, it was observed that the Tr decreased when the pH of the hydrolysate was higher than the pI of the peptide, i.e., when peptides were negatively charged. This occurs with peptides KIIAEK (f(70–75)), ALPMHIR (f(142–148)) and LIVTQTMK (f(1–8)). The non compliance of peptide ALK (f(139–141)) with this trend may be related to its relatively low molecular mass. The results demonstrate that permeation of this peptide was not particularly affected by pH suggesting that size rather than charge was the predominant factor affecting its Tr. In the case of TKIPAVFK (f(76–82)), this peptide has the highest pI of all the peptides identified and has either a positive or neutral charge at the pH values tested. This may explain why its highest Tr value was reached at pH 10.0, i.e., corresponding to conditions where its net charge was zero. Peptide TKIPAVFK (f(76–82)) also presents an unusual behaviour between pH 6.0 and 10.0 since, contrary to expectation, its Tr is decreased at pH 8.0 without any apparent justifiable reason.

Fig. 4 also shows that, generally, when peptides are positively charged (pH < pI) their Tr is lower than in the case of the neutral specie. Peptides KIIAEK (f(70–75)) and ALPMHIR (f(142–148)) are the best representatives of this behaviour. Contrary to what happens in the case of acidic peptides, the positively charged peptides and the negatively charged membrane leads to the formation of electrostatic attractive forces. Contrary to expected, these forces don't facilitate peptide transmission but also this parameter is decreased. Donnan theory could be used to explain this observation. The counterion of positive charged peptides is rejected by the membrane because of electrostatic repulsive forces; therefore, the peptide is also rejected in order to maintain electroneutrality. This was previously enounced by Tsuru et al. (1994) when filtrating single amino acid solutions through NF membranes.

Peptides within the basic grouping have some interesting bioactivities. For example, ALPMHIR (f(142–148)) is one of the most potent ACE inhibitory peptides in β -Lg as reported by Mullally et al. (1997). In addition, peptides KIIAEK (f(70–75)) and TKIPAVFK (f(76–82)) are precursors of IIAEK (f(71–75)) and IPA-VFK (f(78–82)), peptides which are reported to have hypocholesterolemic and antimicrobial activity (Nagaoka et al., 2001; Pellegrini et al., 2001). It is interesting to observe these peptides mainly transmit to the permeate stream during processing at basic pH values (Fig. 4) while under these conditions Tr of acidic peptides is minimized.

3.4. Separation factor

As already mentioned in Section 3.3, β -Lg tryptic peptides can be classified into three groups according to their pI values and each

Table 2

Separation factor between basic and neutral peptides ($S_{B/N}$), basic and acidic peptides ($S_{B/A}$), neutral and acidic peptides ($S_{N/A}$) and bioactive and non-bioactive peptides ($S_{Bio/NBio}$).

	pH 4.0	pH 6.0	pH 8.0	pH 10.0
$S_{B/N}$	0.9	1.1	1.5	3.5
$S_{B/A}$	1.0	15.0	46.2	291.8
$S_{N/A}$	1.2	13.4	31.3	83.4
$S_{Bio/NBio}$	1.2	2.1	2.2	1.8

group behaves differently during membrane processing in a pH dependent manner. Table 2 summarises the separation factors obtained for these three groups of peptides during processing through a 5 kDa PES membrane. Highest values were obtained for $S_{B/A}$ (46.2 and 291.8 at pH 8.0 and 10.0, respectively) indicating that these two group of peptides are easily separable, especially at alkaline pH. It is worth noting that the average molecular mass of acidic and basic peptides in the tryptic digest of β -Lg is 2006.8 and 740.9 Da respectively. Therefore, both charge and molecular mass-based differences may contribute to the good separation observed for peptides in these groupings at alkaline pH. A similar trend occurred (although at lower absolute values) for $S_{N/A}$, this parameter was highest during processing at alkaline pH. Peptide mass may also contribute to the observed separation factor value for these peptide groupings given that the average molecular mass of the neutral peptides is 813 Da. The $S_{B/N}$ values were consistently lower than those observed for $S_{B/A}$ and $S_{N/A}$. This may be related to the fact that the neutral and basic peptides studied herein had similar molecular masses and therefore size differences may not contribute to their fractionation. The highest $S_{B/N}$ value (3.5) was observed at pH 10.0, the only pH value where both groups of peptides are oppositely charged, i.e., basic peptides have negative charges whereas neutral peptides have neutral or positive charges (Tables 1 and 2).

One of the objectives of this research was to evaluate the ability of a 5 kDa PES membrane to fractionate different types of peptides in a well defined, but heterogeneous mixture of peptides in order to obtain a permeate fraction enriched with specific bioactive peptides. As mentioned in Section 3.3, some of the peptides studied herein have previously been shown to possess different biofunctional properties. This includes: ALPMHIR (f(142–148)), GLDIQK (f(9–14)) and IIAEK (f(71–75)) which have ACE inhibitory activity, VLVDLDYK (f(92–100)) with antibacterial activity and IIAEK (f(71–75)) which has hypocholesterolemic activity. In addition, other peptides in the hydrolysate (KIIAEK (f(70–75)), TKIPAVFK (f(76–82)) or VLVDLDYK (f(92–101))) may act as precursors for the generation of the aforementioned bioactive peptides. The separation factor between known bioactive peptides (including precursor peptides) and non bioactive peptides ($S_{Bio/NBio}$) was calculated for all the pH values. Highest $S_{Bio/NBio}$ values (2.1–2.2)

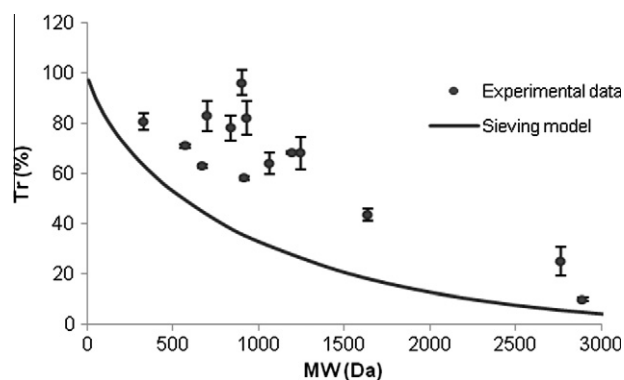


Fig. 5. Comparison between theoretical Tr values calculated according to Zeman and Wales (1981) and experimental values obtained when the peptides have zero net charge ($\text{pH} \approx \text{pI}$), using the 5 kDa PES membrane.

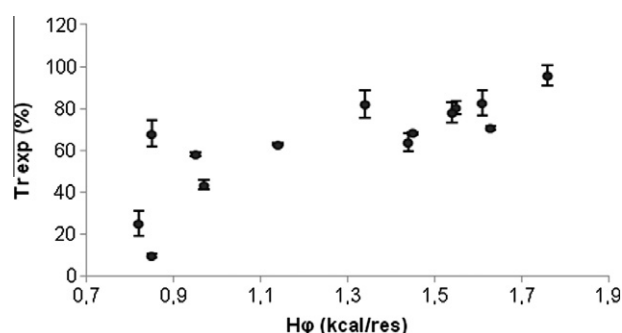


Fig. 6. Relationship between peptide hydrophobicity and experimental Tr when the peptides have zero net charge ($\text{pH} \approx \text{pI}$), using the 5 kDa PES membrane.

were observed between pH 6.0 and 8.0 (Table 2). This may be explained by the fact that most of the currently known trypsin cleaved β -lg bioactive peptides are basic or neutral. Therefore, the Tr of these groups is favoured within this pH range (pH 6.0–8.0), contrary to what happens with acidic peptides. The average molecular masses of bioactive and non bioactive peptides are 792 and 1474 Da, respectively. In this case, molecular mass differences do not seem to contribute to improved separation. In fact, the $S_{\text{Bio/NBio}}$ value at pH 4.0 was only 1.2.

3.5. Influence of peptide hydrophobicity on transmission

Fig. 5 represents experimental Tr values obtained when the pH of the experiment is close to the pI of each peptide, using the 5 kDa PES membrane. Under these conditions charge effects are minimized and it's supposed that Tr is mainly dependent on sieving effects. However, when experimental Tr values are compared with the theoretical values calculated according to a sieving model (Zeman and Wales, 1981), the formers are always higher and, as can be noted in Fig. 5, the greater differences between theoretical and experimental data correspond to the most hydrophobic peptides.

Fig. 6 shows the relation between experimental Tr values of the uncharged peptides and their hydrophobicity, using the 5 kDa PES membrane. As a general rule the highest hydrophobicity implies the highest Tr obtained. When peptides have no net charge, their approach to the membrane wouldn't be impeded by electrostatic interactions and the adsorption on the membrane surface due to hydrophobic interactions is possible. In this sense it could be said that the highest the solute hydrophobicity, the highest the adsorption and therefore, the highest the Tr. There is little literature

regarding the influence of hydrophobic interactions between peptides and NF membranes but Fig. 6 supports this hypothesis. The fact that the graphical relation between Tr and hydrophobicity does not represent a definite shape is due to the physico-chemical characteristics of each peptides, particularly its MW.

4. Conclusions

Good resolution of peptide peaks during UPLC allowed quantification, based on peak area, of the relative amounts of the different peptides present in permeate and retentate samples. Using this methodology, 85.8% sequence coverage was obtained while tryptic peptides corresponding to the main genetic variants of β -lg along with some disulfide linked peptide fragments were identified.

Filtration experiments were carried out at four different pH values (pH 4.0, 6.0, 8.0 and 10.0). The highest Tr values were obtained when the pH of the mixture is close to the pI of each peptide. At higher pH values electrostatic repulsive interactions membrane-peptides leads to a decrease of Tr. When the peptides are positively charged ($\text{pH} > \text{pI}$) the principle of electroneutrality inhibits the increase of Tr.

According to the results obtained for $S_{\text{B/A}}$ and $S_{\text{N/A}}$, fractionation between basic or neutral peptides and acidic peptides can be obtained at pH values higher than 4.0. However, the $S_{\text{B/N}}$ values indicate relatively poor separation between basic and neutral peptides, reaching the highest value (3.5) at pH 10.0. It was concluded that both peptide charge and size effects contributed to these results.

The impact of pH on membrane fouling was also evaluated. A significant decrease in permeate flux was observed when the experimental pH was decreased during processing of the tryptic β -lg hydrolysate through the 5 kDa PES membrane. This result was presumed to arise due to the formation of hydrophobic peptide aggregates at lower pH values.

Using the conditions described herein it was possible to obtain a permeate product enriched in bioactive peptides. The highest separation between bioactive and non-bioactive peptides, i.e., $S_{\text{Bio/NBio}}$ value (2.2) was obtained when the hydrolysate was processed at pH 8.0.

Experimental Tr values obtained when the peptides have zero net charge show that and increase in hydrophobicity represents an increase in peptide Tr, probably due to a better adsorption on the membrane surface.

Further studies are required to confirm that the biofunctional potency of the permeate solution obtained in this way has been increased with respect to the initial hydrolysate. Additionally, the introduction of changes to both the membrane (material, MWCO, and hydrodynamic conditions) and the filtration conditions (ionic strength and concentration) is worthy of further investigation.

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