Original article

Selective isolation of cationic amino acids and peptides by electro-membrane filtration

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Abstract — In the food industry there is a clear trend towards the production of speciality products with a high added value. Electro-membrane filtration (EMF) can be used to separate and concentrate these products from complex solutions. With EMF, lysine was separated from a model solution and a protein hydrolysate both containing leucine. The lysine fraction in the permeate ranged from 0.86 to 0.96. The lysine transport rate and purity were improved by increasing the potential difference from 20 V to 40 V. Reduction of the transmembrane pressure from 2 to 0.5 bar improved the purity at a practically unchanged lysine transport rate. An enriched fraction of antibacterial cationic peptides (e.g. lactoferricin-B) could be produced from a lactoferrin hydrolysate using EMF. Isolation of these bioactive peptides is normally expensive due to the complex nature of the hydrolysate feed. EMF has the potential to become an attractive (partial) isolation technology.

electro-membrane filtration / dairy / food / lactoferricin / separation

1. INTRODUCTION

The current trend in the food industry is to produce speciality products with a high added value, especially nutraceuticals. Consequently, there is an increasing need for isolating and purifying high-value components (e.g. amino acids, bioactive peptides) from complex solutions such as protein hydrolysates. For the production of these components chromatographic techniques are usually required. Since this is capital intensive, alternative easily up-scalable technologies are under investigation.

Membrane filtration (ultrafiltration or nanofiltration) can be used to separate

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ampholytes (amino acids, peptides and proteins) selectively from complex solutions by manipulating the charge interactions between the components and the membrane surface [4, 5, 7, 9, 16–19, 21]. This can be achieved by adjusting the pH or by adapting the salt composition of the feed. Even amino acids of similar size (e.g. leucine and lysine) can be separated with high selectivity [17,18]. The highest selectivity is obtained when size exclusion is (almost) absent and the Donnan exclusion mechanism dominates [18].

The separation efficiency can also be manipulated and improved by the introduction of an external potential difference over the (nanofiltration, ultrafiltration or micro-filtration) membrane [2, 6, 15, 20]. This was originally proposed to reduce concentration polarisation of proteins at the membrane surface, thereby increasing the permeate flux [10, 13, 14, 22]. Recently this has received more and more attention for the production of speciality products due to the effect of the electric field on retention of charged components. Van Nunen [20] estimated, on the basis of pilot-scale (0.25 m^2 membrane area) experiments, that the costs for separating the proteins lipase and lysozyme with a multi-compartment electro-membrane filtration (EMF) system would be around 50 Euro per kg protein produced.

Because of the industrial need for alternative cost-effective separation methods for the production of high-value products and our knowledge on the effects of charge interactions between membranes and these components on the separation efficiency, we have performed initial experiments with EMF to study the possibilities of this process. The separation of the amino acids leucine (Leu) and lysine (Lys) from a model solution and a commercial protein hydrolysate and the isolation of antibacterial cationic peptides (in particular lactoferricin-B, Lfcin-B) from a peptic hydrolysate of lactoferrin (LF) are presented in this article.

2. MATERIALS AND METHODS

2.1. Membrane module

The membrane filtration module (Fig. 1) contains a high-voltage power supply to provide the required electric field strength. The field strength over the membrane can be measured (Fig. 1). During the experiments the anode was situated in the electrode compartment next to the feed/concentrate compartment and the cathode was placed in the electrode compartment next to the permeate compartment. A polymeric NTR 7450 (Nitto-Denko, Shiga, Japan, MWCO 1 kg·mol^{-1}) flat-sheet membrane was used for the separation of leucine and lysine from a model solution and a protein hydrolysate. Isolation of cationic peptides from a LF hydrolysate was done with a more open polysulphone flat-sheet membrane (MWCO 10 kg·mol^{-1}) to minimise size exclusion. The installed membrane area was 0.008 m^2. The electrode compartments were separated from the feed/concentrate and the permeate compartment with a CMX and an AMX ion-exchange membrane, respectively, from Tokuyama Soda, Tokyo, Japan. pH indicators were placed in all collection/supply beakers (Fig. 1) to monitor the pH variations as a result of water splitting reactions at the ion-exchange membranes.

2.2. Feedstock solutions

A model solution containing approximately 5 mmol·L^{-1} leucine (molecular weight 131 g·mol^{-1}) and lysine (molecular weight 146 g·mol^{-1}) was prepared by dissolving analytical-grade leucine and lysine (Sigma, St-Louis, MO, USA) in reverse osmosis permeate of de-mineralised water (RO de-min. water). The pH was adjusted to 5.5 with HCl.

A 1% w/w casein hydrolysate solution containing approximately 3 mmol·L^{-1} lysine and leucine was prepared by dissolving casein hydrolysate (Pepticase, Sheffield
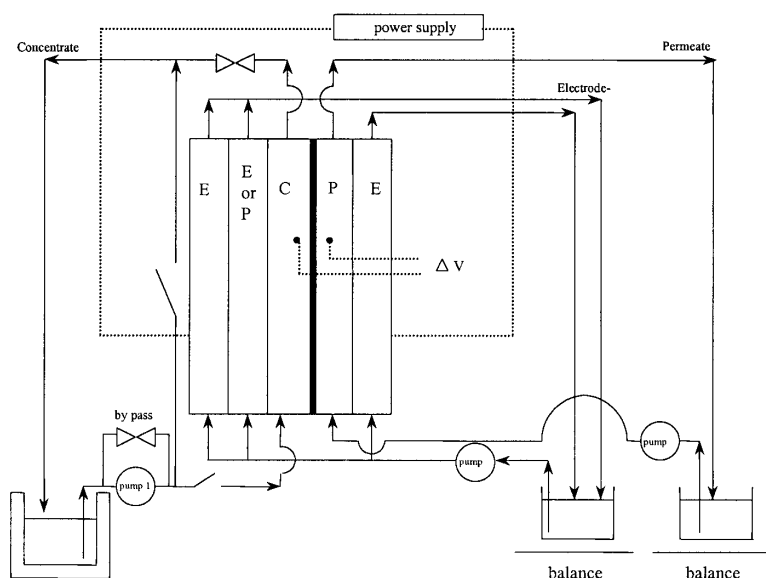
Figure 1. Schematic representation of the EMF test-rig (E electrode compartment, P permeate compartment, C concentrate/feed compartment).

Products, Norwich, USA) in RO de-min. water. The pH of the solution was 7.3.

Bovine LF was kindly supplied by Domo Food Ingredients (Beilen, The Netherlands). A 5% w/v aqueous solution of LF was adjusted to pH 3.0 with 1.0 mol·L^{-1} HCl and digested with 3% w porcine pepsin A (EC 3.4.23.1, 445 U·mg^{-1} solid, from Sigma)/w of substrate for 4 h at 37 °C . Heating at 80 °C for 15 min and adjusting the pH to 7.0 with 1 mol·L^{-1} NaOH terminated the reaction. The digest was centrifuged at 16 000 g for 15 min and the supernatant was retained and freeze-dried. The dried hydrolysate was re-dissolved in twice-distilled water (0.8% w/v final concentration) and the pH was adjusted to 8.5. Subsequently the hydrolysate was centrifuged again. The supernatant was used as feed solution for the EMF system.

2.3. Experiments and methods

All experiments (Tab. I) were carried out at 25 °C with a 0.01 mol·L^{-1} Na$_2$SO$_4$ (ana-

lytical grade, obtained from Merck, Darmstadt, Germany) solution in the electrode compartments. A Na$_2$SO$_4$ solution is preferred over NaCl to avoid the production of Cl$_2$ at the anode. The concentrate, permeate and electrode solution were circulated over beakers at a circulation rate of 175 L·h^{-1}, 50 L·h^{-1} and 50 L·h^{-1}, respectively. The unit was thus operated batch wise not only for the feed/concentrate and electrode solution, but also for the permeate. Due to this operation mode the lysine selectivity can not be determined. The lysine fraction in the permeate, however, gives an indication of the selectivity. This fraction is defined as:
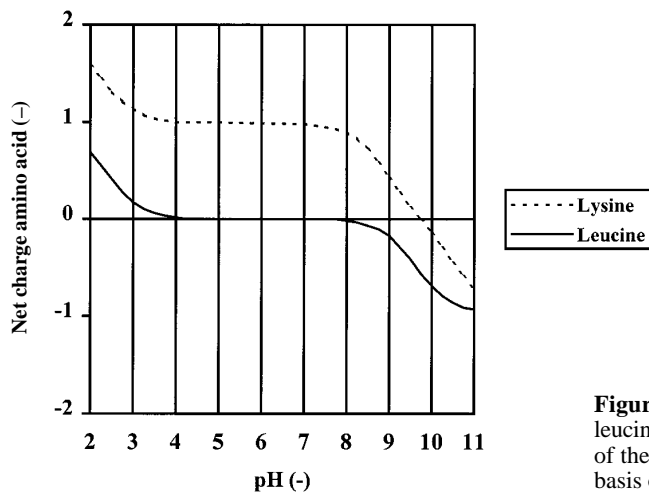
$$f = \frac{c(Lys)_p}{c(Lys)_p + c(Leu)_p} \tag{1}$$

where $c(Lys)_p$ represents the lysine concentration and $c(Leu)_p$ the leucine concentration in the permeate. The pH of the feed (concentrate) and in some cases of the permeate (Tab. I) was adjusted continuously and kept between 4 and 8 for the

Table I. Summary of the experiments (RO demin. water: reverse osmosis permeate of de-mineralised water, V: potential difference set–point of the power supply, C: concentrate, P: permeate).

Exp. No.	Feed	Permeate solution at start of run	V (V)	TMP (bar)	PH adjustment
1	Leu/Lys model solution	0.01 mol·L^{-1} Na$_2$SO$_4$	40	2	C and P
2	Leu/Lys model solution	0.01 mol·L^{-1} Na$_2$SO$_4$	40	0.5	C and P
3	Leu/Lys model solution	RO de-min·water	40	0.5	C
4	Leu/Lys model solution	0.01 mol·L^{-1} Na$_2$SO$_4$	20	2	C
5	1% w/w casein hydrolysate	RO de-min. water	40	0.05	C and P
6	0.8% w/w LF hydrolysate	RO de-min. water	40	0.05	C and P

leucinelysine separation experiments. In this pH range leucine has no net charge, whereas lysine has a net positive charge (Fig. 2). During experiments 3 and 4 (Tab. I) the pH of the permeate exceeded 10 only during the final 10 min and 30 min, respectively. Since the net charge of lysine remained +1 in the concentrate and remained positive in the permeate during the major part of these runs (Fig. 2) neither a reduction in the lysine transport to the permeate compartment nor an inverse lysine transport is expected. For the isolation of cationic peptides from the LF hydrolysate, the pH of the feed/concentrate was kept at 8.5 while the pH of the permeate was kept between 4 and 7.

We operated with transmembrane pressures (TMP) and potential difference set–points for the power supply between 0.05–2 bar and 20–40 V (average electric field strengths between the electrodes between 570–1140 V/m), respectively. At the start of some experiments the permeate section was filled with a 0.01 mol·L^{-1} Na$_2$SO$_4$ solution (also used for the electrode compartments), whereas for other experiments RO de-min. water was used to check the effect of the conductivity of the permeate on the selectivity of the separation. This resulted in a conductivity ratio, defined as the conductivity of the liquid in the permeate compartment over the conductivity of the liquid in the feed/concentrate compart-

Figure 2. The net charge of leucine and lysine as function of the pH, determined on the basis of pK values.

ment of 5 for operation with 0.01 mol·L⁻¹ Na₂SO₄ and below 0.01 for operation with RO de–min. water using the lysine and leucine model solutions. Other operating conditions are listed in Table I.

2.4. Analytical methods

The amino acid analyses were done with an amino acid analyser (type 4151, LKB Biochrom, Cambridge, UK). Peptides of the LF hydrolysate were analysed by reversed-phase HPLC [11]. Mass determination of peptides was performed on a Waters HPLC system connected on-line to a Quatro II triple quadrupole instrument (LC-MS) (Micromass, Cheshire, UK) as described elsewhere [12]. However, in this case a Widepore C_{18} 250×20 mm column (Bio-Rad Laboratories, Richmond, CA, USA) was used. Peptides were eluted with linear gradient of solvent B in A going from 10% to 30% in 25 min at a flow-rate of 1.0 mL·min⁻¹. Solvent A was a mixture of acetonitrile-water-trifluoroacetic acid (100:900:1, v/v/v) and solvent B contained the same components (900:100:0.8, v/v/v). Identification of most of the peptides in the permeate fraction was performed by reversed-phase HPLC and LC-MS using reference peptides prepared at NIZO [11]. The peptide with mass 2096 was identified by electrospray ionisation-tandem mass spectrometry (ESI-MS/MS) using a Quatro II triple quadrupole mass spectrometer (Micromass) under the same conditions as reported previously [12]. The sequence of other newly identified peptides was obtained by combining the N-terminal sequence [11] and molecular mass data and matching these to the known sequence of bovine LF [8].
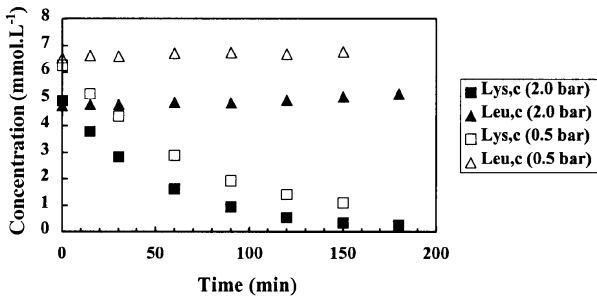
3. RESULTS AND DISCUSSION

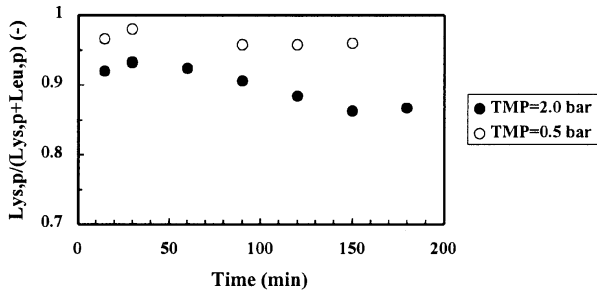#### 3.1. Separation of leucine and lysine from the model solution

A reduction of the *TMP* from 2.0 to 0.5 bar hardly affected the transport of lysine through the membrane, as the reduction rate of the lysine concentration in the concentrate was practically independent of the *TMP* during these batch experiments (Fig. 3a). In contrast, the transport of the neutral leucine molecule decreased when a lower *TMP* was applied probably due to a lower water flux. Operation at a low *TMP* thus resulted in a higher lysine fraction in the permeate (Fig. 3b). At a *TMP* of 0.5 bar a lysine fraction, *f*, as high as 0.96 was obtained (Fig. 3b) starting from a lysine fraction in the feed of 0.5 (Fig. 3a). This implies that the selectivity of the process is very high. Increasing the *TMP* to 2.0 bar resulted in a reduction of the lysine fraction of about 0.1 to approximately 0.86. In both situations the lysine concentration in the permeate was higher than in the feed solution. Lysine could thus be isolated and concentrated simultaneously.

Reduction of the potential difference set-point from 40 V to 20 V reduced the lysine transport more than twofold (Fig. 4a). The relatively high lysine transport rate reduction was in line with a relatively high change in the measured potential difference over the membrane. On the basis of this observation and the independence of the lysine transport on the *TMP*, we conclude that the driving force for transport of the positively charged lysine was mainly governed by the electrical field strength as intended. Daufin et al. [2] described the transport of charged particles in terms of the ratio of the electromigration over the flux (UE/J, where U, E and J are the electrophoretic mobility, the electrical field strength and the flux). On the basis of water fluxes reported for NTR 7450 using 0.01 mol·L⁻¹ and 0.1 mol·L⁻¹ leucine or lysine solutions [18] a UE/J ratio in excess of 45 is estimated for lysine transport at a potential difference set-point of 40 V and 2 bar operating pressure. This supports our observation, that the lysine transport is mainly governed by electromigration and thus the electrical field strength in the feed/concentrate compartment. However, the use of this ratio implicitly assumes that diffusion and the electro-osmotic flow
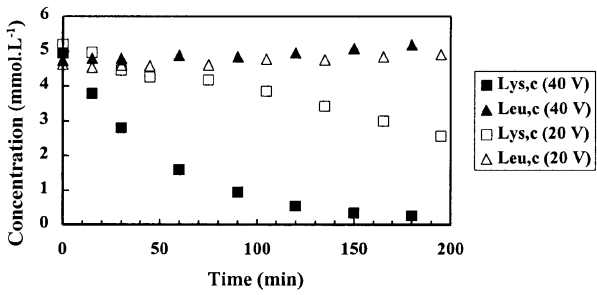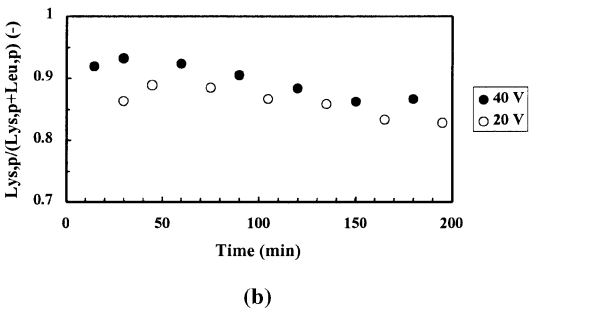
(a)

(b)

Figure 3. The effect of the *TMP* on the leucine and lysine transport from the concentrate to the permeate (**a**) and the lysine fraction in the permeate (**b**). (Leu,c and Lys,c: leucine and lysine concentration in concentrate, Leu,p and Lys,p: leucine and lysine concentration in permeate).

(a)

(b)

Figure 4. The effect of the potential difference on the leucine and lysine transport from the concentrate to the permeate (**a**) and the lysine fraction in the permeate *f* (**b**) at *TMP* = 2.0 bar (Leu,c and Lys,c: leucine and lysine concentration in concentrate, Leu,p and Lys,p: leucine and lysine concentration in permeate).
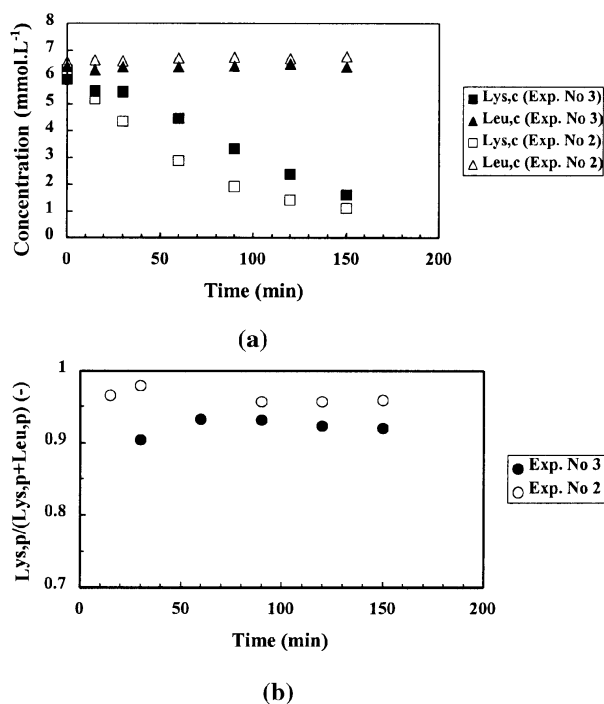
Figure 5. The effect of the initial permeate conductivity on the leucine and lysine concentrations in the concentrate (**a**) and the fraction *f* in the permeate (**b**) at *TMP* = 0.5 bar and 40 V (description exp. 2 and 3 as listed in Tab. I).

can be neglected. Since the observed flux through the membrane at *TMP* = 2 bar for operation without an external electrical field was below 7 L·m^{-2}·h^{-1} [18] it is questionable whether diffusion can be neglected entirely in relation to convection. The relative importance of the electric field over the applied pressure difference for lysine transport may therefore be overestimated by the high ratio UE/J. The leucine transport rate was also slightly lower for operation at low electrical field strength, possibly due to a lower electro-osmotic flow. The reduction in potential difference set point from 40 to 20 V consequently resulted in a lysine fraction decrease of 0.04 (Fig. 4b). This difference in the lysine fraction in the permeate f reduced with progressing of the runs. Due to a higher initial transport rate for lysine at a high electrical field strength, the lysine concentration in the concentrate was reduced faster during batch wise operation. As a result the lysine transport rate for operation at higher electrical field strength was reduced more with progressing of the run. In combination with a relatively stable leucine concentration in the concentrate and leucine transport to the permeate, the lysine fraction in the permeate was reduced with progressing of the run, especially for operation at high potential difference set point. Although the pH of the permeate was not adjusted at the low potential difference, this most likely did not affect the observed change in lysine transport rate and lysine fraction in the permeate, as explained earlier.

Operation with 0.01 mol·L^{-1} Na$_2$SO$_4$ in the permeate compartment at start of the run resulted in a significantly higher lysine transport rate (Fig. 5a) than operation with RO de-min. water. The use of a permeate with higher conductivity enhanced the lysine transport rate as a result of an increase in electric field strength in the feed/concentrate compartment at an unchanged potential difference set-point over the electrodes and thus a higher driving force for transport of
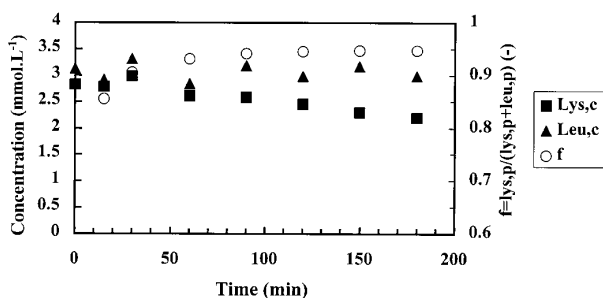
Figure 6. Leucine and lysine concentrations in the concentrate and the lysine fraction in the permeate, f, for EMF of casein hydrolysate at *TMP* = 0.05 bar and 40 V.

charged components (e.g. [20]). The leucine transport rate was hardly affected by the permeate conductivity at start of the run, as expected. Consequently the lysine fraction, f, was significantly higher, when filtration was started with a liquid with high conductivity (0.01 mol·L^{-1} Na$_2$SO$_4$ solution) in the permeate chamber (Fig. 5b). However, since the leucine concentration relative to the lysine concentration in the permeate was very low for both cases, the difference in lysine fraction was less than $f = 0.05$ (Fig. 5b).

3.2. Separation of leucine and lysine from a casein hydrolysate

Lysine and leucine could also be separated from a casein hydrolysate at a lysine fraction in the permeate as high as $f = 0.95$ (Fig. 6). This fraction is similar to the maximum fraction obtained with our leucine/lysine model solution, starting with similar lysine fractions in the feed of 0.5. The permeate concentrations of all the other amino acids, apart from arginine were similar to that for leucine. This observation is in line with conclusions from Daufin et al. [2], who studied the separation of amino acids from a model solution. Lysine can thus be removed from the casein hydrolysate with a high selectivity. However, the lysine transport rate through the membrane was substantially lower than found for our model solution. The transport rate can be improved by optimisation of the process conditions

(e.g. loading the permeate compartment with a solution with higher conductivity before the start of the run).

3.3. Isolation of cationic peptides from a lactoferrin hydrolysate

Cationic peptides could be separated successfully from a LF hydrolysate. The HPLC analysis of the total permeate collected after 180 min of operation revealed a seven-peak profile (Fig. 7). Five peptides (Tab. II) had been previously found in an antibacterial fraction obtained from a LF hydrolysate by cation-exchange chromatography [11], including the potent bactericidal peptide Lfcin-B (fragments 17–41 or 17–42 of LF) [1]. Interestingly, the combination of the N-terminal sequence and MS data revealed that the peptides eluting at 41.2 min also correspond to the fragments identified as Lfcin-B, but in the oxidised form (Met→ MetO). All the identified peptides in the EMF permeate had a net positive charge. At least three of the identified peptides, the heterodimer with mass 2430 (Tab. II) and fragments 17–41 and 17–42 (Lfcin-B), have been shown to be antibacterial [1,3,11]. The Lfcin-B transport rate was relatively low (Fig. 8). Nevertheless, we have shown that with EMF, cationic peptides can be separated selectively from a LF hydrolysate with a product composition similar to that obtained by cation-exchange chromatography [11]. Operating conditions can be further optimised to improve the separation efficiency and transport rate.
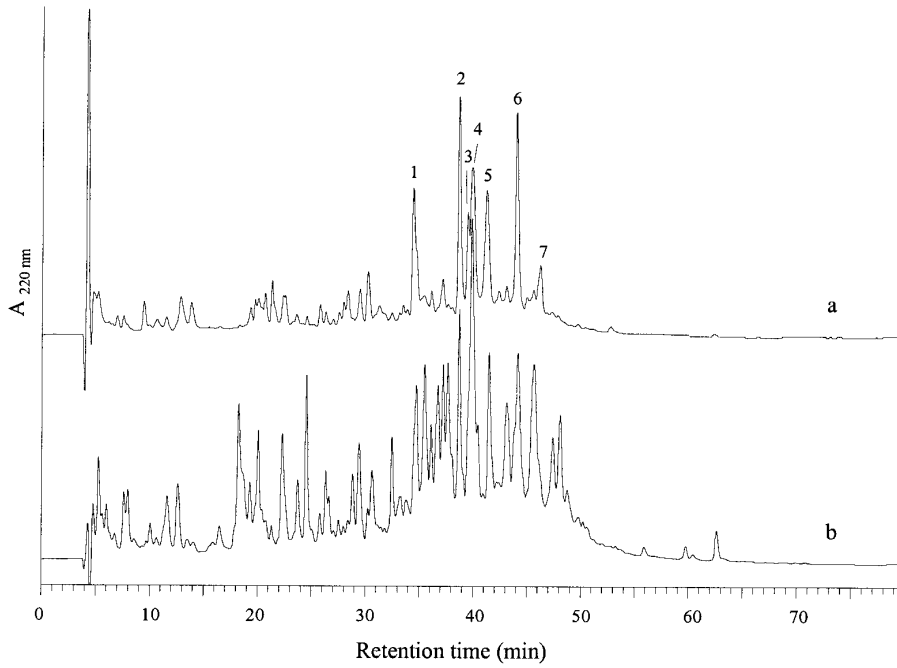
Figure 7. Reversed-phase HPLC chromatograms of the permeate obtained after 180 min EMF (**a**) and the LF feed solution (**b**) (Tab. II).

Table II. Mass determination and peptide identification of the peptic LF fragments obtained in the permeate compartment after EMF during 180 min.

Peak No.[e]	Retention time	Observed mass	Theoretical mass[a]	Net charge	Sequence position[b]	Identified by
1	34.3	2096	2096.4	+4	271–288	This work[c]
2	38.6	1568	1568.8	+2	618–631	This work[d]
		4276	not identified			
3	39.4	2430	2430.8	+3	(1–16)-S-S-(45–48)	[11]
4	39.9	2248	2248.7	+5	267–285	[11]
5	41.2	3140	3139.8	+8	17–41 (oxidised Met)	This work[d]
		3211	3210.9	+8	17–42 (oxidised Met)	
6	44.0	3123	3123.8	+8	17–41	[1, 3, 11]
		3194	3194.9	+8	17–42	
7	46.2	2636	2637.1	+5	267–288	[11]

[a] Average mass values.
[b] LF sequence [8].
[c] Identified by electrospray ionization–tandem mass spectrometry.
[d] Identified by combining the N-terminal sequence and the molecular mass data.
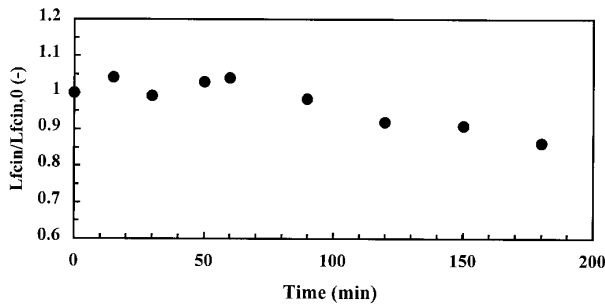[e] See Figure 7.

Figure 8. Reduction of the Lfcin-B concentration in the concentrate relative to the initial Lfcin–B concentration for EMF of LF hydrolysate at *TMP* = 0.05 bar and 40 V.

4. CONCLUSION

Electro-membrane filtration can be used to separate the amino acids leucine and lysine not only from model solutions, but also from a protein hydrolysate at a lysine fraction in the permeate of approximately 0.96. This implies that the selectivity for the separation of leucine and lysine with EMF is very high. A transmembrane pressure increase from 0.5 to 2.0 bar results in a reduction of the lysine fraction of about 0.10 to 0.86 at practically unchanged lysine transport rate. An increase in the applied potential difference from 20 to 40 V results in a more than twofold increase in lysine transport rate and a lysine fraction increase of 0.04 in the permeate. Increasing the conductivity of the solution in the permeate compartment at the start of the run results in a higher lysine transport rate and a higher lysine fraction in the permeate.

With EMF a fraction enriched in antibacterial cationic peptides can be produced from a LF hydrolysate. The composition of the fraction produced is similar to that obtained with classical cation-exchange chromatography.

ACKNOWLEDGEMENTS

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