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Selective Removal of Hydrophobic Peptides from Protein Hydrolysates in a Continuous Supported Liquid Membrane Process

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ABSTRACT

The removal of hydrophobic peptides from a casein hydrolysate solution through a supported liquid membrane (SLM) was investigated in a continuous flow system. The liquid membrane was formulated with an anionic surfactant (Aerosol OT) as the carrier and oleyl alcohol as the organic phase on a Celgard 2500 as support. A mixture of amino acid (tryptophan) and dipeptide (tryptophan-leucine) was examined to determine the pH at which the transport of peptide is preferentially faster than that of the amino acid. At this feed pH the effects of the following variables were examined: hydrolysate concentration, feed and strip phase flow rate, and carrier concentration in membrane preparation. The stability of SLM for long-term continuous operation was studied, and the regeneration of SLM was tried for repeated use. It was demonstrated that the following conditions are favourable for the selective removal of peptides: 1) a feed solution pH of 4.5, 2) a dilute feed concentration (<20 g/L), 3) slower feed and strip flow rates (<20 mL/h), 4) a liquid membrane prepared with 10–20% carrier, and 5) a regeneration scheme after every 24 hours.

INTRODUCTION

Protein hydrolysates are mixtures of peptides and amino acids formed by hydrolysis with either acid or proteolytic enzymes. They have different functional and nutritional properties depending on the method of hydrolysis (1). They are used in the preparation of infant food formulas and speciality products for sports nutrition and some pharmaceutical products (2,

3). The commercial use is mainly limited to whey hydrolysates, which are partly or moderately hydrolyzed (4). Casein hydrolysates could also be utilized for this purpose. However, if the extent of hydrolysis exceeds approximately 5%, hydrophobic peptides are likely to appear and these produce a bitter taste, which limits use of overhydrolysated casein in food products (5–7). The bitterness is considered to be dependent on the hydrophobicity of the amino-acid side chains and believed to be due to small peptides (8–10). The removal of bitter hydrophobic peptides from protein hydrolysates, especially casein hydrolysates, is necessary to widen their application to nutritional food products and medical diets where extensively hydrolyzed proteins are desirable (2, 3, 11).

There are various techniques reported in the literature for separation of peptides from solutions of protein hydrolysates (12–15). These techniques are based on 1) hydrolysis with a mixture of enzymes or acids, 2) use of hydrophobic interaction chromatography, and 3) plastein reaction. These processes suffer from a number of disadvantages:

- Operational problems in the continuous mode
- Difficulties in regeneration of the support media
- Modification of some essential components
- High cost involved in processing

The membrane-based method offers a potentially better alternative for the selective removal of bitter hydrophobic peptides (16–18). In order to explore the applicability of this technology, a comprehensive investigation has been undertaken. In previous papers (19, 20) the effect of hydrophobicity of the amino acid side chains on the transport rate in small-scale batch experiments was determined, and it was shown that:

- Peptide transport can be facilitated by supported liquid membrane (SLM)
- Hydrophobicity of the peptide influences the transport rate
- Selectivity of peptide transport can be controlled by feed pH and membrane composition

Other researchers (21, 22) have found that the selectivity of transport is influenced by such conditions as pH, ionic strength, and type of liquid membrane.

Research was continued to develop an effective separation process to remove peptides and thus to increase the value and quality of protein hydrolysates because of its economic importance. The purpose of the present paper is therefore to present some results of a continuous flow SLM system used to investigate the following variables:

1. pH of feed solution
2. Concentration of hydrolysate in feed samples
3. Flow rate of feed and strip solutions
4. Concentration of carrier in liquid membrane
5. Stability, regeneration, and sanitization of SLM

PROCESS MECHANISM

The overall removal of peptide from the bulk feed to the strip solution can be described by the following transport and reaction processes.

1. Transport of solutes (amino acids and peptides) from the bulk flowing phase to the aqueous boundary layer, and diffusion through it to the feed–membrane interface
2. Chemical reaction at the interface to form a complex (or complexes) with the carrier
3. Diffusion of the carrier–solute complex through the membrane
4. Decomplexation reaction at the strip phase–membrane interface with the liberation of solutes
5. Diffusion of empty carrier back to the feed–membrane interface
6. The released solutes diffuse through the stripside boundary layer to the bulk strip solution where they are extracted from the system

Casein hydrolysate is a complex mixture of amino acids and peptides. Each of the species will undergo all of the above steps in a competitive manner, with the more hydrophobic ones removed selectively at a faster rate in the strip solution.

The reaction between a peptide and a carrier molecule is explained by considering tryptophan-leucine (Trp-Leu as peptide) and Aerosol OT (AOT as carrier). The carrier is negatively charged, and it must be neutralized to pass through the membrane. This is achieved by the carrier's association with either Na^+ or positively charged Trp-Leu. Each peptide has two types of functional groups: carboxylic (pK_1) and amino (pK_2). These groups are affected by the pH, and to ensure that these molecules are positively charged, the pH is chosen such that $pH < (pK_1 + pK_2)/2$. All the peptides in the casein hydrolysate follow his mechanism. The processes for the transfer of $Trp\text{-}Leu^+$ from the feed solution (subscript "f") through the membrane-feed side (subscript "mf") through the membrane-strip side (subscript "ms") to the strip solution (subscript "s") can be represented by the following set of equations:

$$\text{Trp-Leu}_f^+ + (\text{Na}^+\ \text{AOT}^-)_{mf} \rightleftarrows \text{Na}_f^+ + (\text{Trp-Leu}^+\ \text{AOT}^-)_{mf} \quad (1)$$

extraction

$$(\text{Trp-Leu}^+\ \text{AOT}^-)_{mf} \rightleftarrows (\text{Trp-Leu}^+\ \text{AOT}^-)_{ms} \quad \text{membrane transport} \tag{2}$$

$$\text{Na}_s^+ + (\text{Trp-Leu}^+\ \text{AOT}^-)_{mf} \rightleftarrows (\text{Na}^+\ \text{AOT}^-)_{mf} + \text{Trp-Leu}_s^+ \quad \text{stripping} \tag{3}$$

The complex ($\text{Trp-Leu}^+\ \text{AOT}^-$) under its own concentration gradient diffuses to the stripside, where Trp-Leu^+ is replaced by Na^+ ions. The peptide is released in the strip solution and the carrier diffuses back to the feedside of the membrane. The total effect of this shuttle mechanism is the transport of Trp-Leu from the feed solution to the strip solution and countertransport of Na^+ from the strip to the feed solution. The chemical potential gradient (the driving force of the removal process) is ensured by a Na^+ concentration difference between feed and strip solutions.

Amino acids are also transported across the membrane according to the above mechanism. Since pK_1 and pK_2 of an amino acid are different from those of a peptide, it is possible to remove peptides preferentially by controlling solution pH (20).

MATERIALS AND METHODS

The amino acid, L-tryptophan, and the peptide, tryptophan-leucine, were obtained from Sigma Chemical Co. The Chemicals-Aerosol OT (carrier), sodium dihydrogen orthophosphate, and disodium hydrogen orthophosphate were purchased from BDH Co. The membrane solvent oleyl alcohol (Aldrich Co.) and the phosphate buffer materials—sodium acetate and 85% orthophosphoric acid—were from Ajax Chemicals, Australia. Absolute alcohol was from Rhone-Poluene Lab Products (Australia), and sodium chloride (Regular) was from Prolabo (France). Casein hydrolysate was a gift from New Zealand Dairy Board. The commercial membrane support Celgard 2500 was a gift from Hoechst Celanese separation products, Charlotte, NC, USA.

Preparation of Supported Liquid Membrane

The preparation procedure was exactly the same as previously described (19, 20). The SLMs were prepared by soaking Celgard 2500 support in 10% AOT solution in oleyl alcohol for 5–10 minutes followed by placing the contents under vacuum for about 30 minutes. The membrane was then rinsed with deionized water and gently blotted with tissue paper.

For regeneration, the SLMs were washed in 20% ethyl alcohol solution for 5–10 minutes followed by soaking in 100% ethyl alcohol solution for about 20 minutes. They were rinsed with deionized water and blotted

with paper. The membrane was impregnated with 10% AOT solution as described above.

Separation Equipment

The continuous membrane module for separation experiments was designed and fabricated according to the literature (23). It consists of two half-cell faceplates (10 cm diameter) between which the SLM is placed. Each faceplate contained a flow channel of 94 cm length, 0.16 cm depth, and 0.32 cm width. The feed and strip solutions were pumped into the respective channels by a Bio-Rad Econo pump. When the effect of flow rate was studied, another pump—a Micro Tube pump MP3 with a speed controller—was used for stripside solution. A schematic diagram of the separation equipment is shown in Fig. 1.

The hydrolysate feed samples were prepared in acetate buffer and clarified by centrifugation at 4000*g* for 6 minutes using a Sorval RC-SB refrigerated superspeed centrifuge (Dupont Instruments). The pH values of the feed and strip solutions were measured from time to time with a PHM-64 Research pH meter (Radiometer Co., Copenhagen). The conductivity of the strip solution at the exit of the module was measured continuously using a Bio-Rad Econo Gradient Monitor with a digital display device.

Transport Measurements

The transport of amino acids and peptides across the membrane was monitored by measuring the change in concentration of the initially pep-

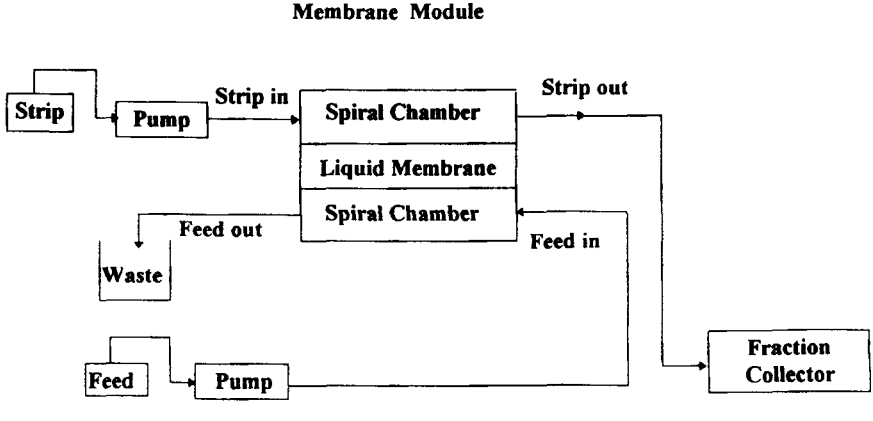

FIG. 1 Schematic of the experimental setup of a continuous supported liquid membrane process.

tide-free strip solution. Samples of strip solution were collected at regular time intervals in a fraction collector (Bio-Rad Model 2110 fraction collector). The absorbance of strip solution was measured spectrophotometrically in the range 200–300 nm using a UV-visible spectrophotometer (Shimadzu UV-160). The feed samples were diluted, and their absorbance values were also measured spectrophotometrically in the same range as above.

HPLC of the Hydrolysate Samples

The samples of the strip solution were analyzed for various components transported across the membrane using a chemstation method on a RP-HPLC system (HP-TI series 1050). The column size was 25 × 0.46 cm, and it was packed with Nucleosil C18 5μ particles (Alltech, USA). The elution system was made up of a combination of two solvents; solvent A: 0.05% aqueous trifluoroacetic acid, pH 1.6, and solvent B: 0.05% trifluoroacetic acid in water/MeCN, 10/90. The solvent gradient was 0–2 minutes 0% B, 11–14 minutes 19% B, 26 minutes 80% B, and 28 minutes 0% B. An operating temperature of 20°C, a flow rate of 1 mL/min, and a sample volume of 50 μL were used for detection at 280 nm.

Calculation of Flux and Percentage of Feed Removed

The flux rate was calculated for the mixture of Trp and Trp-Leu from the measurements of absorbance at the inlet and outlet of the stripping solution using the following equation:

$$J_P = \frac{V_s(C_{P,o} - C_{P,i})}{A} \tag{4}$$

where J_P is the flux rate of Trp-Leu (or Trp) in mmol/(m^2·h), V_s is the strip phase flow rate, $C_{P,o}$ (or $C_{A,o}$) and $C_{P,i}$ (or $C_{A,i}$) are the outlet and inlet concentrations, respectively, of the referred component (Trp-Leu or Trp), and A is the effective surface area of the membrane.

The flux to the strip solution for the casein hydrolysate feed was calculated from the following expression

$$TJ_c = \frac{V_s(Abs_o - Abs_i)C_{F,i}}{Abf_iA} \tag{5}$$

where TJ_c is the total flux of solutes in g/(m^2·h).

The percentage of solutes removed from the feed was calculated from the UV absorbance values of the feed and strip solutions. This is denoted by PR (%) and calculated from the following

$$PR\ (\%) = \frac{(Abs_o - Abs_i)V_s}{Abf_i V_f} \times 100 \quad (6)$$

where V_f is the flow rate of the feed solution.

RESULTS AND DISCUSSION

Initially the transport experiments were carried out to determine the range of pH, where the transport of peptides is faster than that of amino acids. This was done by observing the transport rates of Trp-Leu and Trp from their mixture at various pH values (20). The SLM characteristics and the experimental operating conditions are listed in Table 1. Once the optimum pH was determined, the experiments with casein hydrolysate were conducted at that feed solution pH. All the experiments (except for

TABLE 1

(a) Supported Liquid Membrane Characteristics

Support:	Celgard 2500
Porosity:	37–48%
Pore dimensions:	0.05 × 0.19 μm
Thickness:	20 μm
Liquid membrane:	10% AOT solution in oleyl alcohol

(b) Experimental Conditions for Peptide and Amino Acid Mixture

Feed phase:
1 mM
1 mM Trp or Trp-Leu in 0.1 M acetate-phosphate buffer
pH range = 3–6
Flow rate = 10 mL/h

Strip phase:
0.1 M phosphate buffer in 1 M sodium chloride solution
pH = 5.5
Flow rate = 10 mL/h
Temperature of both phases = 293 K

(c) Experimental Conditions for Casein Hydrolysate (MPH 955)

Feed phase:
5.0–80 g/L casein hydrolysate in 0.1 M acetate-phosphate buffer solution
pH range = 3–6
Flow rate = 20 mL/h

Strip phase:
0.1 M phosphate buffer in 1 M sodium chloride solution
pH = 5.5
Flow rate = 20 mL/h
Temperature of both phases = 293 K

stability experiments) were carried out until an initial steady state was reached, which was confirmed by the little change in absorbance values over a period of time. The effects of various parameters and the HPLC analysis of the removed solutes are presented below.

Feed Solution pH

A Mixture of a Peptide (Trp-Leu) and an Amino Acid (Trp)

The feed solution pH was varied from 3.0 to 6.0 for a 1:1 mixture of Trp and Trp-Leu, and the change in strip phase concentration was followed. The fluxes of Trp and Trp-Leu through the membrane at various pH values are shown in Fig. 2. The transport rate of Trp decreased sharply when the pH was increased more than 3 whereas that of Trp-Leu remained almost constant up to pH 4.5, after which it also decreased. This could be due to the fact that pK_1 (for the carboxylic group) of Trp-Leu is greater than that of Trp (17), so the cations present in the solution are more from Trp-Leu and are transportable by an anionic carrier like AOT. At pH 4.5 the transport rate of Trp-Leu was much higher than that of Trp, and therefore this pH could be used for selective removal of peptides from casein hydrolysate solution.

A Solution of Casein Hydrolysate

The effect of varying the solution pH for 10 g/L of casein hydrolysate feed was assessed by the change of solute flux in the strip solution. The

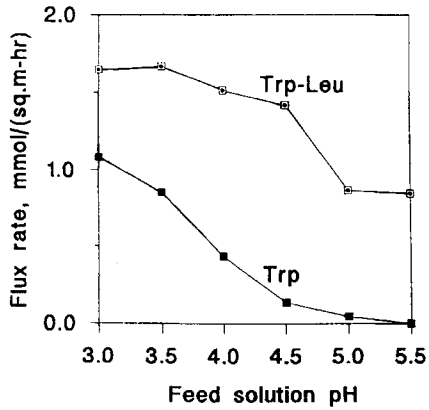

FIG. 2 The effect of feed solution pH on the transport rate of Trp and Trp-Leu from an equimolar mixture.

flux was calculated from the initial steady-state strip solution concentrations, obtained from the UV absorbance values. The change in absorbance with time for various feed concentrations are shown in Fig. 3. The absorbance increased with time and attained a constant value after 40–50 minutes, indicating a steady state has been reached. This steady-state absorbance value increased with initial feed concentration, suggesting that the transport process was unsaturated and that a higher feed solution concentration could be tolerated.

The variation of the solute flux (TJ_C) and the percentage of solutes removed (*PR %*) with feed solution pH are shown in Fig. 4. Both of these process characteristics decreased with pH, possibly due to the decrease in available concentration of amino acids and peptides as the feed pH was increased toward their pI values (about 5.0 for most of them). However, a pH of 4.5 was chosen for the subsequent experiments in order to remove hydrophobic peptides selectively.

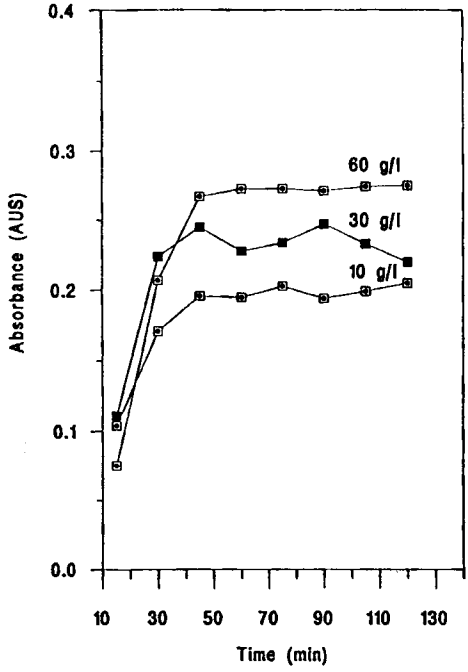

FIG. 3 The UV absorbance–time relationship of the strip solution for a 10 g/L of casein hydrolysate feed at pH 4.5.

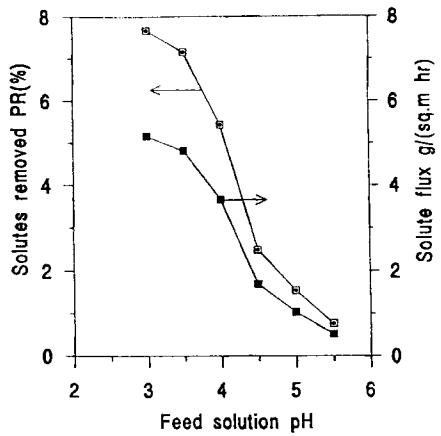

FIG. 4 The effect of feed solution pH on the solute flux and the percentage of solutes removed (*PR* %) for a 10 g/L of casein hydrolysate feed.

HPLC Analysis of the Feed and Strip Solution

The feed and strip solutions were analyzed using RP-HPLC to determine the solutes transported across the membrane. The chromatogram of the initial feed solution (10 g/L) is shown in Fig. 5(a), and the chromatogram of the strip solution (corresponding to the above feed) is shown in Fig. 5(b). From the analysis of the areas of these chromatograms, we calculated percentages of the solutes removed to the strip solution; these are 23.7% for component 1 (retention time 11.6 minutes), 24% for component 2 (retention time 17 minutes), and 56.8% component 3 (retention time 21.0 minutes), and 14.7% for component 4 (retention time 22.2 minutes). From a comparison with the standard chromatograms it can be suggested that the solutes so far determined are tryptophan and tryptophan-leucine with retention times of 11.6 and 17 minutes, respectively. They have been shown to be hydrophobic (19), and the other components removed (yet to be identified!) are believed to be hydrophobic as they elute at a longer retention time.

Effect of Hydrolysate Concentration in the Feed

The effect of feed hydrolysate concentration on the total flux and on the removal of solutes are shown in Fig. 6. In the region of low concentration (<30 g/L), the solute flux increased sharply with the feed concentration, possibly because there were plenty of carrier molecules available to

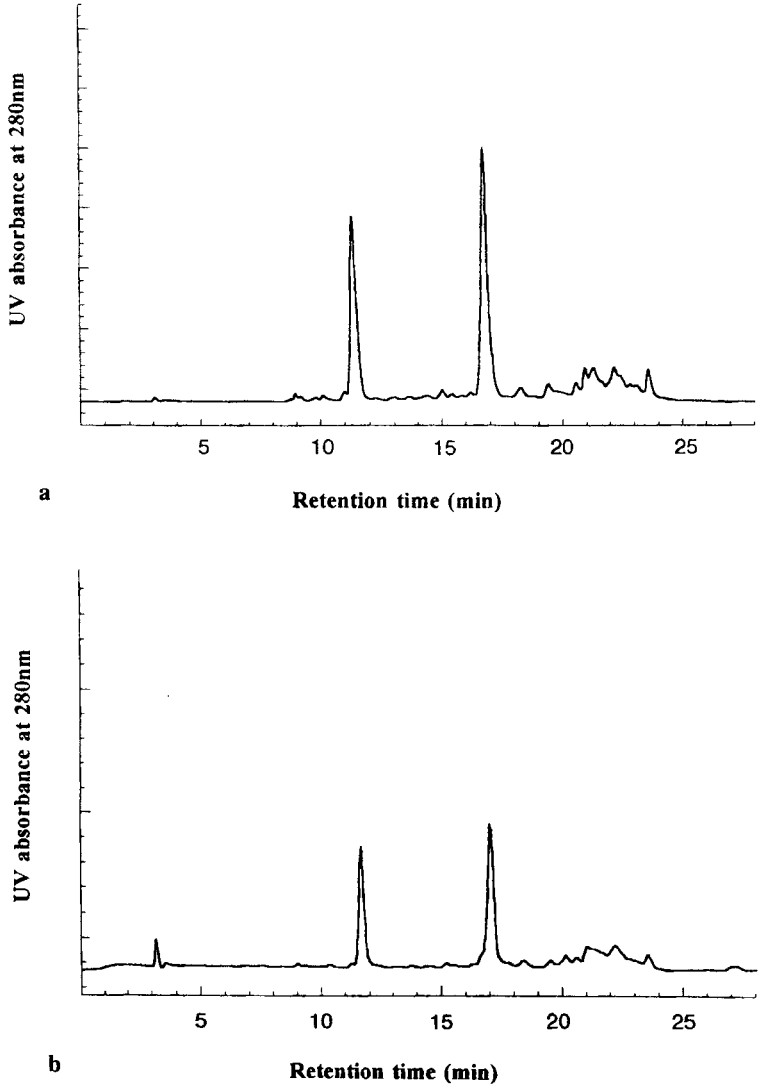

FIG. 5 (a): The HPLC of the initial casein hydrolysate solution (10 g/L). (b): The HPLC of the strip solution after 60 minutes of separation experiment with the feed in (a).

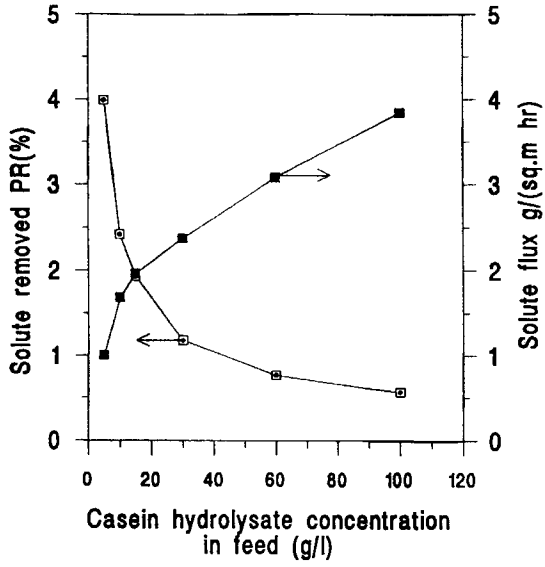

FIG. 6 The effect of feed concentration on the flux through the SLM and on removal of solutes from casein hydrolysate solution.

transport more hydrolysate solutes. Beyond this feed concentration, the rate slowed down to approach a constant value, where the membrane was supposed to be saturated and there were no more carrier molecules left for further reaction. The percentage of solutes removed from the feed, *PR* (%), decreased rapidly with the increasing feed concentration because of the intense competition between the reacting solutes. For feed concentrations of 60 g/L and beyond, the solutes removed were very low, about 0.6–0.8% of total solutes in the feed.

Similar results, i.e., the increase of transport rate with feed concentration, were reported for transport through a SLM in a continuous system (20, 24, 25).

Effect of the Flow Rate

The effect of flow rate on the removal of peptides was studied by varying it as 10, 15, 20, 30, 40, 50 mL/h and maintaining a constant ratio of the feed and strip flow. The flux and the solute removed from the feed, *PR* (%), are plotted in Fig. 7. Both of these characteristics decreased with the flow rate up to 40 mL/h; for higher flow rates, they approached a

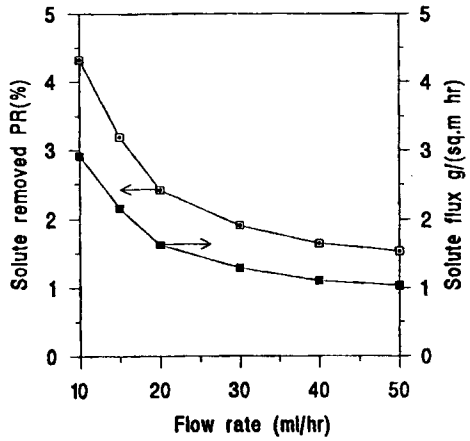

FIG. 7 The effect of flow rate on the removal of solutes at a constant ratio of feed and strip flows for a casein hydrolysate feed of concentration 10 g/L.

constant value. This behavior can be interpreted in terms of the effect of the boundary layer close to the surface of the SLM. At a low flow rate (i.e., <40 mL/h) the overall process is affected by the transport though the boundary layer; at a higher flow rate this effect is negligible and the process is limited by the internal diffusion of the solute–carrier complex through the membrane.

Since the removal process is continuous, it is affected by the variation of the residence time (defined as the ratio of the chamber volume to the flow rate) in the transport system. A change of flow rate from 10 to 40 mL/h corresponds to an alteration of residence time from 28 to 7 minutes. Time to reach steady-state conditions required about 25–30 minutes of continuous operation. Therefore, constant results could not be expected when the flow rate was increased as the steady regime was not attained. An increase in flow rate caused a decrease in the solute flux, and consequently *PR* (%) was reduced. Similar results were reported for the transport of pure components through SLM systems (20, 21, 26, 27).

Effect of Carrier Concentration

The effect of carrier concentration (% w/w AOT in oleyl alcohol) on the solute flux to the strip solution at various feed carrier concentrations is presented in Fig. 8. The flux increased more significantly at lower values of AOT (<20% AOT) and at higher feed concentrations (≥20 g/L). At

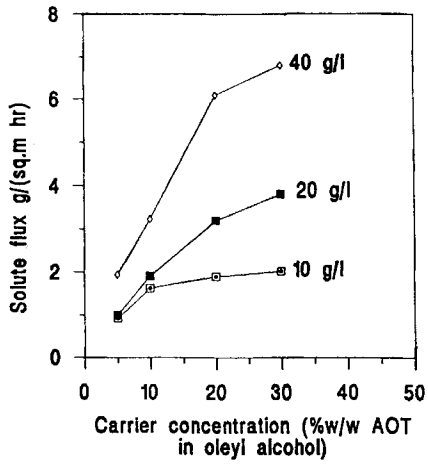

FIG. 8 The effect of carrier concentration (%w/w AOT in oleyl alcohol) on the solute flux to the stripside solution.

lower feed concentrations ($\leq$10 g/L) the flux increase was observed up to 10% AOT, and beyond this it was insignificant. One possible reason could be the saturation of SLM at this combination of carrier and feed concentrations. At higher feed concentrations ($\geq$20 g/L) the flux increase was observed up to 20% AOT, after which the rate slowed down. This increase was due to the increase of the concentration gradient of the carrier–solute complex/complexes inside the membrane with the increasing carrier concentration. At very high carrier concentrations the rate slowed down due to the negative effect of increased viscosity on diffusivity, resulting in reduced flux (28).

Degradation and Regeneration of SLM

Figure 9 shows the stability of the SLM during continuous removal of peptides from hydrolysate samples. The absorbance of the strip solution reached a maximum value and then decreased at a slow rate (about 1% every hour), suggesting a stable SLM over a period of 20 hours. Thereafter they started declining faster and reached a value of one-third the maximum after 42 hours of continuous operation. The decline in flux of casein hydrolysate was much faster than in the case of a single/binary mixture (20). This is the effect of a real feed with more than 20 components!

The regeneration was done by testing two different steps in order to ensure that all the organics are removed before contacting with AOT solu-

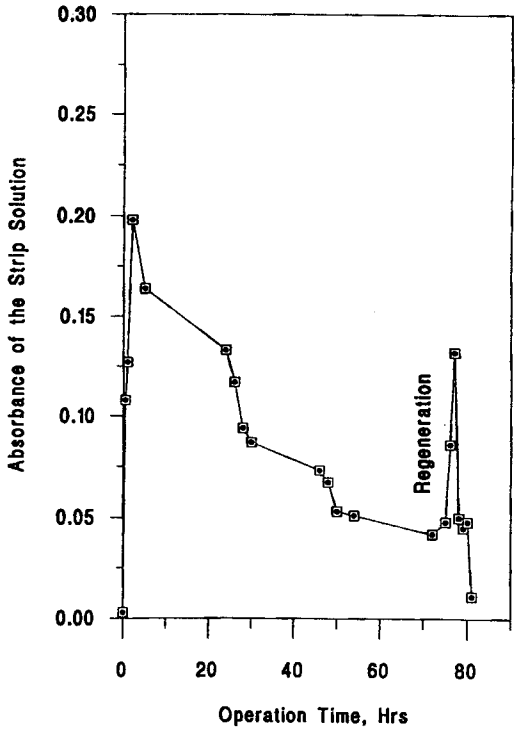

FIG. 9 Stability of the supported liquid membrane as fresh and after regeneration with 20% ethanol for a casein hydrolysate feed of 10 g/L.

tion. For regeneration of the membrane the Celgard support was washed with 20% ethanol and contacted with 10% AOT in oleyl alcohol solution. The regenerated SLM recovered its performance substantially, but only for a short period of time, and within a few hours it was completely degraded (Fig. 9).

The regeneration procedure was considerably improved if the support was washed with 20% ethanol followed by a wash of 100% ethanol (Fig. 10). The regenerated membrane performance was as good as the original one, and this was maintained over a period of about 20 hours.

The instability of SLM could be due to the gradual and continual loss of AOT and/or oleyl alcohol from the support during continuous operation. This loss, along with adsorption of any solute from the casein hydrolysate feed, could have resulted in the decline of flux after some time.

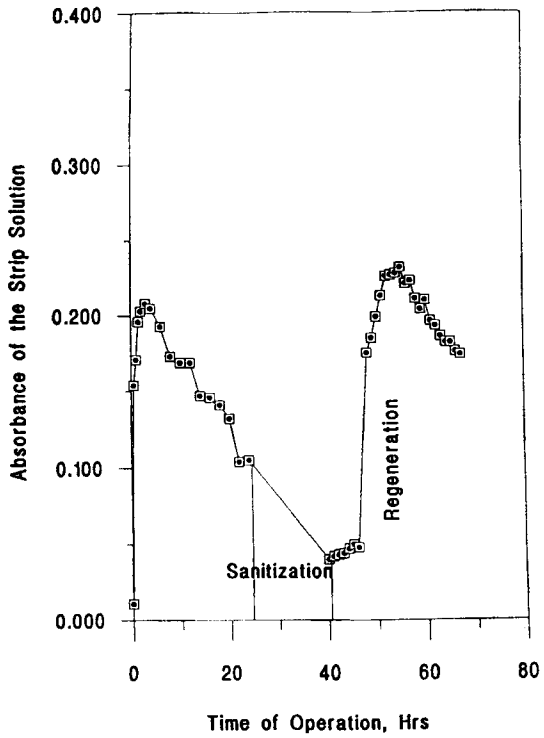

FIG. 10 Stability of the supported liquid membrane as fresh and after regeneration with 20% ethanol followed by 100% ethanol for a casein hydrolysate solution of 10 g/L.

The membrane phase—a solution of oleyl alcohol and AOT—exhibits low solubility in aqueous media, and in the presence of low salt concentrations (1 M NaCl was used as the strip solution) they could leak little by little (29, 30), especially when the feed and strip solutions are not saturated with these chemicals (as in our experiments). The loss of AOT and oleyl alcohol can be quantified by colorimetric methods based on ion-pair extraction (31, 32).

The loss in performance of SLM could also be due to the formation of small emulsion droplets when the feed solution is flowing along the surface (33, 34). The stability of SLM can be improved by applying plasma polymerization and liquid gelling techniques to the liquid membrane system (35).

Experiments are being carried out to study and improve the stability of SLMs by 1) determining the leakage rate of solvent and carrier from

the membrane phase and 2) applying a gel network in the pores of the support or applying a gel layer to the feed side. The results of these investigations will be published in a later communication.

The system was sanitized overnight with 0.1 M NaOH from 24–40 hours (Fig. 10). The loss of performance was similar to the situation of normal operating conditions of processing where it was not sanitized (Fig. 9). The sanitization prevented the growth of any microorganisms inside the system.

CONCLUSIONS

A continuous-flow countercurrent membrane module (a supported liquid membrane with a spiral flow path) with an AOT–oleyl alcohol membrane supported on Celgard 2500 has been demonstrated to successfully remove peptides from casein hydrolysate solution. The SLM preparation procedure used food industry acceptable chemicals (a very low concentration of AOT in food products is allowed by FDA). The removal performance can be enhanced by choosing the following operating conditions

- A feed solution pH of 4.5.
- A slow flow rate of feed and strip solutions (<20 mL/h)
- An increasing carrier concentration (% AOT in oleyl alcohol) up to a value of 20%
- A dilute feed concentration (<20 g/L)

The SLM system can treat casein hydrolysate feed (continuously) and stable over a period of 24 hours. It can be easily regenerated, and the regenerated SLM performs as good as the original one over a similar period of time.

ACKNOWLEDGMENT

The financial assistance of the Foundation for Research Science and Technology (FRST), New Zealand, is gratefully acknowledged.

SYMBOLS

A	surface area (m^2)
Abf	UV absorbance of feed solution
Abs	UV absorbance of strip solution
AOT	Aerosol OT, sodium di-2-ethylhexyl sulfosuccinate
C_A	concentration of (Trp) amino acid (mmol/mL)
C_F	concentration of casein hydrolysate in feed solution (g/L)

C_P	concentration of (Trp-Leu) peptide (mmol/mL)
J	flux for amino acid or peptide feed based on the area of spiral chamber, defined in Eq. (4) (mmol/m^2/h)
TJ	flux for casein hydrolysate feed, defined in Eq. (5) (g/m^2/h)
Trp	tryptophan
Trp-Leu	tryptophan-leucine
V	flow rate (mL/h)

Subscripts

A	amino acid
C	casein hydrolysate solution
f	feed solution
i	inlet of the membrane module
mf	membrane–feed side interface
ms	membrane–strip side interface
o	outlet of the membrane module
P	peptide
s	strip solution

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