Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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To cite this Article Tsuru, Toshinori , Shutou, Takatoshi , Nakao, Shin-Ichi and Kimura, Shoji(1994) 'Peptide and Amino Acid Separation with Nanofiltration Membranes', Separation Science and Technology, 29: 8, 971 — 984
To link to this Article: DOI: 10.1080/01496399408005611
URL: http://dx.doi.org/10.1080/01496399408005611

SEPARATION SCIENCE AND TECHNOLOGY, **29**(8), pp. 971–984, 1994

Peptide and Amino Acid Separation with Nanofiltration Membranes

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ABSTRACT

Several nanofiltration membranes [UTC-20, 60 (Toray Industries), NF-40 (Film-Tech Corporation), Desal-5, G-20 (Desalination Systems), and NTR-7450 (Nitto Electric Industrial Co.)] were applied to separate amino acids and peptides on the basis of charge interaction with the membranes since most of them contain charged functional groups. Nanofiltration membranes having a molecular weight cutoff (MWCO) below 300 (UTC-20, 60, NF-40 and Desal-5) were not suitable for separation of amino acids. On the other hand, separation of amino acids and peptides with nanofiltration membranes having a MWCO around 2000–3000 (NTR-7450 and G-20) was satisfactory based on a charge effect mechanism; charged amino acids and peptides were rejected while neutral amino acids and peptides permeated through the membranes. Separation of peptides having different isoelectric points with nanofiltration membranes was possible by adjusting the pH.

INTRODUCTION

Low-pressure-type reverse osmosis membranes, which are commonly called nanofiltration membranes or loose reverse osmosis membranes (loose RO), have been developed recently in order to separate or concentrate solutes having molecular weights of 200–1000. Conventional reverse osmosis membranes have been developed to reject all solutes in the feed solutions, while ultrafiltration membranes are used for the concentration

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and separation of proteins and colloids which are relatively large molecules. The range of molecular weight cutoff (MWCO) of nanofiltration membranes is one of the features. Moreover, nanofiltration membranes have another striking difference from RO and ultrafiltration (UF) membranes; most of them have a charged layer as the separation zone since their skin layer is made of polyelectrolytes such as polyamide, sulfonated polyether sulfone, and so on. Typical nanofiltration membranes have been reported elsewhere (1–5).

Therefore, nanofiltration membranes have two separation mechanisms: molecular sieve effect and charge effect. Important parameters for solute separation include not only the differences of sizes between membrane pores and solutes, but also the charge polarities of membranes and solutes. Figure 1 shows the schematic separation mechanism of a nanofiltration membrane which is negatively charged. The driving force in nanofiltration separation is the pressure difference across the membrane similarly to RO or UF membranes, causing a volume flux through the membranes. This is extremely different from dialysis and electrodialysis using ion-exchange membranes, which have been developed in such a way as not to allow water to permeate through the membranes. In nanofiltration, solutes having sizes larger than the pore size of membranes cannot permeate through the membranes and are consequently rejected, while smaller solutes can permeate through the membranes. On the other hand, ions have electrostatic interaction with charged membranes; this phenomenon is called Donnan equilibrium. A repulsive force on anions and an attractive force on cations occur with negatively charged membranes. However, it should be noted that anions and cations cannot permeate the membrane independently but permeate the membranes while maintaining electroneutrality.

We have been working on charged membranes in reverse osmosis. First, we have developed charged UF membranes, and proposed transport equations based upon irreversible thermodynamics (6–8). Second, we have investigated separation of ion mixtures theoretically and experimentally

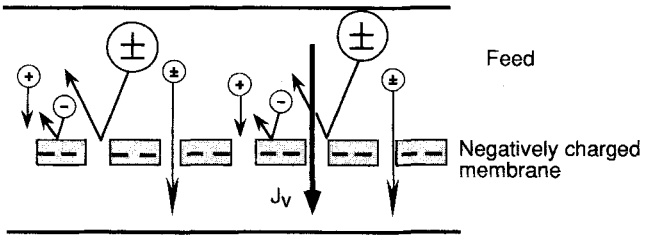

FIG. 1 Separation mechanism of charged reverse osmosis membranes.

(9–11). Third, we have proposed and developed bipolar reverse osmosis membranes having permselectivity of mono-ions over divalent ions (12).

Moreover, we have shown that amino acids and proteins can be separated by charged UF membranes (13, 14). Amino acids are electrically neutral at their isoelectric point (pI) and can be negatively or positively charged at higher or lower pH, respectively, since they have ionic functional groups such as carboxylic and amino groups. We have ultrafiltrated single solutions of amino acids and proteins and verified that the rejections changed according to pH. At pI, the rejections were almost zero since the solutes are electrically neutral and the size is much smaller than the pore sizes of the membranes used. At higher pH, where the solutes are negatively charged, they were rejected by the membranes. Mixtures of proteins and amino acids were also separated by negatively charged ultrafiltration membranes.

In this study the separation of peptides and amino acids was attempted experimentally using nanofiltration membranes. For this purpose the following were set. 1) Ultrafiltration experiments of single solutions, 2) pH variation experiments of single solutions, and 3) separation experiments of mixed peptides.

Peptides are intermediate materials between amino acids and proteins from the viewpoint of molecular weight; they can be defined as products of condensation between more than two amino acids. Some of them are very valuable as bioactive agents such as hormone and insulin. Since they are usually obtained in very dilute concentrations as bioproducts, it is very important to improve the separation or concentration process. This study will provide useful information for choosing the methods to separate or concentrate peptides, since very few papers have reported on the separation of peptides and amino acids with commercial nanofiltration membranes.

EXPERIMENTAL

Membranes

Several kinds of nanofiltration membranes were applied to separate peptides and amino acids as shown in Table 1; all of them are flat-sheet types of membranes. They are commercially available and were kindly supplied by their manufacturers. UTC-20 and 60 have skin layers made of polyamide, which are reported to be manufactured by an interfacial condensation reaction between acid and amine. The charges of these two membranes are characterized as amphoteric and negative, respectively, depending on residual unreacted functional groups (1, 5, 15). On the other hand, NTR-7450 has sulfonated polyether sulfone (2).

TABLE 1
Membranes Used in This Study

Membranes	UTC-20	UTC-60	Desal-5	NF-40	NTR-7450	G-20
Manufacturer	Toray Industries, Inc.	Toray Industries, Inc.	Desalination Systems, Inc.	Film Tec Co.	Nitto Electric Industrial Co.	Desalination Systems, Inc.
Materials of separation layer (5)	Polyamide	Polyamide	Thin film composite	Polyamide	Sulfonated polyether sulfone	Sulfonated polysulfone
pH	3–8	3–8	2–11	2–11	2–11	2–11
Maximum temperature (°C)	40	45	50	45	40	50
Maximum pressure (MPa)	4.2	2.8	2.8	4.1	3.0	4.2
Charge[a] at neutral pH	Amphoteric	Negative	Negative	Amphoteric	Negative	Negative
$L_p \times 10^5$ ($m \cdot s^{-1} \cdot MPa^{-1}$)	2.5	2.5	1.3	0.8	2.0	1.5
MWCO	≈200	≈200	≈300	≈300	≈1000	≈3000
NaCl:[b] R	0.23	0.58	0.49	0.35	0.35	0.13
J_v	10	13	4.2	2.6	8.2	6.2
Na_2SO_4:[b] R	0.93	0.97	0.99	0.98	0.85	0.54
J_v	6.2	9.0	2.9	1.8	6.0	5.0
$MgCl_2$:[b] R	0.77	0.65	0.56	0.75	0.10	0
J_v	7.0	11	3.4	1.9	7.0	6.0

[a] Charge was estimated from rejection data of NaCl, Na_2SO_4, and $MgCl_2$.
[b] Rejection (R) and permeate volume flux (J_v) were measured at the feed concentration of 30 $mol \cdot m^{-3}$ under a pressure difference of 0.5 MPa.

Permeation experiments of neutral solutes of various molecular weights were carried out at a pressure of 0.5 MPa: ethanol (MW = 46), isopropanol (MW = 60), *tert*-butanol, glucose (MW = 180), sucrose (MW = 342), raffinose (MW = 594), and α-cyclodextrin (MW = 972). A concentration of 100 ppm was chosen to prevent flux change caused by the osmotic effect. Various types of electrolytes, such as NaCl for the 1-1 type, Na_2SO_4 for the 1-2 type, and $MgCl_2$ for the 2-1 type, were employed simply to estimate the charge properties of the membranes.

Amino Acids and Peptides

The amino acids and peptides used are summarized in Table 2. All chemicals (Wako Pure Chemical Industries and Sigma) were used without further purification. There are three types of amino acids and peptides:

TABLE 2
Amino Acids and Peptides

Solutes	Abbreviation	pI (pK_i)[a]	MW
Amino acids:			
Neutral: Glycine	Gly	5.97 (2.35, 9.78)	75
L-Leucine	Leu	5.98 (2.36, 9.60)	131
L-Alanine	Ala	6.00 (2.34, 9.69)	89
L-Glutamine	Gln	5.65 (2.17, 9.13)	146
Acid: L-Glutamic acid	Glu	3.22 (2.19, 4.25, 9.67)	147
Basic: L-Lysine	Lys	9.74 (2.20, 8.90, 10.28)	183
Peptides:			
Neutral: Glycyl-glycine	Gly-Gly	5.7 (3.14, 8.25)	132
Glycyl-glycyl-glycine	Gly-Gly-Gly	5.5 (3.2, 7.89)	189
Glycyl-glutamine	Gly-Gln	5.9 (2.17, 9.78)	208
Glycyl-leucine	Gly-Leu	5.6 (3.09, 8.14)	188
Alanyl-glutamine	Ala-Gln	5.9 (2.17, 9.69)	217
Acid: Glycyl-glutamic acid	Gly-Glu	3.2 (2.19, 4.25, 9.78)	213
Basic: Glycyl-lysine	Gly-Lys	10 (2.35, 9.78, 10.28)	203

[a] pK values of peptides except Gly-Gly, Gly-Leu, and Gly-Gly-Gly are assumed to be the same as those of the original amino acids.

neutral, acidic, and basic. Since they contain both acidic and basic functional groups, their charge can be changed according to the pH. For example, they are neutral around their pI (isoelectric point), and are positively or negatively charged at pH below or above their pI, respectively. In this study, dipeptides and tripeptides were used for separation experiments.

Permeation Experiment

Two membrane modules of the thin-flow-channel type were used in this experiment; the membrane area of one module was 40.7 cm^2 and that of the other was 35.3 cm^2. Feed flow was set at 6 L/min so as to minimize concentration polarization. The experimental setup is shown in Fig. 2. Both permeated and retentated streams were recycled to the feed tank to keep the feed concentration constant. Applied pressure across the membranes ranged from 0.3 to 0.8 MPa. The temperature was kept at 25°C; the pH of the feed solutions was adjusted from 3 to 11 by using hydrochloric acid or sodium hydroxide. The concentrations of amino acids and peptides were measured with a total organic carbon analyzer (TOC 500, Shimadzu) in a single-component system. Mixtures were analyzed by high-performance liquid chromatography equipped with an ODS column.

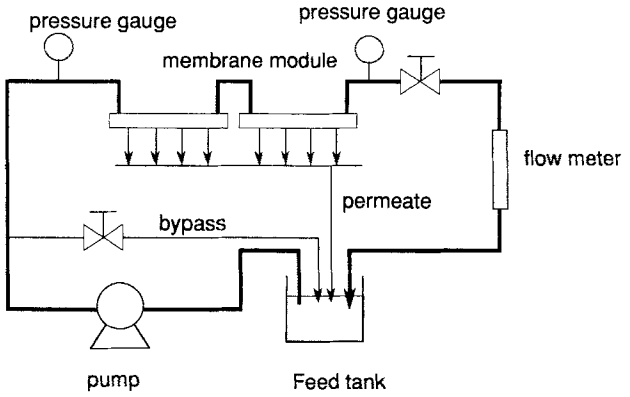

FIG. 2 Schematic experimental apparatus for reverse osmosis.

RESULTS AND DISCUSSION

Basic Performance of Nanofiltration Membranes

Rejections of organic solutes obtained under constant applied pressure are plotted as a function of molecular weight of the solutes in Fig. 3. It should be noted that they indicate rejections only for the given conditions, since rejection is dependent upon permeate flux; a higher flux causes higher rejection. Nanofiltration membranes used in this study can be cate-

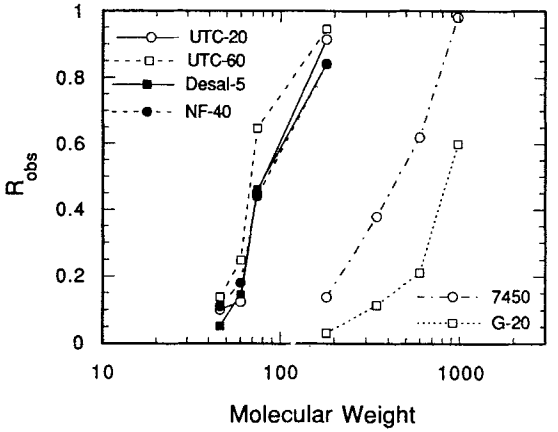

FIG. 3 Rejection of organic solutes as a function of molecular weight. ΔP = 0.5 MPa, concentration = 100 ppm. The following solutes were used: ethanol (46), IPA (60), *t*-BuOH (74), glucose (180), sucrose (342), raffinose (594), and α-cyclodextrin (972).

gorized into two groups with respect to their rejection ability. UTC-20, -60, Desal-5, and NF-40 rejected solutes of molecular weights over 300, while NTR-7450 and G-20 have molecular weight cutoffs (MWCO) of more than 1000. Their rejection performance for neutral organic solutes is between those of conventional reverse osmosis membranes and ultrafiltration membranes (4, 5). Table 1 summarizes the estimated values of MWCO.

As explained in the Introduction, most nanofiltration membranes have fixed charges since they have unreacted carboxylic or amino groups originating from their raw materials. Rejections of electrolytes having typical valence types are also shown in Table 1. Rejections were obtained at a constant feed concentration of 30 $mol \cdot m^{-3}$; it should be noted that rejection of electrolytes with charged membranes is strongly dependent on both permeate flux and feed concentration (9, 10). Sodium sulfate is most highly rejected by the membranes because of electric repulsion between the membrane charge and the sulfate ion. NTR-7450 and G-20 rejected sodium sulfate in spite of their MWCOs which are much larger than the molecular weights of the electrolytes. On the other hand, rejection of $MgCl_2$ as an electrolyte having a divalent cation is smaller than that of NaCl as a 1-1 type electrolyte. These two membranes clearly show the performance of negatively charged membranes; the charge effect is predominant in the rejection mechanism of the two membranes.

As for the other nanofiltration membranes employed in this study, it seems that not only charge effect but also sieve effect is significant in contributing to rejections of electrolytes with consideration of the MWCO.

Permeation Experiment of Amino Acids

Rejection of three amino acids with UTC-60 is shown in Fig. 4 as a function of applied pressure. L-Glutamic acid, which is negatively charged at pH 6, was rejected almost completely. Rejection of L-leucine as a neutral solute was between L-glutamic acid and L-lysine, the latter being positively charged. However, one cannot expect good separation among the three amino acids only on the basis of the charge effect, since the contribution of the sieve effect is not negligible due to the MWCO of UTC-60 being close to the molecular weights of the amino acids used. Therefore, NTR-7450 and G-20, which have MWCOs in the 2000–3000 range, were employed in the following investigation.

Figure 5 shows the rejection and permeate fluxes of NTR-7450 and G-20. Rejection of glycine was almost zero for the two membranes, which indicates that the sieving effect was negligible for the systems. On the other hand, rejection of L-glutamic acid was above 0.8. Rejection in-

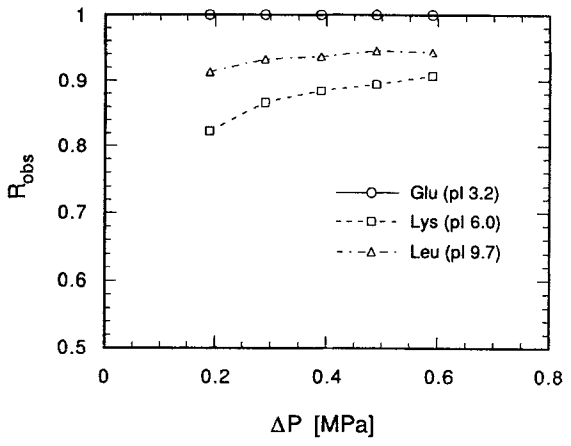

FIG. 4 Rejection of amino acids using UTC-60. Concentration = 2 mM, pH 6.

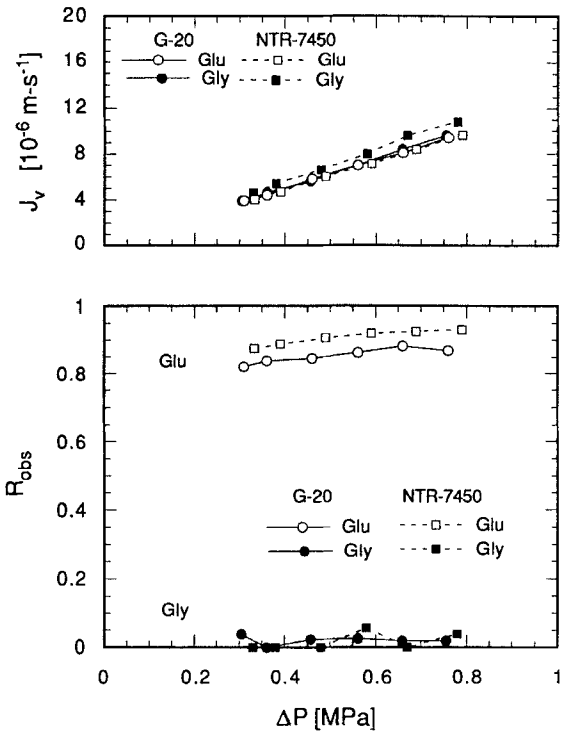

FIG. 5 Rejection and volume flux as a function of pressure. Membranes: NTR-7450 and G-20, pH 6, single solute solutions of Gly and Glu.

creased with applied pressure; this is the same dependency of neutral solutes (10). Table 3 summarizes rejection using various nanofiltration membranes. It should be noted that rejection of L-lysine as a basic amino acid is higher than that of neutral amino acids due to electric repulsion of the coion (chloride ion).

Permeation Experiment of Peptides

The permeation experiment of single peptides was carried out as shown in Fig. 6. Rejection of Ala-Gln, whose pI is 5.9, was dependent on pH. Rejections at pH 6 near the pI were almost zero, while Ala-Gln was rejected at pH 9 where it is negatively charged due to the dissociation of its carboxylic acid. One can see that the charge effect on rejection is most important in this system. Volume flux was linear to applied pressure, irrespective of pH.

Rejections of peptide at pH 6 are summarized in Table 4 for membranes of NTR-7450 and G-20. The tendency of the rejections was quite similar to that for the amino acids shown in Table 3.

pH Dependency of Rejection

As is shown in Fig. 6, rejection of peptides is dependent on pH. Figures 7, 8, and 9 show rejection of peptides as a function of pH for Gly-Glu, Gly-Gly, and Gly-Lys, respectively. The curves in the figures indicate the percentage of existence of dissociated peptides. It is clearly seen that rejections of the neutral form were low, and rejections increase both above and below the isoelectric point, similar to the tendency of rejection of amino acids with negatively charged membranes. Peptides which are nega-

TABLE 3
R_{obs} of Various Amino Acids with Nanofiltration Membranes[a]

	Membranes (MWCO)			
Amino acid	NTR-7450 (≈1000)	G-20 (≈2000)	UTC-20 (≈200)	UTC-60 (≈200)
Neutral: Gly	0.03	0.02		
Ala	0.03	0		
Leu	0.1	0.01	0.93	0.94
Gln	0.03	0.03		
Acid: Glu	0.93	0.85	0.96	1.0
Basic: Lys	0.78	0.6	0.93	0.91

[a] Experimental conditions: ΔP = 0.6 MPa, pH 6.

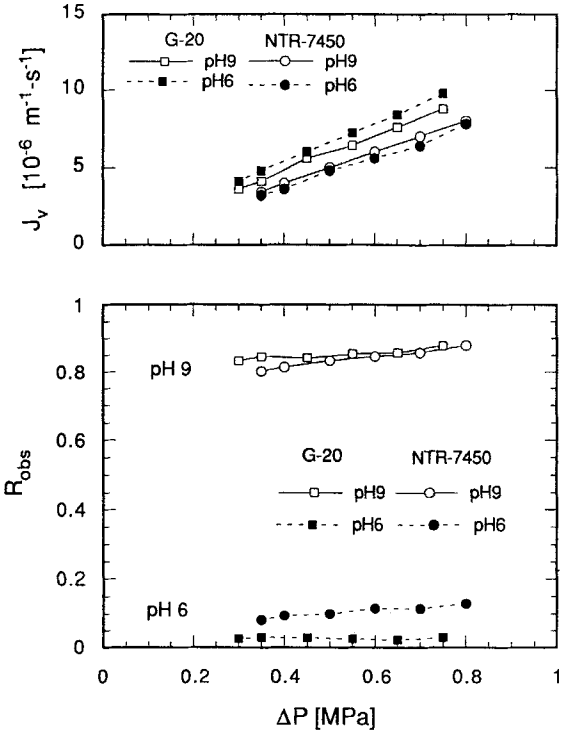

FIG. 6 Rejection and volume flux as a function of pressure. Membranes: NTR-7450 and G-20, solute = Ala-Gln, pH 6 and 9.

TABLE 4
R_{obs} of Various Peptides with Loose RO Membranes[a]

	Membranes	
Amino acid	NTR-7450	G-20
Neutral: Gly-Gly	0	0
Gly-Gly-Gly	0.03	0
Gly-Gln	0.28	0.03
Gly-Leu	0.1	0.04
Ala-Gln	0.11	0.03
Acid: Gly-Glu	0.7	0.7
Basic: Gly-Lys	0.5	0.5

[a] Experimental conditions: ΔP = 0.6 MPa, pH 6.

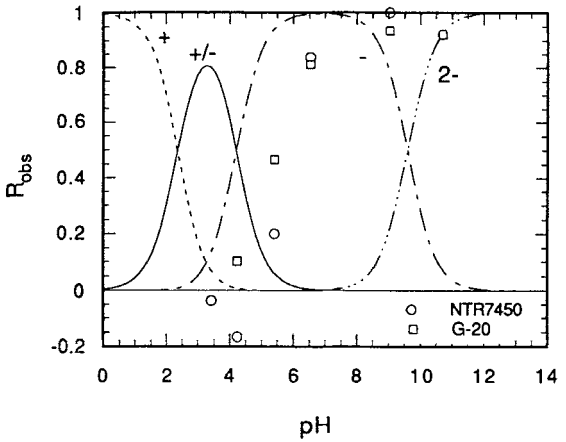

FIG. 7 Rejection as a function of pH. Gly-Glu 200 ppm; membranes: NTR-7450 and G-20; $\Delta P = 0.6$ MPa.

tively charged at a pH above the pI were rejected because of the electric repulsion due to the negative charge of the membranes used. In the case of a pH below the pI, more knowledge of the electric phenomenon occurring in the membranes is required to understand why the peptides were rejected by the membranes. This can be explained as follows. The counterion of positive charged peptides, the chloride ion, was rejected by the

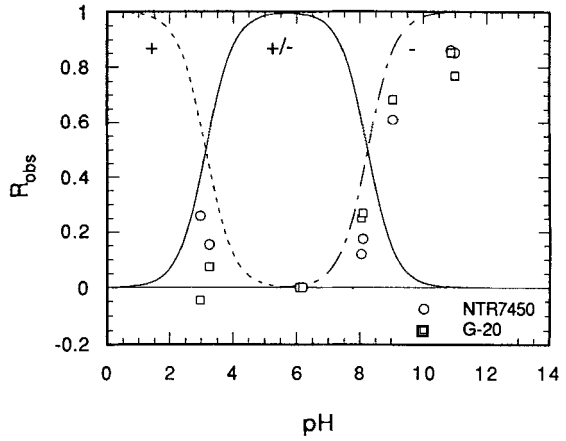

FIG. 8 Rejection as a function of pH. Gly-Gly 200 ppm; membranes: NTR-7450 and G-20; $\Delta P = 0.6$ MPa.

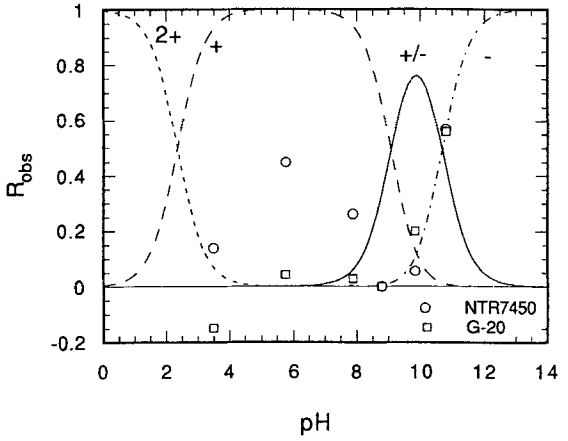

FIG. 9 Rejection as a function of pH. Gly-Lys 200 ppm; membranes: NTR-7450 and G-20; ΔP = 0.6 MPa.

membranes; therefore, the peptide was also rejected in order to maintain electroneutrality.

Our experimental range of pH was from 3 to 11 for the following reasons. First, an increase in the ionic strength of the feed solution would decrease rejection since the ratio of the membrane charge over the feed solution would be reduced. Second, a proton or hydroxide ion, which has a much

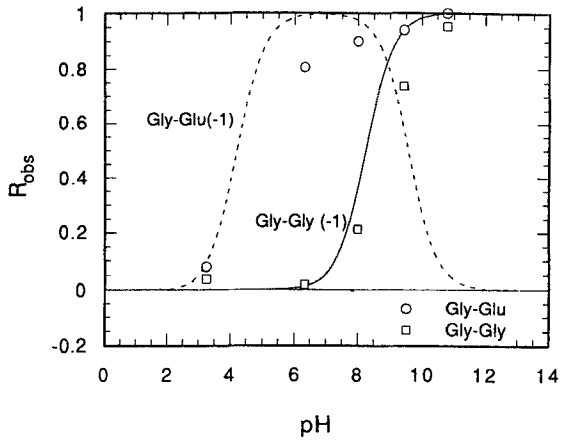

FIG. 10 Rejection as a function of pH. Mixture of Gly-Glu (100 ppm) and Gly-Gly (100 ppm), membrane = NTR-7450, ΔP = 0.6 MPa.

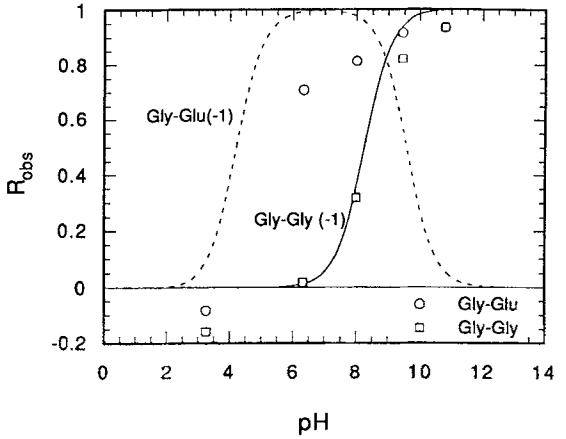

FIG. 11 Rejection as a function of pH. Mixture of Gly-Glu (100 ppm) and Gly-Gly (100 ppm), membrane = G-20, ΔP = 0.6 MPa.

larger ionic mobility than the peptides, could affect rejection of the peptides since the concentration of a proton or hydroxide ion in comparison with the peptides cannot be neglected.

Separation of Peptides in Mixture

Rejections of Gly-Glu and Gly-Gly in a mixed solution are shown as a function of pH in Figs. 10 and 11. The curves in the figures show the percentage of peptides existing in the form of monovalent anions. It seems possible to separate peptides with different isoelectric points by using nanofiltration membranes.

CONCLUSIONS

Several nanofiltration membranes (SU-200, 600, NF-40, Desal-5, G-20, and NTR-7450) were applied to the separation of amino acids and peptides on the basis of charge interaction with the membranes since most of them contain charged functional groups.

1. Nanofiltration membranes having a molecular weight cutoff below 300 (SU-200, 600, NF-40, and Desal-5) were not suitable for the separation of amino acids.
2. Separation of amino acids and peptides with nanofiltration membranes having an MWCO around 2000–3000 (NTR-7450 and G-20) was satis-

factory based on the charge effect; charged amino acids and peptides were rejected while neutral amino acids and peptides permeated the membranes.

3. Separation of peptides having different isoelectric points with nanofiltration membranes was possible by adjusting the pH.

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Received by editor September 3, 1993