Optical Resolution of Phenylalanine Using D-Phe-Imprinted Poly(acrylic acid-co-acrylonitrile) Membrane—pH Effect on performance

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Summary: The molecular imprinting technique was used for chiral resolution of phenylalanine (Phe). The template was introduced during polymerization and removed after polymerization by leaving imprinted cavities in the polymer matrix correspondence to template. The D-Phe imprinted membrane selectively adsorbed D-Phe, facilitated permeation of D-Phe and rejected L-Phe. These results are evidence of the availability of the chiral environment in the membrane. The membrane was found to be pH sensitive, with 0.30 rejection selectivity, 2.40 adsorption selectivity and 2.03 permselectivity being achieved at pH-2. FT-IR and FE-SEM analyses revealed that the membrane was nano-porous and very thin.

Keywords: Chiral separation, Molecularly imprinted polymer, Membrane, Phenylalanine, Rejection, Separation.

Introduction

The remarkable molecular imprinting technique can be used for chiral resolution [1, 2]. In this method, a template is introduced during the polymerization process and removed after formation of the polymer template, leaving imprinted cavities in the matrix of polymer that correspond to the shape of template [2–4]. Wulff and Sarhan introduced imprinting of template in polymer matrix using covalent interactions [5]. Mosbach *et al*. proposed a non-covalent imprinting technique in which non-covalent bonds, hydrogen bonding and electrostatic interactions can be used to imprint template in highly cross-linked polymer [6]. The complementary imprinted cavities after removal of the template possess significant molecular recognition. The discrimination by molecularly imprinted polymer have enabled its use in separation of amino acids, carbohydrates, coenzymes, drugs, hormones, nucleotide bases, pesticides and proteins [7–14].

Yoshikawa *et al*. employed dry phase inversion (solvent evaporation) for the formation of molecularly imprinted membranes using peptide recognition groups with polystyrene resins in a blend with a crosslinked polymer [15, 16]. The permselectivity of the membrane became much higher with respect to blank membranes. Kobayashi *et al*. was a pioneer of developing molecularly imprinted membranes by the wet phase inversion method [17–19]. The solidification and precipitation of polymer was achieved by replacement of solvent with non-solvent in a coagulation bath. Copolymer materials (using different templates) and methodology have recently been successfully adapted by other groups [20–22].

In the present study, the method described by Kobayashi *et. al*. was extended and a D-Phe imprinted membrane was prepared for the separation of phenylalanine using wet phase inversion. Kobayashi *et. al*. imprinted theophylline (THO) and caffeine (CAF) in the matrix of P(AA-co-AN) [17], L-Glutamine in nylon [23] and bisphenol (BIS) in cellulose acetate (CA), nylon 6,6, and polysulfone (PSf) [24] membranes. Trotta *et. al*. imprinted naringin [17] and tetracycline hydrochloride (TCH) in a matrix of P(AA-co-AN) [21]. In this study, D-Phe was imprinted in a matrix of P(AA-co-AN) using a non-covalent approach that is more favorable for binding template and easy to break. Rejection phenomena have been overlooked by other researchers [15–22]; however, in the present study, the rejection was found to be selective and a new term of "rejection selectivity" was introduced [3]. The ultrafiltration technique was then used for chiral resolution of phenylalanine. Following are the new findings of the current research:

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- Morphological studies revealed that the membrane was nanoporous and very thin.
- The membrane showed relatively high adsorption selectivity.
- The membrane showed facilitated permeation. Continuous enantioselective transport with relatively high selectivity was achieved.

This study included an investigation of the influence of pH on the separation ability of the membrane. Additionally, an attempt to identify the mechanism through which pH influenced membrane performance was made.

Results and Discussion

Morphological Characterization of D-Phe imprinted P(AA-co-AN) Membrane

The surface morphology, pore structure (barrier pore size), layer topology (internal symmetric structure versus asymmetric structure of pores and surface), nature of polymer, imprinted cavities and channels have a decisive impact on selective solute transportation using molecularly imprinted membranes (MIMs) [25]. The structure of the base membrane can be used to adapt both pores size permeability and internal surface area binding capacity to the desired application. Previously, the thickness of a Nylon membrane imprinted by L-Glu was found to be 30 μm [23]. Additionally, the thickness of P(AA-co-AN) membrane imprinted by THO and CAF was 80 μm, while the thickness of the dense top layer was 10 μm [17]. The Naringin imprinted P(AA-co-AN) membrane was 500 μm thick [20], and the thickness of the dense top layer and membrane thickness of UA imprinted membrane were 10-20 μm and 200-300 μm [22], respectively, while the pore diameter was found to be 0.1. The BPA imprinted membranes had a thickness of 180 μm [26] and pore diameter >0.5 μm. The D-Phe imprinted membrane had no micro voids, and an average membrane thickness and thickness of the dense top layer of 26 μm and 6 μm, respectively. The pore size of the D-Phe imprinted membrane was up to 27 nm.

FT-IR Characteristics of D-Phe imprinted P(AA-co-AN) membrane

The FT-IR technique was used to study the chemical structures of D-Phe imprinted P(AA-co-AN) and non imprinted membranes. The spectra were found to be similar to those previously reported for P(AA-co-AN) [17, 21]. C=O stretching reflecting acrylic acid in the blank and D-Phe imprinted membranes appeared at 1734 cm^{-1}, which was close to the expected location of 1730 cm^{-1}. The stretching of dimerized OH usually appears at a wide range of 2500-3300 cm^{-1}. In the present study, dimerized OH stretching of the COOH group of blank polymer appeared at 2593 cm^{-1} and 3426 cm^{-1}, while stretching of the OH dimer in the D-Phe imprinted membrane appeared at 2615 cm^{-1} and 3360 cm^{-1}. The presence of an OH dimmer is evidence of hydrogen bonding between COOH groups in blank and imprinted membranes. C-O stretching in blank polymer appeared at 1166 cm^{-1} and 1228 cm^{-1}, while C-O stretching in D-Phe imprinted membrane appeared at 1170 cm^{-1} and 1229 cm^{-1}. The CH_2, CN and CH bending of both blank and D-Phe imprinted membrane appeared at 1455 cm^{-1}, 1734 cm^{-1} and 2939 cm^{-1}, respectively. The free OH bending in the D-Phe imprinted membrane showed absorption at 3243 cm^{-1} and 3461 cm^{-1}, which demonstrates the presence of recognition sites in the imprinted membrane.

Permeation properties of D-Phe imprinted P(AA-co-AN) membrane

During the filtration experiment permselectivity increased gradually. The prepared membrane fulfills the theory of facilitated permeation, which may occur due to the presence of imprinted cavities between channels functioning as gates that allow template to pass through while retarding permeation of unwanted enantiomers. The effect of pH on permeate flux and selective transmission of solute was examined at pH 2, 4 and 6 using racemate solution with a feed concentration of 100 mg-Phe/l in respective buffers (Fig. 1). At pH 2, the respective flux of D-Phe and L-Phe was 0.2386 $mg/m^2.s$ and 0.2283 $mg/m^2.s$ respectively, while the flux of D-Phe and L-Phe obtained at pH-4 was 0.2399 $mg/m^2.s$ and 0.2306 $mg/m^2.s$, respectively. When racemate solution with pH-6 was passed through membranes, the flux of D-Phe was 0.2424 $mg/m^2.s$ and that of L-Phe was 0.2343 $mg/m^2.s$. The permeate flux trend was $[\alpha_F]_{pH\text{-}2} < [\alpha_F]_{pH\text{-}4} < [\alpha_F]_{pH\text{-}6}$.

These results demonstrate that flux increased with pH. Similar results were reported by Ghosh *et. al*. during purification of Lysozyme using ultrafiltration [24]. The charge on membranes has been reported to decrease as pH increases, resulting in increased permeates flux. The permselectivity at pH-2, pH-4 and pH-6 were 2.01, 1.96, and 1.94, respectively, and the trend of permselectivity was $[\alpha_P]_{pH\text{-}6} < [\alpha_P]_{pH\text{-}4} < [\alpha_P]_{pH\text{-}2}$. The permeate flux was relatively less sensitive to pH [24]. The decrease in permselectivity with pH may also be caused by

electrostatic repulsive forces between the membrane and solute. The increase in permeation selectivity is the result of a decrease in the rejection selectivity (cf. 3.5) and gate that allows the template to pass through and retard permeation of other enantiomers [25].

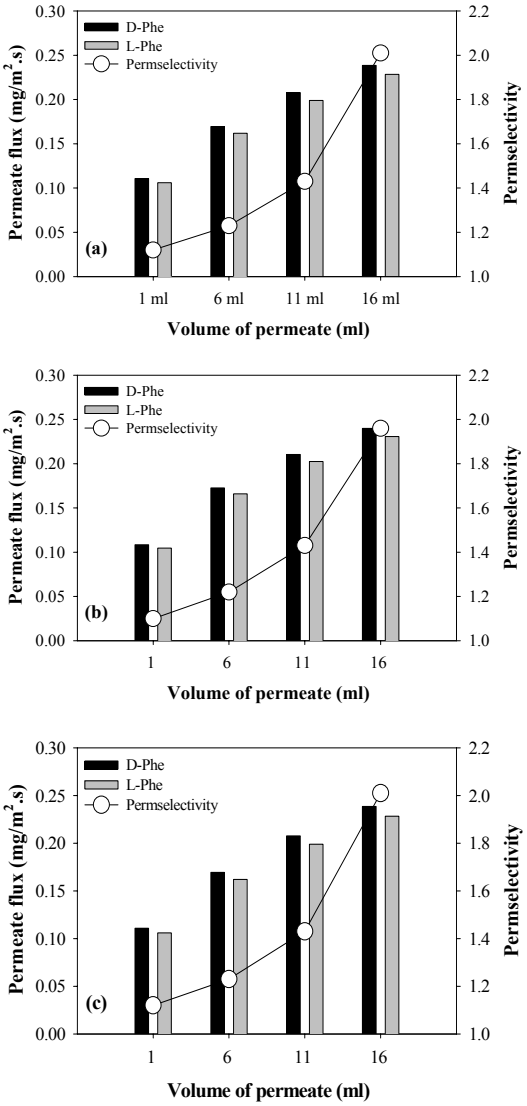

Fig. 1: Permeation profile of Phenylalanine using racemate solutions of (a) pH-2, (b) pH-4 and (c) pH-6.

Adsorption properties of D-Phe imprinted P(AA-co-AN) membrane

Template recognition by the imprinted polymeric membrane is due to the existence of electrostatic forces, hydrogen bonding and charge transfer between carboxylic groups and imprinted membranes [25]. The influence of pH is higher between colloids and analytes present in racemate solution, and the imprinted membrane. When racemate solution of pH-2 was filtered, the adsorption selectivity was 2.40 and the amounts of D-Phe and L-Phe adsorbed were 0.1674 mg/gram membrane and 0.0698 mg/gram membrane, respectively. The amounts of D-Phe and L-Phe adsorbed on the membrane after filtration of 16ml of racemate solution of pH-4 were 0.1878 mg/gram membrane and 0.0819 mg/gram membrane, respectively, and the adsorption selectivity was 2.29. The racemate solution of pH-6 showed 2.25 adsorption selectivity, and adsorption of 0.1979 mg/gram membrane D-Phe and 0.088 mg/gram membrane L-Phe. The adsorption profile is illustrated in Fig. 2. The trend of adsorption capacity of solute (D-Phe and L-Phe) on membrane was $[A]_{pH\text{-}2} < [A]_{pH\text{-}4} < [A]_{pH\text{-}6}$ and the trend of adsorption selectivity was $[\alpha_A]_{pH\text{-}6} < [\alpha_A]_{pH\text{-}4} < [\alpha_A]_{pH\text{-}2}$.

It has been reported that the optimum interaction of template molecule with complementary recognition sites occurs at high pH. The conversion of the carboxyl group (COOH) of solute (D-Phe and L-Phe) into COO^- increases with pH, but there is no change in NH_3^+ [27]. At pH-2, the conversion of COOH into COO^- was 21%, while at pH-2 the conversion reached 99.9%. These findings indicate that binding of solute increases with pH due to electrostatic attractive forces between negatively charged solutes and positively charged membrane. At low pH, adsorption selectivity was high because membrane and solute were both negatively charged and transport of solute through the imprinted pore and channel in the membrane matrix occurred. The increase in adsorption selectivity was caused by selective rejection (cf. 3.5) and availability of recognition sites in the membrane matrix.

Rejection properties of D-Phe imprinted P(AA-co-AN) membrane

During optical resolution of phenylalanine using the ultrafiltration technique, the solute not only adsorbed onto the membrane, but was also rejected. The rejection of L-Phe was found to be higher than the rejection of D-Phe, which is evidence that imprinting channels and cavities in the membrane do not allow L-Phe to pass through easily. After filtration of 16 ml of racemate solution with pH-2, the rejection selectivity was 0.3 and rejection of D-Phe and L-Phe was 4.53% and 15.26%, respectively. The rejection of D-Phe and L-Phe after filtration of 16 ml racemate solution with a pH of 4 was 3.02% and 14.29% respectively with a rejection selectivity of 0.21. When racemate solution of pH-6

was used, after 16 ml of filtration the rejection of D-Phe was 1.76% and the rejection value of L-Phe was 12.92% with a rejection selectivity of 0.14. The trend for rejection selectivity was $[\alpha_R]_{pH\text{-}6} < [\alpha_R]_{pH\text{-}4} < [\alpha_R]_{pH\text{-}2}$ (Fig. 3). The minimum rejection value shows that membrane rejection of other enantiomers was greater than that of the template. At low pH, membrane rejection of solute was greater than at high pH, resulting in high accumulation of template in imprinted cavities and low permeates flux. The selective rejection of solute is a result of selective permeation and adsorption, while the top layer of the imprinted membrane works as a barrier to solute transport [25]. Conversely, decreased rejection is caused by deformation of pores and increased concentration with ultrafiltration time.

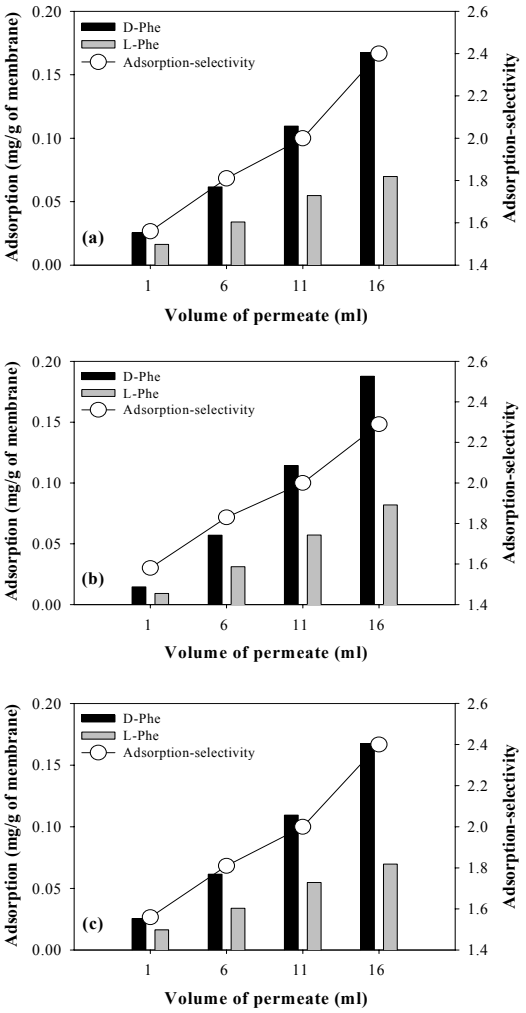

Fig. 2: Adsorption profile of Phenylalanine using racemate solutions of (a) pH-2, (b) pH-4 and (c) pH-6.

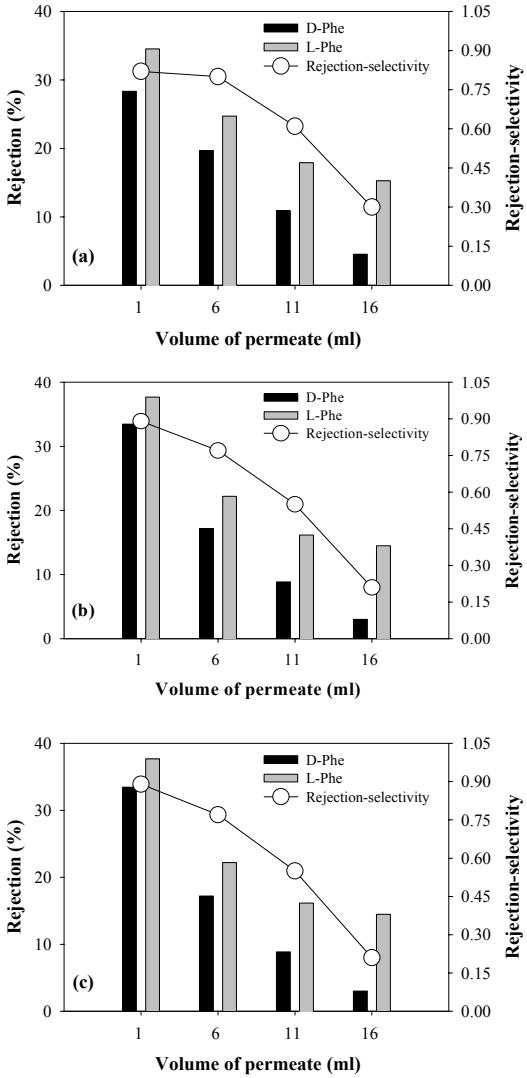

Fig. 3: Rejection profile of Phenylalanine using racemate solutions of (a) pH-2, (b) pH-4 and (c) pH-6

Experimental

Materials

D-Phe, racemate mixture of Phe, 2,2-Azobiisobutyronitrile (AIBN) and Trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (USA), Dimethyl sulfoxide (DMSO) was purchased from Kanto (Japan), while acrylic acid (AA) and acrylonitrile (AN) were obtained from Junsei (Japan) and Yakuri (Japan) respectively. All reagents were of analytical grade.

Synthesis of D-Phe Imprinted P(AA-co-AN) copolymer

In 50 ml DMSO, 0.5 g D-Phe, 2 mg TFA and 7.51 g AA were mixed for 2 hours at 50°C in a polymerization reactor. Next, 37.72 ml AN and a solution of 0.22 g AIBN dissolved in 50 ml DMSO were poured into polymerization reactor, after which nitrogen gas was bubbled through the solution for 5 minutes. The polymerization reactor was then sealed and nitrogen was supplied to the solution to create an inert environment, after which the solution was stirred at 200 rpm for 6 hours at 60°C to give a viscous polymer. Next, 100 ml DMSO was added to the polymer solution and stirred for 20 hours at room temperature to reduce its viscosity. Micro air bubbles were then removed by placing the diluted polymer solution in a vacuum oven for 24 hours at – 60 cmHg pressure. A reference non-imprinted polymer was prepared in the same way, but in the absence of the template molecule.

Preparation of D-Phe imprinted P(AA-co-AN) copolymer membrane

The casting of polymer solution on a glass plate was done using a doctor blade then the polymer film was coagulated by immersing in deionized water at 25 °C DMSO was removed by extensive washing of the membrane with distilled water. The template was removed by washing with 5% (V/V) acetic acid solution for 2 hrs, after which the membranes were rinsed with de-ionized water. Finally, the membranes were put in de-ionized water for more than 60 days to remove shrinkage and swelling.

Characterization

The morphology of the membrane was observed with a Hitachi model S-4300 Field Emission Scanning Electron Microscope (FE-SEM). FT-IR spectra of the non imprinted and D-Phe imprinted P(AA-co-AN) membrane were recorded using a Mattson Galaxy 7020A FT-IR spectrophotometer with a resolution of 0.025 cm^{-1} and a wavelength range of 4000–400 cm^{-1}. The HPLC instrument consisted of an M 930 solvent delivery pump and M 720 UV absorbance detector produced by Young-Lin Instrument Co. Ltd. (Korea).

Ultrafiltration Experiment

The prepared membranes were cut into circles with a diameter of 43 mm and washed thoroughly with distilled water before post treatment. The membranes were then dipped into buffer solutions with a pH of 2, 4 and 6. The pH value was checked every two hours and the buffer was changed until the pH value was fixed. Next, 30 ml of 100 mg Phe/l solutions with pH values of 2, 4 and 6 were eluted through membrane to analyze the separation performance of membranes using an UF kit at 1 kg.f/cm^2 pressure. Five sheets of membranes were used for each ultrafiltration experiment.

Solute rejected by D-Phe imprinted P(AA-co-AN) membrane

The rejection (R) of solute by the membrane is defined as follows [28]:

$$R = \frac{ln\left[\frac{C_F}{C_I}\right]}{ln\left[\frac{V_I}{V_F}\right]} \times 100 \qquad (1)$$

where V_I and V_F are the volumes (ml) of feed solution and retentate, respectively, and C_I and C_F are the concentrations of Phe (mg/l) in feed solution and retentate, respectively. The rejection selectivity α_R can be calculated according to following equation:

$$\alpha_R = \frac{R_D}{R_L} \qquad (2)$$

where R_D and R_L represent rejections of D-Phe and L-Phe, respectively.

Adsorption and adsorption selectivity

The amount of D-Phe adsorbed (A_D) per gram of membrane was calculated by:

$$A_D = \frac{[M_M]_D}{W_M} \qquad (3)$$

where $[M_M]_D$ is the amount of D-Phe (mg) on the membrane and W_M is the dry weight (g) of membrane. The adsorption selectivity of membrane αA was defined as:

$$\alpha_A = \frac{A_D}{A_L} \qquad (4)$$

where A_L is the amount of L-Phe adsorbed per gram of membrane.

Solute flux and permselectivity:

The solute flux J_D (mg/m^{2}s) of D-Phe, permeability coefficient P_D (m^2/s) of D-Phe and permselectivity α_P was calculated by the following equations [29]:

$$J_D = \frac{[M_P]_D}{A_O T} \quad (5)$$

$$P_D = \frac{[J]_D \partial}{[C_I]_D - [C_P]_D} \quad (6)$$

$$\alpha_P = \frac{P_D}{P_L} \quad (7)$$

where $[M_P]_D$ is the amount of D-Phe in permeate (mg), A_O is the effective area (m^2) of membrane, T represents the time (sec) required for the solution to pass through the membrane; ∂ is the membrane thickness (m), $[C_I]_D$ and $[C_P]_D$ are the concentrations of D-Phe (mg/l) in the feed solution and permeate, respectively, and P_L is the permeability coefficient (m^2/s) of L-Phe.

Conclusions

D-Phe imprinted P(AA-co-AN) membrane prepared by the wet phase inversion method can be successfully used for chiral resolution of the DL-Phe racemic mixture. Morphological studies revealed that the membrane was very thin and the pore diameter was in nanometers. The adsorption selectivity indicates that the membrane successfully recognizes molecules corresponding to imprinted cavities in size and shape. The membrane facilitated permeation, and permselectivity of the membrane increased gradually. Selective rejection was also observed and counter enantiomers were rejected by the membrane. Finally, pH of the solution played an important role in selective permeation, adsorption and rejection, with better performance occurring at low pH.

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