Separation of DL-Phenylalanine by Chiral Selector-Supported Ultrafiltration

Sema Salgın*, Seda Bahadır Öner and Uğur Salgın

Faculty of Engineering, Department of Chemical Engineering, Cumhuriyet University, Sivas, Turkey

Abstract: In this study, the enantiomeric separation of racemic phenylalanine using hydrophobic polyethersulfone and hydrophilic regenerated cellulose membranes was performed. BSA and lipase were used as chiral selector. The complexes of DL-PHE-chiral selector were ultrafiltrated by the stirred ultrafiltration cell. During the ultrafiltration, the samples taken from permeate were analyzed with liquid chromatography system using Chiralpak AD-H column for the determination of D-PHE and L-PHE enantiomers. The effectiveness of chiral separation was characterized in terms of the values of enantiomeric excess (*ee%*). Chemical structures and zeta potentials of membranes were determined by Fourier transforms infrared spectroscopy, and by an electrokinetic analyzer, respectively. BSA showed affinity to D-PHE, while lipase showed affinity to L-PHE. The higher enantiomeric excess was obtained using lipase chiral selector and PES membrane.

Keywords: Chiral separation, DL-phenylalanine, BSA, lipase, ultrafiltration membrane.

1. INTRODUCTION

Great number of organic compounds such as amino acids, drugs, pesticides, and insecticides are optically active molecules and exist as pairs of enantiomer. Generally, only one enantiomer has the desirable biological effect, whereas the other is inert or even toxic. Therefore, the separation of enantiomers from the racemic mixture is ever needed in agrochemicals, pharmacology, and food industry. The aim of this case is to increase the importance of chiral separations. The traditional chiral separation methods are diastereomeric or preferential crystallization, chemically or enzymatic kinetic resolution, and column chromatography. These conventional methods are limited by some technical problems, such as difficult to scale up, not easily amenable to continuous operation, energy-intensive, inefficient and costly. The increasing demand of enantiopure compounds in respect purities, and amounts in the industries requires the development of new separation and production techniques. The membrane-based chiral separation has been considered to have great potential for separation of enantiomers. Notably, membrane-based separations have been recognized as among the top 10 green engineering research focuses by the American Chemistry Society Green Chemistry Institute Pharmaceutical Roundtable. In this context, the membrane-based chiral technology offers several advantages such as high throughput, energy saving, continuous process, easy scale-up, low time cost, simplicity of operation, and is more economical than many other separation processes [1-5]. The chiral separation with membranes can be divided into two broad types, namely (i) affinity membrane system using chiral selectors or ligands in feed solution (non-chiral membrane), (ii) supported liquid membranes including enantiomer-recognizing carriers and solid membranes (chiral membrane) [4]. The latter is known to have a shorter operating lifespan due to membrane stability issues [5].

For chiral separations with the non-chiral membrane, a specific complex is formed between chiral selector, and only one enantiomer in the feed side of the membrane. Thus, the chiral separation can be achieved by means of permeation of the free enantiomer to the permeate side, due to its small molecular weight which is smaller than the membrane molecular weight cut off. As the chiral selector-enantiomer complex cannot pass through permeate, enantiomeric separation is achieved [6]. In membrane-based chiral separation processes, the most commonly used chiral selectors are cyclodextrins and their derivatives, BSA, DNA, crown ether and macrocyclic antibiotics [4,7-9].

The enantiomeric separation of D, L-phenylalanine (DL-PHE) is important because the effectiveness of D- and L- PHE enantiomers is different. L-PHE is capable of strongly inhibiting the alkaline phosphatase of the intestine, and stimulating pancreatic secretion, whereas D-PHE is much more promising as an inhibitor of enzyme activity [5]. The aim of this study is to investigate the enantiomeric separation of DL-PHE using chiral selector-enhanced ultrafiltration. We analyzed the effect of chiral selectors such as BSA and lipase on the control of the preferential permeation of

*Address correspondence to this author at the Faculty of Engineering, Department of Chemical Engineering, Cumhuriyet University, Sivas, Turkey; Tel: 00.90.346.219 10 10/ 2804; Fax: 00.90.346.219 11 79;
E-mail: ssalgin@cumhuriyet.edu.tr

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enantiomers through the membrane. We also examined the effect of different types of membranes on the enantioselectivity.

2. MATERIALS AND METHODS

2.1. Materials

DL-PHE, BSA, *Candida rugosa* lipase and trifluoroacetic were obtained from Sigma-Aldrich Co. LLC. KCl, ethanol and *n*-hexane were purchased from Merck KGaA. In preparation of all aqueous solutions, deionized water (Milli-Q, Gradient, Millipore) with a conductivity of 18.2 MΩ.cm was used. Hydrophobic polyethersulfone (PES) and hydrophilic regenerated cellulose (RC) membranes (Sartorius AG) with nominal cut-off value of 30 kDa and diameter of 25 mm were used in the experiments.

2.2. Chiral Separation of DL-PHE

The complexes were prepared by mixing equal amounts of DL-PHE and chiral selectors in 0.001 M KCl solution at pH=7.2. The ultrafiltration (UF) of complex solution was carried out using a dead-end stirred UF cell (8010, Amicon). The non-chiral membrane discs were fixed tightly in UF cell which possessed 10 mL volume, and the prepared complexes were used as feed solution. The constant pressure (0.3 MPa) was applied to the UF cell through using nitrogen gas. The batch UF setup is shown in Figure **1**.

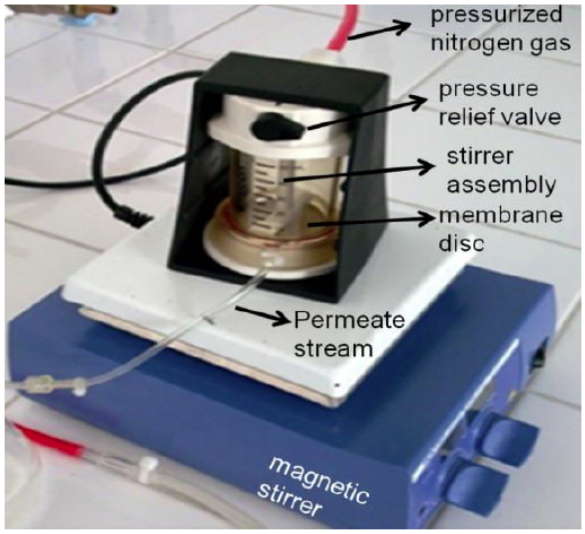

Figure 1: Schematic diagram of the ultrafiltration experimental setup.

The samples were taken in permeate and analyzed by liquid chromatography system (LC 10, Shimadzu) using ChiralPAK AD-H (Daicel) column. In the analyses, the mixture of n-hexane (85 %)-ethanol (15 %)-trifluoroacetic acid (0.1%) was used as a mobile phase at the flow rate of 0.5 mL/min. UV detection of D-PHE and L-PHE was done at the wavelength of 210 nm [10]. The enantioselectivity of transport through non-chiral UF membranes was expressed as the enantiomeric excess (*ee*%). Eq. (1) was used to calculate the *ee*% value that is defined as the ratio of the concentration difference over the total concentration of both enantiomers in the permeate [11].

$$ee,\% = \frac{(C_{high} - C_{low})}{(C_{high} + C_{low})} \cdot 100 \quad (1)$$

where C_{high} and C_{low} denote concentration of major enantiomer and minor enantiomers of racemic PHE in the permeate stream, respectively.

2.3. Instrumentation

The chiral selector concentrations were determined using a linear calibration curve, UV-vis spectroscopy (UV1800, Shimadzu). Structural characterizations of PES and RC membranes before and after UF experiments were obtained by FTIR spectroscopy (Spectrum 100, Perkin Elmer). The FTIR spectra was recorded at room temperature with a resolution of 4 cm^{-1} over the range of 400-4000 cm^{-1}. The ATR accessory of the FTIR instrument contained a ZnSe crystal. The zeta potential of PES and RC membranes was analyzed by an Electrokinetic Analyzer (EKA SurPASS, Anton Paar). The apparent zeta potential of membranes was calculated through the streaming potential data, using the Helmholtz-Smoluchowksi equation. The details of the system and procedure have been described by Salgın *et al*. [12].

3. RESULT AND DISCUSSION

PES and RC membranes studied have 30 kDa MWCO which is less than the average molecular weight of chiral selectors BSA (~67 kDa) and lipase (~58 kDa) [13, 14]. Due to size exclusion properties of the UF membranes, theoretically chiral selectors can not pass through these membranes. To confirm this, the feed solutions containing 0.5 g/L chiral selector were ultrafiltrated by each membrane. The concentrations of BSA and lipase chiral selectors in the permeate solution were obtained from absorbance measurement at 278 nm and 257.5 nm respectively, using a UV spectroscopy. Both of chiral selectors were not dedected in permeate stream.

3.1. Chiral Separation of DL-PHE using PES Membrane

A racemic mixture of PHE was separated by 30 kDa PES membrane using chiral selectors such as BSA and lipase. The specific complex solution was prepared by mixing 0.5 g/L DL-PHE, and 0.5 g/L chiral selectors in 0.001 M KCl solution at pH=7.2. In the UF process using BSA chiral selector, it was determined that the concentration of L-PHE was high in the permeate stream. It can be concluded that the BSA binds with D-PHE to form a larger complex that gets retained by the membrane. The enantiomeric excess value of L-PHE was calculated as ee_L,% = 6.0. In enantiomeric separation using lipase chiral selector, it was determined that the concentration of D-PHE was high in the permeate. Therefore, it is conceivable that the lipase enzyme binds with L-PHE to form a larger complex that gets retained by the membrane. The enantiomeric excess value for D-PHE was calculated as ee_D,% = 32. PES membrane surface was analyzed, using an FTIR-ATR spectroscopy to detect the structural changes originated from the larger complexes (DL-PHE-BSA and DL-PHE-lipase) adsorption. The FTIR-ATR spectra of the PES

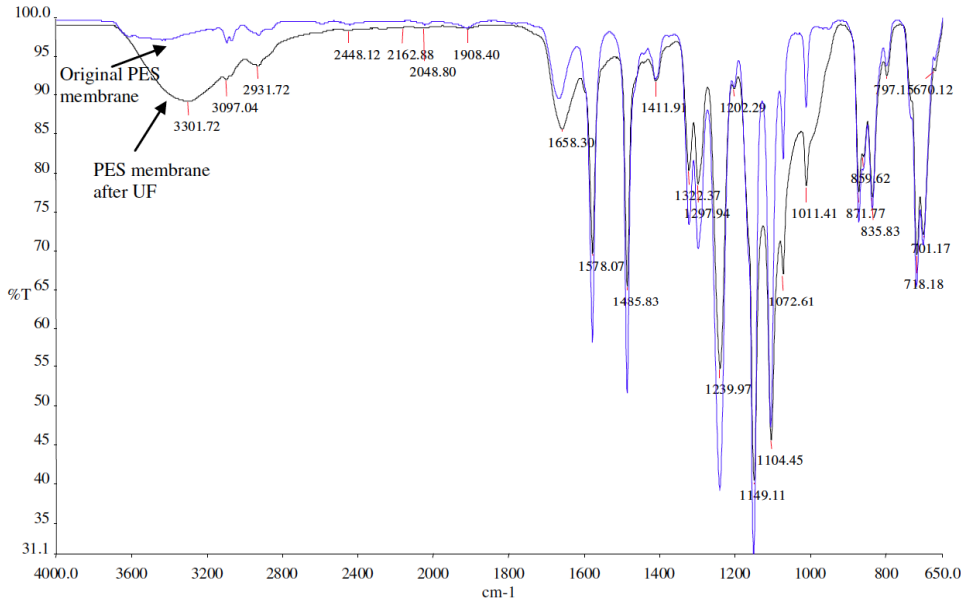

Figure 2: Comparative FTIR-ATR spectra of the PES membrane before and after chiral separation of DL-PHE-BSA complex.

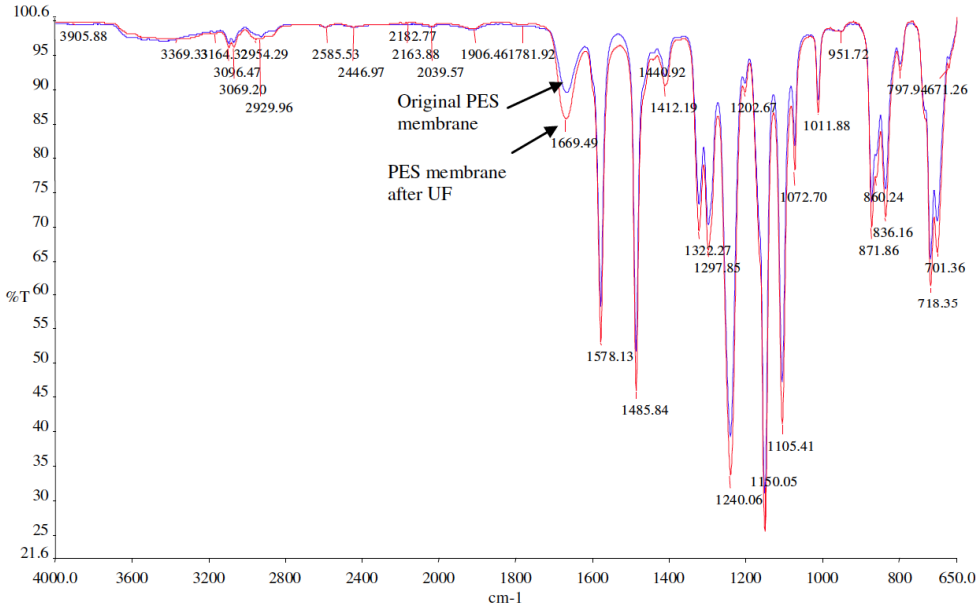

Figure 3: Comparative FTIR-ATR spectra of the PES membrane before and after chiral separation of DL-PHE-lipase complex.

membrane before and after the UF of DL-PHE-BSA are shown in Figure **2**. The FTIR-ATR spectra of the PES membrane before and after the UF of DL-PHE-lipase are also shown in Figure **3**.

In general, two characteristic bands of the amide I and amide II are inspected as the evidence of the adsorption of protein on the membrane surfaces. The amide I band originates predominantly from the C= O streching vibration of the peptide groups, and the amide II band is characteristic of the bending of NH groups in the plane, and the C-N streching modes of the polypeptide chains [15, 16]. The spectra of the PES membranes after UF of the chiral complex solutions revealed the presence of the amide I at the wavelength of 1658 and 1669 cm^{-1}.

3.2. Chiral Separation of DL-PHE using RC Membrane

A racemic mixture of PHE was separated by 30 kDa RC membrane using BSA and lipase chiral selectors. The specific complex solution was prepared by mixing 0.5 g/L DL-PHE, and 0.5 g/L chiral selectors in 0.001 M KCl solution at pH=7.2. In the UF process using BSA chiral selector, it was determined that the concentration of L-PHE was high in the permeate stream. The enantiomeric excess value of L-PHE was calculated as ee_L,% = 12. In enantiomeric separation using lipase chiral selector, it was determined that the concentration of D-PHE was high in permeate. The enantiomeric excess value for D-PHE was calculated as ee_D,% = 24. The FTIR-ATR spectra of the RC membrane before and after UF of DL-PHE-BSA and DL-PHE-lipase solutions are shown in Figures **4** and **5**, respectively.

The spectrum of RC membranes after chiral separation of DL-PHE revealed the presence of amide II band (~ 1545 cm^{-1}) in addition to the amide I band.

Both of chiral selectors exhibited different interaction with the racemic mixture of DL-PHE. While BSA showed affinity to D-PHE, lipase showed affinity to L-PHE. This can be explained by the different interactions such as steric, hydrophobic, and hydrogen-bonding between the chiral selector and racemic mixture [17]. A number of enzymes have the ability to discriminate between enantiomers of racemic mixture. Stereospecific enzymes are especially hydrolases [18]. Lipase enzyme is in the group of hydrolases. Due to stereospecific properties, the highest value of *ee*,% was obtained with lipase chiral selector. While BSA chiral selector performed better with the RC membrane, lipase chiral selector performed better with the PES membrane. The presence of the amide I and amide II peaks in FTIR spectra indicates that the conformation of the polypeptides on the membranes.

In the studied ionic environment (0.001 M KCl and pH=7.2), the zeta potentials of the membranes were measured by EKA, and were given in Table **1**. After UF of the complex solution, the absolute zeta potential of membranes increased. This increase in zeta potentials of chiral membranes when compared to the original membranes indicated that a new group bonded to the membrane surface.

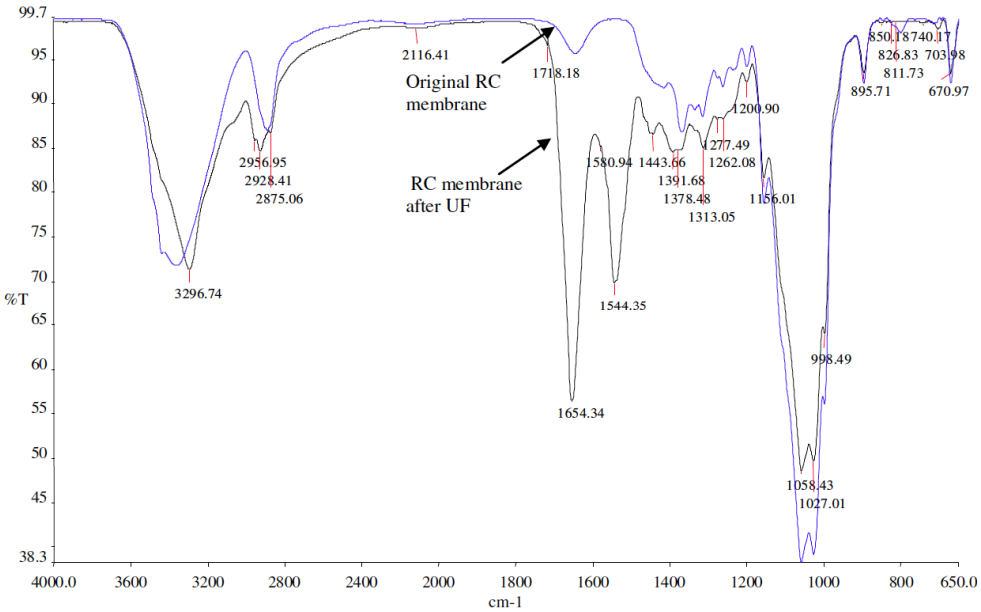

Figure 4: Comparative FTIR-ATR spectra of the RC membrane before and after chiral separation of DL-PHE-BSA complex.

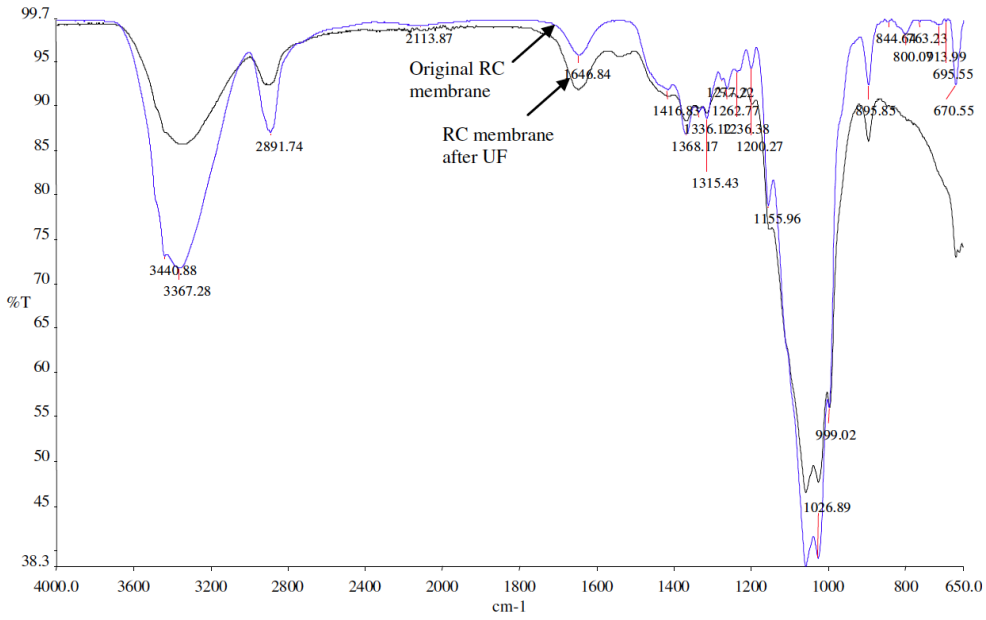

Figure 5: Comparative FTIR-ATR spectra of the RC membrane before and after chiral separation of DL-PHE-lipase complex.

Table 1: The Zeta Potentials of Membranes before and after UF at pH=7.2 and 0.001 M KCl

	Zeta potentials, mV
Original PES membrane	-88.39
PES membrane after UF of DL-PHE-BSA	-107.20
PES membrane after UF of DL-PHE-lipase	-124.00
Original RC membrane	-60.00
RC membrane after UF of DL-PHE-BSA	-84.60
RC membrane after UF of DL-PHE-lipase	-94.50

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