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Novel Optical Resolution of Phenylalanine Racemate Utilizing Enzyme Reaction and Membrane Extraction

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ABSTRACT

A novel optical resolution method for D,L-phenylalanine in which an enzyme reaction and a membrane extraction are combined has been designed. In the first stage only the L-isomer of the racemic phenylalanine methyl ester was selectively hydrolyzed by the enzyme α-chymotrypsin. Further, the unreacted ester was selectively recovered from the mixture of the transferred amino acid and the ester form by a membrane extractor. Effects of operation conditions in the enzyme reaction and the membrane extraction on the separation efficiency were investigated. The pH in the material sources is found to be an important factor in the activity and selectivity for the enzymatic resolution. A novel immobilized enzyme enables the expensive enzyme to be reused in the feed solutions. The hollow-fiber membrane extractor was a good separator for an amino acid and its ester derivative, because only the ester was selectively extracted into an organic phase. The novel separation system becomes a very useful process for the optical resolution of amino acids by combining the enzyme reaction with a membrane extraction.

INTRODUCTION

Amino acids are optically active materials having an asymmetric carbon in their molecular structures, and both enantiomers often show different properties, especially in a medical or pharmaceutical field. Therefore, the development of an efficient separation process for such isomers has been expected over the last several years. The chemical and physical character-

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istics of their optically active isomers are so similar that the separation of racemates, which is an equivalent mixture of both enantiomers, is very difficult. Among amino acids, the resolution of the phenylalanine racemate has been noted from the viewpoint of availability of L-phenylalanine at low cost as the starting material for aspartame, whose production has increased year by year and has now reached over several thousands tons.

The utility of enzymes has a high possibility of recognizing an optically active substrate (1). Matson and Quinn investigated the optical resolution of racemic amino acid using a membrane bioreactor which is a combination of an enzyme-immobilized membrane with a permselective membrane (2). Scheper et al. (3), Ha and Hong (4), and Chang et al. (5) reported an enzymatic resolution of amino acids using a liquid emulsion membrane in which α-chymotrypsin was immobilized. The reactions proceeded in a continuous mode, and the activity loss of the enzyme was about 60–70% under optimum conditions. However, the emulsion technique has an inherent problem of emulsion stability, and many experimental parameters, e.g., surfactant, carrier, organic solvent, and pH in an aqueous phase, greatly affect the emulsion stability, enzyme activity, and transport rates of substrates and products. Therefore, it is often difficult to set up optimum conditions. A simpler technique that overcomes the difficulties in the liquid membrane technique was proposed (6) in which L-phenylalanine was produced from a solution of D,L-phenylalanine ethyl ester utilizing enzymatic resolution in the presence of an organic solvent. In the two-phase system the organic phase was employed as a reservoir for the ester compound. Further, in 1992, Ricks et al. developed a novel resolution method for phenylalanine racemate with an enzyme utilizing a hollow-fiber membrane reactor (7). Most enzymatic processes currently in industrial use are conducted in batch reactors. However, batch reactors suffer from a number of well-documented limitations, such as batch-to-batch oscillations, high operation costs, etc., and the need to recover enzymes after each batch. On the other hand, a hollow-fiber membrane is very useful as a bioreactor and as a separator for practical application (8) because it has a large interfacial area and offers additional services such as product separation at the same time as the reaction.

To perform the recycling of enzymes, a water-in-oil microemulsion or reversed micelle has been utilized for the hydrolysis of D,L-phenylalanine methyl ester using α-chymotrypsin and for the in-situ separation of the hydrolysis product L-phenylalanine (9, 10). The microemulsion system offers the advantage of greater control of the partitioning of the substrates, products, and catalysts. Also, unlike a liquid emulsion membrane system, the leakage of enzymes is not a serious problem because the enzymes are confined to the microemulsion phase at equilibrium. The only problem in

the microemulsion system was the difficulty of phase separation between the material aqueous source and the microemulsion organic phase.

In the present work we introduce a novel resolution method for amino acids combining an enzyme reaction and a membrane extraction. In the enzyme reaction we employ a newly developed immobilized enzyme with surfactants. The surfactant-coated enzymes are easily soluble in organic solvent; however, they are insoluble in water (11). Therefore, the immobilized enzyme with surfactants enables an expensive enzyme to be recycled from material sources by easy filtration. In the membrane extraction process we use a hollow-fiber membrane as a separator for selectively produced L-phenylalanine, which partitions to the aqueous phase, and unreacted D-ester, which is extracted with an appropriate extractant in an organic phase. The membrane extraction system has no problems concerning emulsification or flooding because the aqueous and organic phases are not mixed directly.

In this study we discuss the optimum conditions in the novel resolution system utilizing an enzyme reaction and a membrane extraction. α-Chymotrypsin was chosen as a model system for the resolution of D,L-phenylalanine methyl esters. The effects of some experimental variables on the selective hydrolysis in the enzyme reaction and on the extraction of unreacted D-ester were mainly investigated.

EXPERIMENTAL

Hydrolysis of D- or L-Phenylalanine Methyl Ester by Enzymes

D- and L-Phenylalanine methyl ester hydrochlorides (D-, L-OMe) were purchased from Sigma Co., Ltd., and they were used as received as substrates for the optical resolution. α-Chymotrypsin (EC 3.4.21.1) was obtained from Sigma Co., Ltd., and lipase from *Candida cylindracea* was supplied by Amano Pharmaceutical Co., Ltd.; they were provided as biocatalysts for the optical resolution. The other reagents were analytical grade. Table 1 shows the general experimental conditions for the hydrolysis of phenylalanine methyl ester. The pH and ionic strength in the aqueous solution were adjusted with phosphate buffer solutions and potassium chloride. The substrate and the enzyme of known concentration were dissolved in an aqueous solution and shaken in a thermostated water bath (303 K). The enzyme reaction was analyzed by HPLC (UVIDEC-100-V, ODS column, JASCO) equipped with a UV detector. The eluent of the HPLC was prepared as following: 0.1 M sodium acetate and acetonitrile (volume ratio 4:1) were mixed, and concentrated acetic acid was added to the solution to regulate to pH 4.2.

TABLE 1
Experimental Conditions for Hydrolysis of Phenylalanine Methyl Ester

Enzyme: α-Chymotrypsin (*EC 3.4.21.1*)
Lipase (*Candida cylindracea*)
Enzyme concentration: 0.01–0.1 g/L
Substrate: Phenylalanine methyl ester
Substrate concentration: 1–10 mM
pH: 5.5–11.0
KH_2PO_4 (100 mM) + K_2HPO_4 (50 mM) + KOH (100 mM)
Coexisting ion: KCl (100 mM)
Temperature: 303 K

Extraction Equilibrium of Phenylalanine and Methyl Ester with an Extractant

L-Phenylalanine (L-Phe, P.I.: 5.48) purchased from Ishizu Pharmaceutical Co., Ltd., and L-OMe were used as substrates for extraction. Tri-*n*-octyl methyl ammonium chloride (TOMAC), di-2-ethylhexyl phosphoric acid (D2EHPA), and sodium di-2-ethylhexyl sulfosuccinate (AOT), obtained from Dojin Chemical Co., Ltd., Tokyo Kasei Co., Ltd., and Kishida Chemical Co., Ltd., respectively, were employed as extractants for the ester extraction. 2-Ethylhexanol purchased from Ishizu Pharmaceutical Co., Ltd., was added as a reforming agent to improve the solubility of TOMAC in an organic solvent. The other reagents were of analytical grade. Table 2 presents the detailed experimental conditions for the extraction equilibrium of phenylalanine and its methyl ester. Analytical-grade isooctane was used as the organic phase. The pH and ionic strength

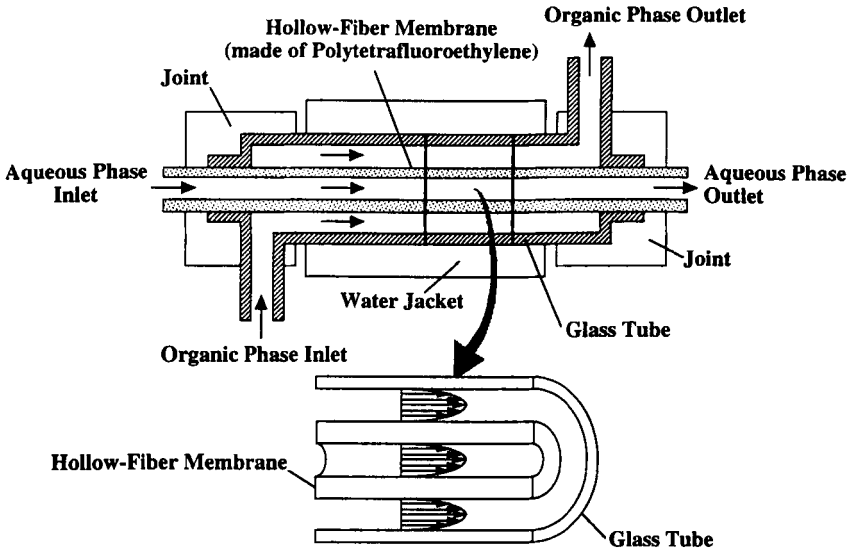
TABLE 2
Experimental Conditions for Extraction of Phenylalanine and Phenylalanine Methyl Ester

Aqueous phase:
Substrate: Phenylalanine (5 mM)
Phenylalanine methyl ester (5–16 mM)
pH: 5.5–10.0
KH_2PO_4 (100 mM) + K_2HPO_4 (50 mM) + KOH (100 mM)
Coexisting ion: KCl (100 mM)
Organic phase:
Solvent: Isooctane
Extractants: TOMAC (Tri-*n*-octyl methyl ammonium chloride): 10 mM
AOT (Sodium di-2-ethylhexyl sulfosuccinate): 10 mM
D2EHPA (Di-2-ethylhexyl phosphoric acid): 4–14 mM
Reforming agent: 2-Ethylhexanol (25 mM)
Temperature: 303 K

of the aqueous solution were adjusted with the phosphate buffer solutions and potassium chloride. The organic phase involving an extractant and the reforming agent of known concentration and the aqueous phase containing the substrate were mixed equivalently. The mixed solution was equilibrated after shaking hard and incubating it in a thermostated water bath (303 K) for half a day. A sample was analyzed by a UV spectrophotometer (UVIDEC-670, JASCO), and the substrate concentration in the aqueous phase was determined by the absorbance at 258 nm. The extraction efficiency was evaluated by the ratio of substrate concentration before and after equilibrium in the aqueous phase.

Separation of Phenylalanine and Methyl Ester Utilizing a Hollow-Fiber Membrane Extractor

TOMAC and D2EHPA were employed as the extractant. The separation experiment was performed at 303 K utilizing a hollow-fiber membrane



Properties of Hollow-Fiber Membrane	
Outer Diameter : d_o = 1.86mm	Inner Diameter : d_i = 1.02mm
Length : L = 0.25m	Pore Size : d_p = 2.00 μm
Porosity : ε = 0.43	Tortuosity Factor : τ = 1.14

FIG. 1 Schematic diagram of hollow-fiber membrane extractor.

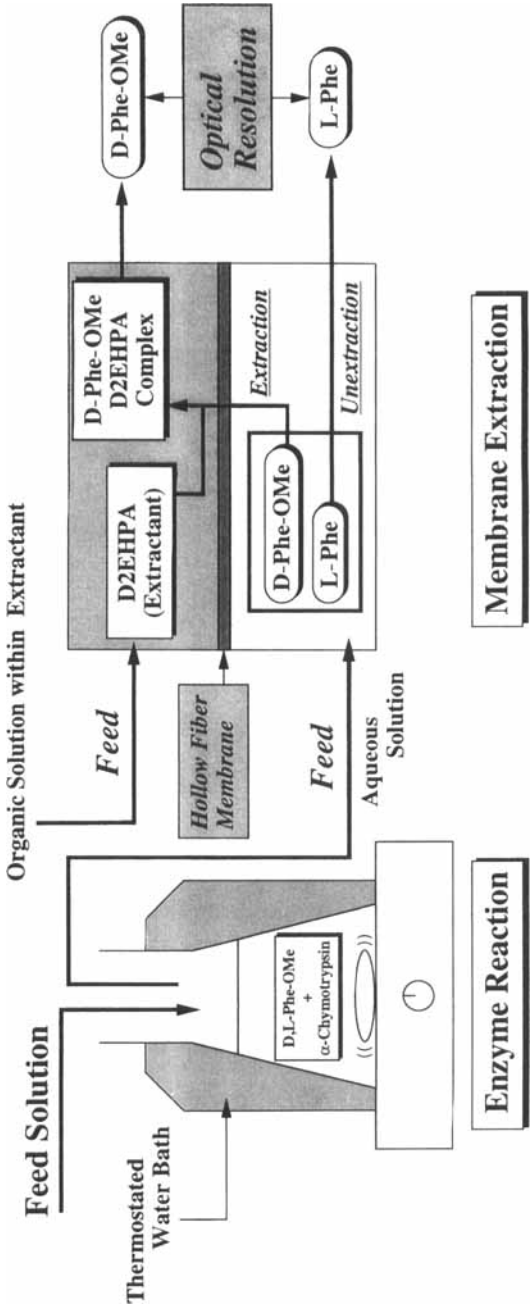


FIG. 2 Optical resolution system of amino acids.

extractor composed of a glass tube and a hollow fiber made of polytetrafluoroethylene as shown in Fig. 1. The aqueous and organic solutions were deaerated in advance and were fed cocurrently into the inner and the outer sides of the hollow fiber using a microtube pump. The volume fluxes of the aqueous and organic solutions in the extractor were 2.41×10^{-9} [m^3/s] and 2.70×10^{-9} [m^3/s]. The OMe and Phe concentrations in the aqueous solution were determined by the UV spectrophotometer.

RESULTS AND DISCUSSION

Figure 2 illustrates a novel optical resolution system for the racemate of amino acids, in which the enzyme reaction and the membrane extraction are hybridized. In the first reaction stage, only the L-isomer is transferred to the amino acid by an enzyme leaving the D-isomer. The material aqueous solution which involves the L-amino acid and D-ester is fed to a hollow-fiber membrane extractor. In the next extraction stage the unreacted ester is selectively extracted with a suitable extractant in the membrane extractor.

Hydrolysis of D- and L-Phenylalanine Methyl Esters by Enzymes

We first investigated the effect of some operation parameters for the hydrolysis of D- and L-phenylalanine methyl esters with an enzyme. In the presence of α-chymotrypsin, L-OMe was selectively hydrolyzed faster than D-OMe (2).

Although several kinds of enzymes were investigated for the hydrolysis reaction, α-chymotrypsin was the best candidate as a currently available enzyme. Thus, we used α-chymotrypsin as the biocatalyst for the hydrolysis of OMe in subsequent experiments.

The price of enzymes is so great for practical use that it is very important to develop a recycling method for the enzymes. We have already reported a preparation method for surfactant-coated enzymes utilizing W/O emulsions (12), which are almost soluble in organic solvents, but are not soluble in aqueous solution. Therefore, we have prepared a surfactant-coated α-chymotrypsin and investigated its reaction properties. Surfactant-coated α-chymotrypsin is insoluble in aqueous media as described above; therefore, the enzyme is expected to be very easy to recover and reuse by a simple operation such as filtration. In fact, we conducted the stability test and confirmed that the surfactant-coated enzyme can be reused by membrane filtration and that it still maintains a high catalytic activity when reused. The optical resolution system could be more effective by introducing the surfactant-coated enzyme in the first reaction step.

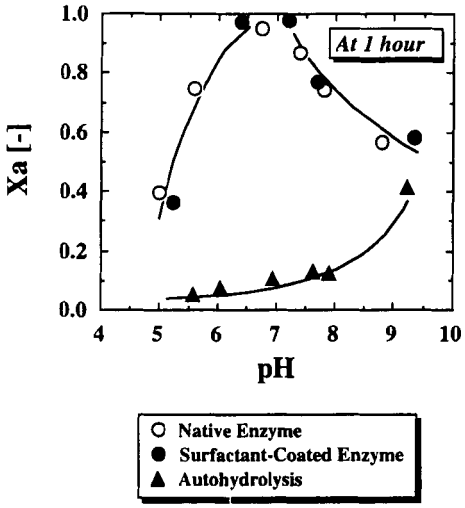


FIG. 3 pH dependency of the hydrolysis of L-phenylalanine methyl ester. Enzyme concentration is 0.01 g/L, substrate concentration is 5 mM.

Figure 3 exhibits the pH dependency of the hydrolysis of L-OMe in the presence (enzymatic hydrolysis) or absence (autohydrolysis) of the enzyme, in which Xa [—] indicates the conversion of OMe. The pH values in the figure are those after the reaction. The pH decreased by one unit after the reaction because the hydrochloric salt of OMe was employed in this study. Based on the results in Fig. 3, it is considered that α-chymotrypsin shows maximum activity around an intermediate pH (between 6.0 and 7.0). The rate of hydrolysis is reduced in higher and lower pH regions. On the other hand, in the absence of the enzyme, the conversion and reaction rate significantly increased at a pH of more than 8 due to the autohydrolysis reaction. From the viewpoint of both enzymatic hydrolysis and autohydrolysis, the optimal pH for the selective hydrolysis of OMe racemate is between 6.0 and 7.0.

Figure 4 shows the effect of the enzyme concentration on the reaction yield of L-Phe. It is clear that the higher the enzyme concentration, the higher the reactivity obtained. A quick hydrolysis is required to depress the autohydrolysis as much as possible.

The effect of ionic (KCl) concentration on the reaction yield of L-Phe was investigated. Although the ionic strength in the reaction media was considered to be an important factor (as was pH), the ionic concentration did not affect either the conversion or the reaction rate. Khmelnisky et al. reported that an enzyme in a salt matrix showed a dramatic enhance-

ment of enzymatic activity in an organic solvent because the matrix provides a protective effect (13). In aqueous media, however, the salt is completely solubilized and dissociates into ion pairs so that it is not able to form the matrix referred to above.

We found that surfactant-coated α-chymotrypsin has a reaction specificity similar to that of the native one. Further, to clarify the kinetics of the enzyme reaction, we analyzed the data obtained with the Michaelis–Menten mechanism. On the basis of the Lineweaver–Burk plot, the K_m [mM] and V_{max} [mol/g·min] values obtained are 13.5 and 29.4 for the surfactant-coated α-chymotrypsin, and 2.42 and 12.7 for the native α-chymotrypsin. Based on the result of the K_m values, it was found to be more difficult for the surfactant-coated α-chymotrypsin to entrap the substrate than for the native one. The surfactants surrounding the enzyme are considered to inhibit the entrapping of substrates. Moreover, on the basis of the results of V_{max} values, it appears that the surfactant-coated α-chymotrypsin would hydrolyze the substrate faster than the native one if the enzyme has formed an intermediate complex with the substrate.

Extraction Equilibrium of Phenylalanine and Methyl Ester

We investigated the extraction equilibrium of Phe and OMe to obtain fundamental information used to determine optimal conditions for the selective extraction of Phe and OMe. Figure 5 shows the results of the extraction equilibrium for OMe with TOMAC, D2EHPA, or AOT used as the extractant. The distribution ratio of OMe, D [—], was defined by

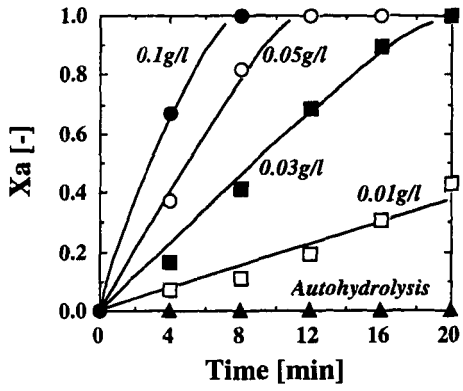


FIG. 4 Enzyme concentration dependency of hydrolysis of L-phenylalanine methyl ester. Substrate concentration is 5 mM, KCl concentration is 100 mM, and the pH of the reaction solution is 7.0.

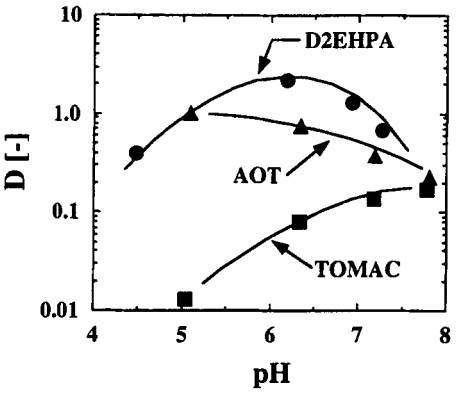


FIG. 5 Extraction equilibrium of Phe and OMe. Substrate concentration is 5 mM, KCl concentration is 100 mM, and extractant concentration is 10 mM. In the case of using TOMAC, di-2-ethylhexanol is added as the reforming agent.

$[OMe]_{org}/[OMe]_{aq}$. We could not detect Phe in the organic phases by a UV-Vis spectrophotometer when every extractant was used. To elucidate the reason, we calculated the existence ratio of the Phe species corresponding to the pH in the aqueous solution as shown in Fig. 6. Because an amino acid forms a zwitterion in aqueous solution, the formation of

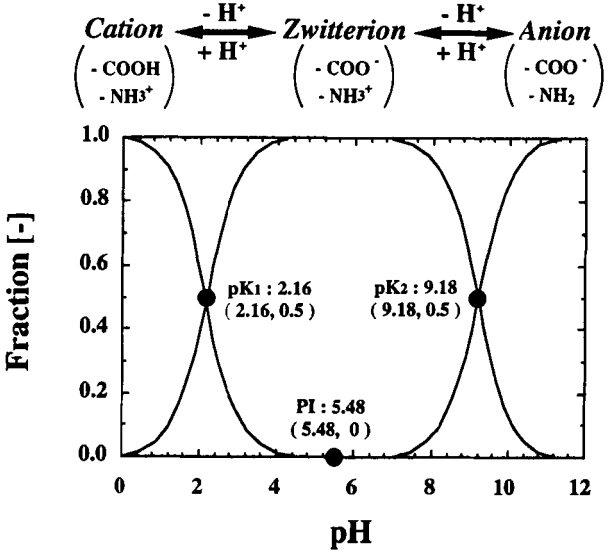


FIG. 6 Fraction ratio of phenylalanine.

cations, anions, and zwitterions strongly depends on the aqueous pH. Hano et al. investigated the extraction equilibrium of amino acids with TOMAC and reported that amino acids could be extracted into an organic phase when anionic species of amino acids and TOMAC created a 1:1 complex by an electrostatic interaction (14). However, most Phe was found to exist as the zwitter form at an intermediate pH under the present experimental conditions, so that it was not extracted due to the high hydrophilicity. Though the extraction behavior of amino acids with D2EHPA (15) or AOT (16, 17) has also been reported, the extraction of Phe was not performed under the intermediate pH condition because of the same reason.

It appears that every extractant can extract OMe; nevertheless, there are differences in extraction efficiency among the three extractants. The extraction efficiency of TOMAC is 20% at maximum, and those of D2EHPA and AOT are around 70 and 50%, respectively. Based on the results shown in Fig. 5, it is clear that the pH in the feed solution is one of the significant factors in the effective extraction of OMe. It seems that the extraction of OMe strongly depends on the ratio of the cationic species which corresponds to the cationized amino group in the OMe molecule.

It is concluded that complete separation of Phe and OMe is possible using a liquid–liquid extraction system. We found that D2EHPA is the most suitable extractant for the separation of Phe and OMe from the viewpoint of the optimal pH in the enzyme reaction, which has to be combined with a membrane extractor. Thus, we provided D2EHPA as the extractant in the following experiments.

In a low salt concentration (below 50 mM), both aqueous and organic phases seem to be easily emulsified. Therefore, the salt concentration should be maintained at a relatively high level. In this study we adjusted the salt concentration to 100 mM with KCl.

We investigated the dependency of extractant concentration on the extraction equilibrium and the continuous variation method [Job Ostomisslensky's method (18)] to elucidate the complex formation between OMe and D2EHPA. Figure 7 shows the results of the dependency of the extractant concentration on the extraction equilibrium of OMe. In Fig. 7 $[(Ex)^2]$ represents the dimer concentration of the extractant. This result suggests that the ester compound is extracted with three monomer extractants. Figure 8 exhibits Job's plot as a result of the continuous variation method. In Fig. 8, E [—] indicates the degree of extraction. Based on the results in the figure, because the maximum peak appeared at 0.75, three extractant molecules are considered to coordinate to one substrate molecule. Teramoto et al. reported that most of the D2EHPA exists as a dimer in dodecane (19), and it can be assumed that the main species of D2EHPA is a

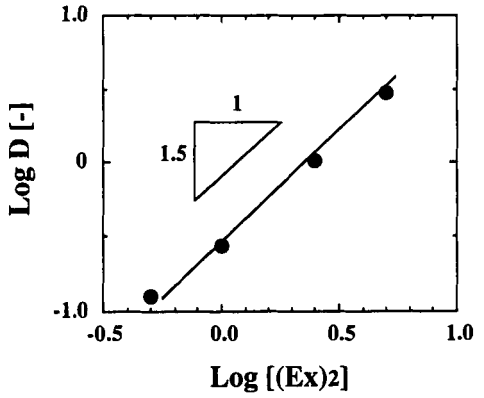


FIG. 7 Dependency of extractant concentration on extraction equilibrium. Substrate concentration is 5 mM, KCl concentration is 100 mM, and the pH of the aqueous solution is 6.5. Extractant is D2EHPA.

dimer in the isooctane used in this study. Based on the results, we are led to the conclusion that 1.5 molecules of extractant dimer (three molecules of extractant monomer) coordinate to one substrate molecule. This result is in agreement with the results of the continuous variation method. Sutherland et al. examined the extraction of an amino acid ester with a crown ether (20), and an extraction model was proposed. A similar model as illustrated in Fig. 9 can be suggested for D2EHPA.

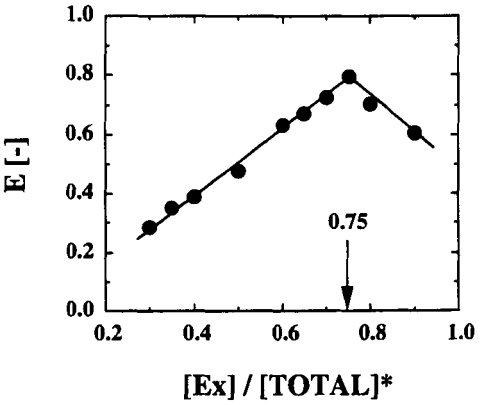


FIG. 8 Result of continuous variation method (Job's plot). Substrate concentration is 6–16 mM, KCl concentration is 100 mM, and the pH of the aqueous solution is 6.5. Extractant concentration is 4–14 mM. * [TOTAL] = [Ex] + [OMe] = 20 mM.

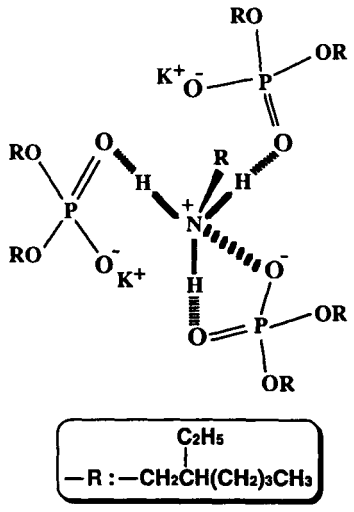


FIG. 9 Schematic illustration of the interaction between amino acid ester and D2EHPA.

Separation of Phenylalanine and Methyl Ester by a Hollow-Fiber Membrane Extractor

We investigated the selective extraction of Phe and OMe utilizing a hollow-fiber membrane extractor under the optimal conditions on the basis of the results of the extraction equilibrium of OMe with D2EHPA. From the experimental results the apparent permeability of OMe, P_{OMe} [m/s], was obtained as the following equation (21):

$$P_{OMe} = J_{OMe}/C_{OMe,0} = C_{OMe,org} \cdot Q_{org}/(\pi \cdot d_i \cdot L) \quad (1)$$

where J_{OMe} [mol/m^2·s] is the average extraction rate of OMe, $C_{OMe,0}$ [mol/m^3] is the initial concentration of OMe in the aqueous feed solution at the inlet of the membrane reactor, $C_{OMe,org}$ [mol/m^2] is the concentration of OMe in the organic solution at the outlet of the membrane extractor, Q_{org} [m^3/s] is the volumetric flow rate of the organic solution, d_i [m] is the inner diameter of the hollow fiber, and L [m] is the effective length of the membrane extractor. Figure 10 shows the dependency of pH in the feed solution on the permeation rate of OMe. Phe could not be detected in the organic phase even in the presence of an extractant. The hollow-fiber membrane extractor was very useful as a separator of amino acids and their ester derivatives because only the ester compound was selectively transferred to the organic phase. In comparison with a solvent extraction process, the membrane process has several advantages; for example, a

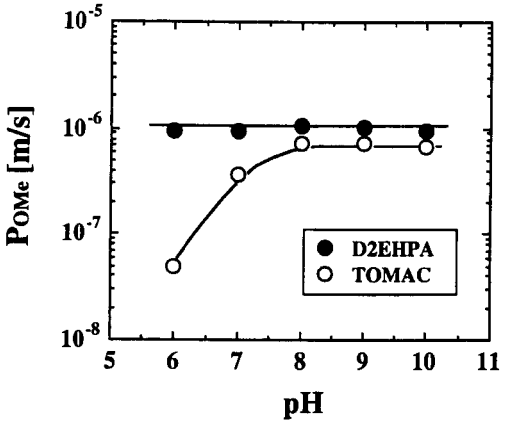


FIG. 10 The dependency of the permeation coefficient. Substrate concentration is 5 mM, KCl concentration is 100 mM, and the extractant concentration is 10 mM. In the case of using TOMAC, di-2-ethylhexanol is added as the reforming agent.

large interfacial area per unit extractor volume and a decreased operation energy. D2EHPA shows a better result as the extractant than does TOMAC.

The membrane permeability of OMe, P_{OMe}, was 1.1×10^{-6} [m/s] at the maximum for D2EHPA, which is of the same order as that found when extracting metal ions by a similar membrane extractor (22). For the application of this separation system on an industrial scale, a membrane separation process using hollow-fiber modules would be effective and is a promising method for the optical resolution of amino acids.

CONCLUSIONS

A novel optical resolution system for amino acids, which combines an enzyme reaction and a membrane extraction, has been proposed. The effects of some operation conditions in the separation system were investigated, and the following results were obtained.

1. In the racemic ester of D- and L-OMe, L-OMe was selectively hydrolyzed by α-chymotrypsin, and the pH in an aqueous solution is an important factor in the enzymatic activity and selectivity. The optimal pH for the selective hydrolysis of the racemate was between 6 and 7.
2. Introducing a surfactant-coated enzyme, which is insoluble in an aqueous solution, in the first step in the hydrolysis reaction enables an expensive enzyme to be reused in water.

3. A hollow-fiber membrane extractor was very useful as a separator for amino acids and their ester derivatives because only the ester compound was extracted with an extractant, leaving the amino acid.

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