0273-2289/00/88/0097/$12.50

Enantioselective Hydrolysis of a Schiff's Base of D,L-Phenylalanine Ethyl Ester in Water-Poor Media Via the Reaction Catalyzed with α-Chymotrypsin Immobilized on Hydrophilic Macroporous Gel Support

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Abstract

The application of immobilized α-chymotrypsin for the purpose of enantioselective hydrolysis of a Schiff's base of D,L-Phe-OEt (D,L-SBPH) in the mixed water-acetonitrile media with the different content of water is described. The immobilized biocatalyst was prepared by the chemical coupling of the enzyme to poly(vinyl alcohol) (PVA) cryogel—the macroporous hydrogel prepared by means of the freezing–thawing techniques. SBPH is water insoluble, and, therefore, acetonitrile (MeCN) with minor water additives was used as a solvent for the reaction of enantioselective hydrolysis of the racemic substrate. The process was conducted for 96–200 h, and L-Phe with the purity up to 98% e.e. precipitated in both the reaction medium and gel-carrier bulk. The product was recovered by washing the organo-insoluble sediment with aqueous ammonia. D-Phe with the purity up to 85% e.e. was recovered from the organic solution of D-ester after its acidic hydrolysis. The PVA-cryogel-attached enzyme was effective in SBPH hydrolysis in MeCN/water mixtures. The immobilized biocatalyst was active for more than 1 mo of application and could be successfully used after another 4 mo storage at +10°C.

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Index Entries: Biocatalysis in MeCN water-poor media; immobilized α-chymotrypsin; macroporous poly(vinyl alcohol) cryogel carrier; L-Phe; D-Phe.

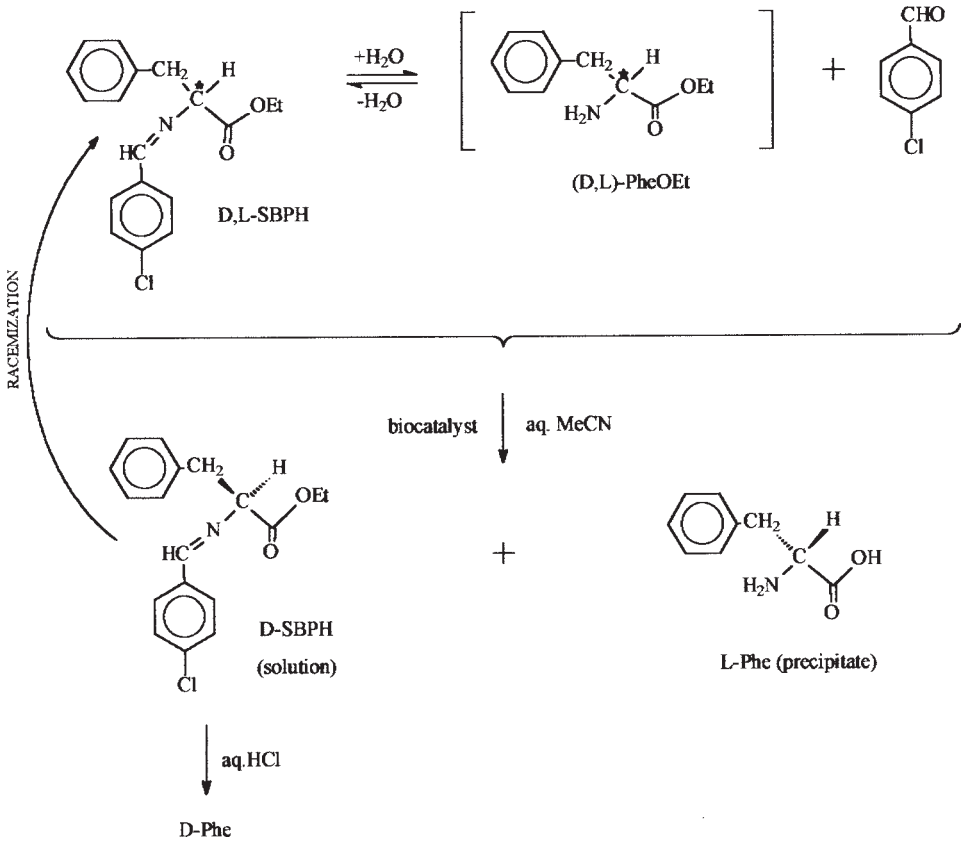
Introduction

Proteases are widely used for enantioselective hydrolysis of organic compounds. Unfortunately, a number of important problems, such as low enzyme stability, complicated procedures of separation of reaction products from the by-products resulting from the enzyme autodigestion, and low solubility of routine organic substrates in water, restrict the use of these biocatalysts in preparative organic chemistry. Recently, the application of enzymes as catalysts in organic solvents has become a popular method for asymmetric synthesis and resolution of water insoluble compounds in many laboratories *(1–3)*. However, enzyme activity in neat organic solvents is rather often low mainly due to protein denaturation and/or aggregation *(4)*. Thus, there is a general need to improve efficiency of enzymatic catalysts in both water and organic solvents.

Immobilization could be proposed as a way to stabilize biocatalysts. At the same time, dispersion of enzyme macromolecules within a solid support avoids their aggregation in the nonaqueous media. Therefore, supports for enzyme immobilization as well as support/enzyme conjugates should have universal stability in both water and nonaqueous media.

The well-known polymeric hydrogel supports (likewise polyacrylamide gels, sephadexes, sepharoses, polyacrylates, etc.) usually efficient in water media cannot be taken as competent carriers in nonaqueous or mixed solutions because of possible collapse of respective polymeric network in these solvents. Recently, a number of efficient approaches to the elaboration of immobilized biocatalysts for the use in nonaqueous media have been made *(5,6)*, including the preparation of crosslinked enzyme crystals *(7,8)*. However, only a few reports on preparations of the protein-containing polymers with high activity in both organic solvents and organic/water mixtures are known, e.g., *see* ref. *9*. One of the recent developments was the use of poly(vinyl alcohol) (PVA) cryogels, the macroporous hydrogels prepared by freezing–thawing techniques *(10)*, for the immobilization of cells and enzymes *(11–13)*. Some preliminary results have clearly demonstrated that the carrier held a great promise for enzyme stabilization in water-poor media. Besides, the original procedure for the preparation of these carriers is based on the use of a cheap and available gelling agent, namely PVA, which is nontoxic, biocompatible, exhibits a high chemical and biological resistance, and finally, the polymer is produced by many companies worldwide.

This work describes the application of α-chymotrypsin immobilized by coupling to hydrophilic macroporous PVA-cryogel support for the enantioselective hydrolysis of a Schiff's base derived from *p*-chlorobenzaldehyde and D,L-Phe-OEt (D,L-SBPH) in a mixed water-acetonitrile media with different content of water (*see* Scheme 1). The enantioselective



Scheme 1. The enantioselective hydrolysis of a Schiff's base of D,L-phenylalanine ethyl ester (D,L-SBPH) in water-poor media via the reaction catalyzed by the immobilized α-chymotrypsin.

hydrolysis of a set of Schiff's bases of amino acids esters (including SBPH) catalyzed by powdered lipase and α-chymotrypsin in water-poor or two-phase reaction media has been investigated earlier *(14)*.

Materials and Methods

Materials

The following substances were used in the work: α-Chymotrypsin (EC 3.4.21.1) from bovine pancreas, a lyophilized powder, was obtained from Fluka (Buchs, Switzerland). *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTNA) was from Sigma (St. Louis, MO, USA). Poly(vinyl alcohol) of trademark 20/1 (MW 82,000) was obtained from NPO "Polivinilatsetat" (Yerevan, Armenia). Preparation of D,L-SBPH was performed according to the method in ref. *14*. Diethyl ester of *N*-acetylaminomalonic acid (DEAMA) was purchased from Reakhim (Moscow, Russia) and additionally recrystallized from ethanol.

Acetonitrile (MeCN) was obtained from Reakhmin (Russia) and additionally purified by double distillation (b.p. 81–82°C) over P_2O_5. Bi-distilled water was used for the preparation of all aqueous solutions and buffers.

Enantiomeric Analysis

Gas chromatography was performed using a chiral glass capillary column (l = 41 m, i.d. = 0.21 mm) with a diamide polysiloxane phase type "Chirasyl-Val" (synthesized in the authors' Institution) and an FID detector. The carrier gas was helium, 147°C, the flowrate was 1 mL/min, and the inlet pressure was 1.6 bar. L-Phe and D-Phe were analyzed as N-trifluoroacetyl derivatives of their isopropyl esters.

Chymotrypsin Immobilization by Covalent Coupling to the Beads of Aldehyde-Bearing Poly(Vinyl Alcohol) Cryogel

Beaded PVA cryogel (particles' diameter approx 1 mm) was prepared with a cryogranulating set-up "CryoMat" (BioChemMac Co., Moscow, Russia) in accordance with the patented procedure *(15)*. Then the beads obtained were treated with 7% aqueous solution of glutaric aldehyde at pH 1.0 for 1 h at the room temperature. The activated gel was rinsed with water to remove the dialdehyde excess, the aldehyde-bearing beads (1 g of wet wt) were mixed with 5 mL of α-chymotrypsin solution (1 mg/mL) in 0.1 *M* Na-phosphate buffer, pH 7.4, at room temperature. The insoluble material was stirred for 5 h, washed twice with the same buffer solution, and treated with 0.1 *M* Tris/HCl, pH 8.0, for 2 h to block the remaining free CHO groups. The immobilized biocatalyst was washed with 0.1 *M* Na-phosphate buffer, pH 7.4, and used in further experiments. The amount of the enzyme bound to the carrier (1.95 mg of protein per 1 g of the wet gel carrier) was determined spectrophotometrically on Hitachi UV-VIS Spectrophotometer 557 (Hitachi, Tokyo, Japan) as the difference in the absorbance at 280 nm of the initial solution of the enzyme used for the immobilization and that of the combined washings of the immobilized material ($A^{1\%}_{280\ nm}$ was equal to 20.4 *[16]*).

α-Chymotrypsin Activity Assay

The enzyme activity was determined using two substrates: DEAMA (ester compound) and BTNA (amide substance). In the former case the standard titrimetric procedure *(16)* with the use of a TTT-1c autotitrator (Radiometer, Copenhagen, Denmark) was employed. In the latter case, a spectrophotometric assay was used to follow the cleavage of the specific substrate in both the aqueous and organic media. For this purpose the weighed amount of the wet beads of immobilized biocatalyst containing 0.2 mg of the protein was suspended in 3 mL of 0.05 *M* Tris-HCl buffer solution, pH 8.0, then 0.01 mL of 1 m*M* solution of BTNA in MeCN was added, and the system was stirred at 22°C for 50 min with simultaneous recording of the absorbance of the supernatant solution at 405 nm (a molar absorptivity for *p*-nitroaniline liberated was taken equal to 9620) *(17)*. Virtually the

same procedure was used to study BTNA hydrolysis in MeCN/water solution. The biocatalyst beads were soaked with an aqueous buffer solution for a night, then equilibrated for 1 h with MeCN (2 mL/0.1 g of the beads) and thereafter were used in the experiments.

Stability of the Immobilized Enzyme

To determine the stability of immobilized enzyme in the organic media, the α-chymotrypsin-bearing beads were stored in MeCN until the appropriate time. The wet beads (1 g) were equilibrated with MeCN (1 mL) containing <0.1% of water and filtered, and a fresh portion of the solvent (3 mL) was added. The bead suspension was incubated for various times at 25°C with periodic stirring. The water content in the MeCN/water mixture obtained was approx 20%. The aliquots of these beads (100 μL) were taken periodically to determine the enzyme activity. The water content of the supernatant was monitored by Karl Fisher's procedure. To get the lower water content (8.9 and 0.6 %), the above described procedure was used, but the volume of MeCN solutions was increased to 3 and 9 mL instead of 1 and 3 mL, respectively.

Typical Procedure of the Enzymatic Hydrolysis with Immobilized α-Chymotrypsin

D,L-SBPH dissolved in MeCN/water mixtures (5–20% H_2O) was added to a suspension of the immobilized biocatalyst in the same solvent, and the reaction bulk was stirred at the room temperature for a stipulated period of time. A sediment containing the precipitated L-Phe and the immobilized α-chymotrypsin was filtered and washed twice with water-free MeCN to remove all substances soluble in organic solvents. When the water concentration was higher than 10%, 5 mL of dry, containing <0.1% of water, MeCN was added at the end of the process. Then the sediment was washed with 1% aqueous ammonia (twice) and filtered, and the filtrate was evaporated *in vacuo*. The residue obtained was purified by ion-exchange chromatography on DOWEX-50W(H^+) resin. L-Phe was eluted from the resin with 5% ammonia, the solution was evaporated, and the residue was analyzed. In the case of D-Phe isolation, the combined MeCN extract was evaporated *in vacuo*, and the residue was hydrolyzed with 6 *N* HCl at 20°C for 10 min. The solution obtained was extracted with toluene to remove *p*-chlorobenzaldehyde. The water layer was refluxed for 5 h and evaporated. The residue containing D-Phe was purified on DOWEX-50W (H^+) column and analyzed. The chemical purity of Phe enatiomers was examined with ^{1}H NMR spectroscopy and TLC.

Results and Discussion

Characteristics of the Immobilized Biocatalyst

The evaluation of the activity of immobilized enzymes in the reaction of hydrolysis of ester and amide substrates in aqueous buffers monitored by pH-stat and spectrophotometrically showed that the residual catalytic

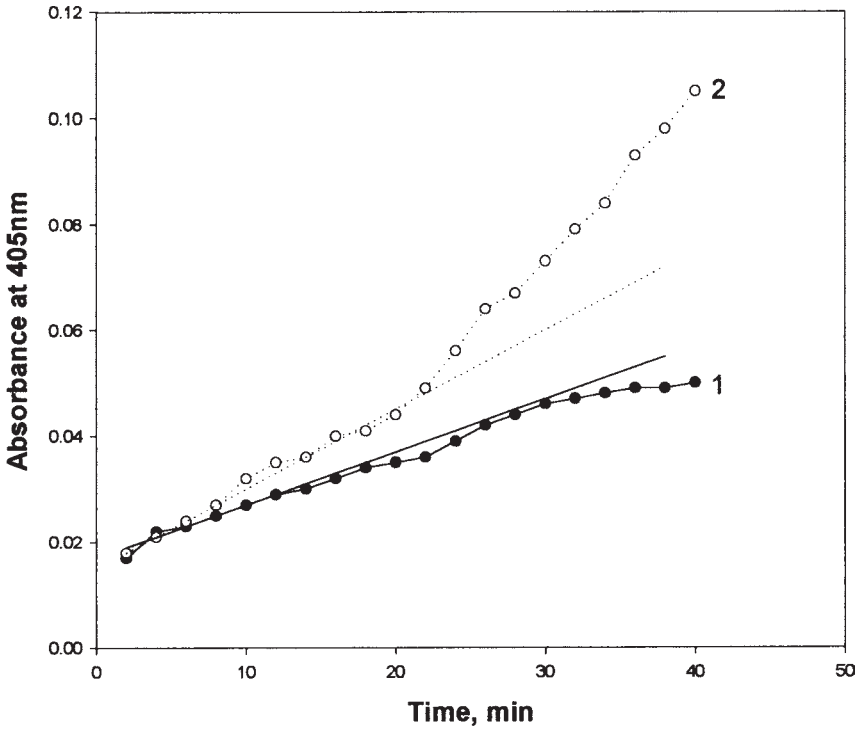


Fig. 1. The kinetics of α-chymotrypsin-catalyzed cleavage of BTNA in MeCN (5% H_2O) medium (straight lines show linearization of the initial regions of the curves): 1, powdered enzyme; 2, PVA-cryogel-immobilized enzyme.

activity of the immobilized α-chymotrypsin in water media depended on the substrate type and was 85.5% of the native enzyme activity in the hydrolysis of DEAMA (moderate specificity substrate) and 10.3% in the case of BTNA (high specificity substrate). Obviously, the catalytic activity of the enzyme active site was somewhat modified as a consequence of the immobilization procedure.

The spectrophotometric method was used to compare the activities of native and immobilized α-chymotrypsin in MeCN/water mixtures, where both the initial enzyme and its PVA-cryogel-coupled form were insoluble. Figure 1 illustrates the increase in optical density at 405 nm of the supernatant solution of the reaction mixture due to *p*-nitroaniline formation from the BTNA hydrolyzed by the powdered enzyme (curve 1) and the freshly prepared PVA-cryogel-attached α-chymotrypsin (curve 2). The protein concentration and the enzyme-to-substrate ratio were identical in the two cases.

The efficiency of the immobilized biocatalyst was somewhat higher compared to the powdered enzyme. And, second, such an enzyme-catalyzed reaction in the organic medium was sufficiently slow: initial rates were equal to $1.3\,10^{-3}$ μmol/min mg (from curve 1) and $1.9\,10^{-3}$ μmol/min mg (from curve 2).

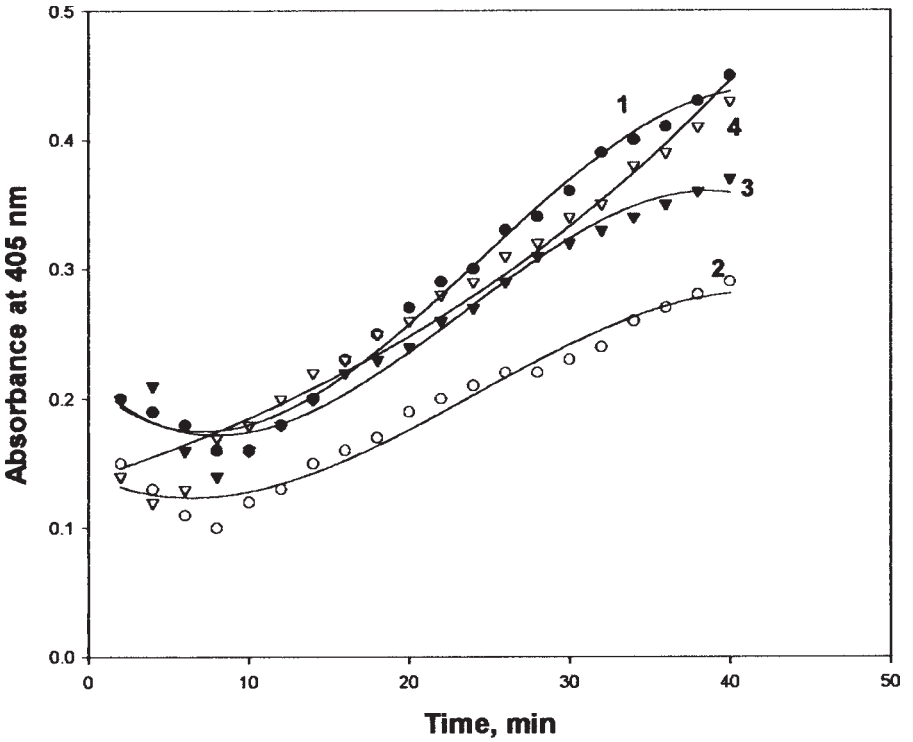


Fig. 2. Hydrolysis of BTNA in 0.05 *M* Tris-HCl buffer solution, pH 8.0, with PVA-cryogel-immobilized α-chymotrypsin preliminarily incubated for various times in MeCN (5% H_2O) medium: 1, immobilized biocatalyst just after preparation; 2, biocatalyst incubated in MeCN for 1 h; 3, biocatalyst incubated in MeCN for 6 d; 4, biocatalyst incubated in MeCN for 38 d.

The stability of the PVA preparations in water-poor media was tested by incubation of the beads in MeCN/water mixtures (95/5 [w/w]) for defined periods (1–912 h) of time followed by the kinetic studies of BTNA hydrolysis with the beads in an aqueous buffer. It can be seen from the data in Fig. 2 that the prolonged storage (up to 38 d) of the beads in the water-poor media had a slight effect on the initial rate of the substrate hydrolysis.

Enantioselective Hydrolysis of a Schiff's Base of D,L-Phenylalanine Ethyl Ester (SBPH)

The racemic SBPH was synthesized from the ethyl ester of racemic phenylalanine and *p*-chlorobenzaldehyde as described elsewhere *(14)*. The salient feature of the substrate was its high storage stability, the ease of its hydrolysis by α-chymotrypsin in MeCN/water mixtures, and the possibility of recycling the remaining D-form of the substrate by racemization (*see* Scheme 1) *(14)*.

The immobilized biocatalyst was stored in water, and the water-saturated beads were used to hydrolyze the substrate solution in aqueous

Table 1
Enantioselective Hydrolysis of D,L-SBPH in MeCN Water-Poor Media Catalyzed by α-Chymotrypsin Immobilized on PVA-Cryogel[a]

Hydrolysis conditions			Hydrolysis products		
Run (number of cycles)[b]	Amount of substrate, mg	Time, h	Chemical yield of L-Phe, %	L-Phe, % e.e.	D-Phe, % e.e.
1	37	96	70	94.0	63.8
2	32	96	68	97.7	55.1
3	33	144	82	88.8	81.7
4	35	144	72	95.6	68.8
5	32	192	88	98.6	84.5

[a]1.5 mg of α-chymotrypsin/1 g of PVA-cryogel, 0.7 g of the water-saturated beads, 1.5 mL of MeCN solution of SBPH, the water content of the equilibrated supernatant MeCN solution was 5%.

[b]The same beads recovered from the reaction mixture were repeatedly used in the subsequent cycles.

MeCN with the ratio of bead weight to that of acetonitrile equal to 1:1.5–2. The water content of the equilibrated supernatant MeCN solution was 5–30%. The reaction proceeded for 96–200 h at the ambient temperature and was terminated by the addition of a large excess of water-free MeCN. L-Phe formed was precipitated in the course of reaction and remained in the resultant system either as a "free" solid or as a solid matter in the inner areas of the biocatalyst gel beads. To recover the amino acid, the sediment (L-Phe precipitated and gel beads together) was filtered and washed with aqueous ammonia to dissolve L-Phe. D-Phe, in turn, was isolated from the organic filtrate after MeCN solution evaporation and hydrolysis of the residue with aqueous HCl. The chemical yield of the final products was determined by weighing, and their optical purity was examined with gas–liquid chromatography on a chiral phase (*see* Materials and Methods). The beads after being rinsed with water were used repeatedly. Table 1 summarizes the results of these preparative experiments.

It was found that PVA-cryogel-immobilized α-chymotrypsin was effective in hydrolysis of the ester racemic substrate, SBPH, in MeCN/water mixture. This immobilized biocatalyst was active for a month and could be successfully used after another 4 mo storage at +10°C.

The enantiomeric purity of L-Phe depended on the water concentration in the reaction media, therefore we have studied the influence of water content in MeCN on the enantioselectivity of the substrate hydrolysis catalyzed by enzyme immobilized on PVA beads. With the increase in the water concentration in the reaction medium from 6.5 to 20%, the enantioselectivity decreased from 96 to 88% (*see* Table 2). A drop in enantioselectivity might be a result of an increase in the rate of spontaneous hydrolysis of ester group yielding the racemic Phe.

Table 2
The Effect of Water Content of MeCN on Enantioselectivity of D,L-SBPH Hydrolysis of Catalyzed by α-Chymotrypsin Immobilized on PVA-Cryogel[a]

Hydrolysis conditions			Hydrolysis products		
Water content (%) of the supernatant MeCN[b]	Amount of substrate, mg	Time, h	Chemical yield of L-Phe, %	L-Phe, % e.e.	D-Phe, % e.e.
20.5	40	72	75	88,2	70.9
10.5	40	72	77	93.4	73.2
6.7	40	72	75	95,8	70.2

[a]The initial preparation was recovered after several cycles in aq. MeCN and stored for several months in a refrigerator before use. 1.5 mg of α-chymotrypsin/1 g of PVA, initially 1 g of water-saturated beads and 2 mL of MeCN solution of SBPH (run 1).

[b]In runs 2 and 3, the recovered beads were washed with water, then with water-free MeCN, and a solution of SBPH in MeCN added to the beads.

To clarify the influence of the matrix structure on the efficacy of immobilized enzyme operation in water-poor media, α-chymotrypsin was chemically coupled to another hydrophilic matrix, Sephadex G-75 (macrohomogeneous gel carrier), and the biocatalyst obtained was used for the hydrolysis of SBPH under the experimental conditions indicated in the footnotes to Table 1. Immobilization procedure in this latter case was identical to the experimental protocol described above for the PVA-cryogel carrier. The immobilized biocatalyst prepared using Sephadex G-75 contained 2.2 mg of protein/1 g of wet gel beads. The results of the enantiospecific cleavage of the racemic ester substrate were disappointing with this latter enzyme catalyst, since the gel carrier collapsed in MeCN media, and both the chemical yield (approx 15% only) and enantiomeric purity (e.e. 13%) of L-Phe were considerably lower than those presented in Table 1 for α-chymotrypsin immobilized in macroheterogeneous PVA-cryogel carrier. These results evidently testified to the importance of correct selection of appropriate gel matrix for the immobilized enzymes to be used in water-poor organic solutions. It should also be pointed out that L-Phe precipitation in the water-poor media could destroy the gel matrix, because the mechanical stresses caused by the crystallization is significant. In fact, a partial destruction of the microporous Sephadex carrier was observed (Light Microscope MB-3, LOMO, Russia), whereas the macroporous PVA-cryogel matrix was not affected most likely because L-Phe crystals had enough space to grow inside the macropores of the cryogel preparation.

High stability and activity of PVA-chymotrypsin preparations in water-poor media had to be discussed. One reason for it might be the macroporous morphology *(10)* of the PVA-cryogel matrices with a very rigid mutual arrangements of the polymer chains. The matrix is additionally stabilized by crosslinking process, taking place simultaneously with the introduction of reactive aldehyde groups into the carrier in the course

of its treatment with a large excess of glutaric aldehyde *(12)*. Such a chemical modification further preserves (to a certain extent) the gel support against collapsing in the water-poor organic solvents, in the concentrated MeCN media in particular. At the same time, very high stability of the enzyme chemically attached to the aldehyde derivative of PVA-cryogel beads in the reactions performing in organic media obviously indicate to the multi-point binding of α-chymotrypsin macromolecules to the CHO carrier.

Conclusions

Application of enzyme catalysis allowed the separation of a racemic mixture of D,L-SBPH yielding individual enantiomers of Phe to be achieved. The developed procedure of α-chymotrypsin immobilization gave rise to the biocatalyst efficiently functioning in water-poor media for months. PVA-cryogel demonstrated a promising potential of such a carrier for the production of immobilized enzymes destined for the use in preparative organic synthesis.

Acknowledgment

Financial support from the Russian Foundation of Fundamental Investigations (grant no. 96-03-33424) and Department of Science and Technology of India Government are gratefully acknowledged.

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