Pure Appl. Chem., Vol. 77, No. 1, pp. 227–236, 2005.
DOI: 10.1351/pac200577010227

Gel-immobilized enzymes as promising biocatalysts: Results from Indo-Russian collaborative studies*

Elena A. Markvicheva[1,‡], Vladimir I. Lozinsky[2], Fatima M. Plieva[2], Konstantin A. Kochetkov[2], Lev D. Rumsh[1], Vitali P. Zubov[1], Jyotirmoy Maity[3], Rajesh Kumar[3], Virinder S. Parmar[3], and Yury N. Belokon[2]

[1]Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya St. 16/10, Moscow 117871, Russia; [2]Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, Vavilov St. 28, Moscow, V-334, 117813, Russia; [3]Bioorganic Laboratory, Department of Chemistry, University of Delhi, Delhi 110 007, India

Abstract: Chemo-enzymatic methods constitute a promising approach to obtain various biologically active compounds, including enantiomerically pure substances. Entrapment in gels is one of the most convenient methods to stabilize enzymes for their application in water/organic media. Proteases and lipases are widely used for enantioselective transformations of various organic compounds in water-poor media. In this study, chymotrypsin was entrapped into a composite poly(*N*-vinyl caprolactam)-calcium alginate (PVCL-CaAlg) and covalently attached to poly(vinyl alcohol) (PVA) cryogel beads. Lipase was immobilized by covalently attaching to aldehyde-bearing PVA cryogel beads. The activities of the entrapped biocatalysts were studied. Both entrapped α-chymotrypsin and lipase retained high activity in acetonitrile/water medium (water content 0.5–20 %) and displayed high storage stability for several months. The high operational stability of immobilized α-chymotrypsin and lipase in a cyclic process (up to 912 h in total) was also demonstrated. Gel-immobilized enzymes were successfully used to obtain optically pure L-phenylalanine (ee 98.6 and 83 % in the case of α-chymotrypsin and lipase, respectively) by enantioselective hydrolysis of Schiff's base of amino acid ethyl ester in an acetonitrile/water system.

INTRODUCTION

As recently as 30–40 years ago, the idea of performing an enzymatic reaction in nonaqueous media seemed almost heretical, but during the last few years, applications of enzymes for catalyzing various chemical processes in water/organic media has become a widely used approach to obtain biologically active compounds. However, despite numerous examples of successful use of biocatalysts in nonaqueous media, elimination of vicious effects of organic solvents on enzymes still remains a problem. As is well known, direct interaction of enzyme molecules with an organic solvent affects the catalytically ac-

*Paper based on a presentation at the 24th International Symposium on the Chemistry of Natural Products and the 4th International Congress on Biodiversity, held jointly in Delhi, India, 26–31 January 2004. Other presentations are published in this issue, pp. 1–344.

‡Corresponding author

tive conformation of the enzyme. Immobilization of enzymes is one of the most promising techniques to prevent enzymes from inactivation in organic solvents.

Proteases and lipases are widely used for enantioselective transformation of various organic compounds in water-poor media [1–4]. In this study, two hydrogel carriers for immobilization of bovine α-chymotrypsin and hog pancreas lipase have been used. One of them is a composite temperature-sensitive poly(*N*-vinyl caprolactam)-Ca alginate (PVCL-CaAlg) hydrogel which has been successfully used in water medium earlier [5]. Another one is macroporous poly(vinyl alcohol) cryogel carrier prepared by freezing–thawing technique using the patented procedure [6]. Both carriers stabilized enzyme activity within broad concentration ranges of organic solvents. Chymotrypsin was immobilized [7–11] in both PVCL-CaAlg and PVA cryogel beads and was successfully used for enantioselective hydrolysis of the Schiff's base of D,L-phenylalanine ethyl ester (SBPH) in a water/acetonitrile medium. Hog pancreas lipase covalently attached to PVA macroporous cryogel beads was also tested in this reaction and allowed us to obtain optically pure L-phenylalanine (ee 83 %) in 144 h.

MATERIALS AND METHODS

Chemicals

PVCL (MW 900 000) was obtained by polymerization of *N*-vinyl caprolactam as described earlier [7]. Sodium alginate (medium viscosity) was from Sigma, USA. Aromatic polyamide POLAR (MW 25 000) was obtained by copolymerization of isophthalic acid and 4,4'-diaminodiphenyl-2,2'-disulfonic acid [12]. *N*-Benzoyl-L-tyrosine-*p*-nitroanilide (BTNA) and *N*-acetyl-L-tyrosine ethyl ester (ATEE) were from Sigma, USA. α-Chymotrypsin (EC 3.4.21.1), 122 U/mg, was from Biolar, Latvia. Hog pancreas lipase, PPL (EC 3.1.1.3), 3.6 U/mg, was from Fluka, Switzerland. Poly(vinyl alcohol) preparations of MW 69 000 and MW 82 000, were from NPO "Azot" (Severodonetsk, Ukraine) and from NPO Polivinilatsetat (Armenia), respectively. *N*-Carbobenzoxy-glycine *p*-nitrophenyl ester (*Z*-Gly-pNP; Reanal, Hungary) was recrystallized twice from isopropanol. *p*-Nitrophenyl propanoate (pNPP) was synthesized from distilled propanoic acid and *p*-nitrophenol (recrystallized from benzene) via condensation with *N*,*N*'-dicyclohexylcarbodiimide. Diethyl ester of *N*-acetylaminomalonic acid (DEAMA) was purchased from Reakhim (Russia) and additionally recrystallized from ethanol. Acetonitrile was from Sigma, USA. Schiff's base was prepared from *p*-chlorobenzaldehyde and D,L-Phe-OEt (Sigma).

METHODS

Entrapment of α-chymotrypsin in PVCL-CaAlg beads

The enzyme solution (0.1–0.2 ml, 10 mg/ml) was mixed with two polymer solutions: PVCL [1.0 ml, 10 % (w/v)] and POLAR (0.125 ml, 1 % w/v). Then, 0.3 ml of an ovalbumin solution (7 mg/ml) and 2.5 ml of a sodium alginate solution (2 %, w/v) were added. The mixture was dropped into a $CaCl_2$ solution (50 ml, 2 % w/v) at 40 °C, and beads (0.4–0.6 mm) with the entrapped enzyme were obtained with a special sprayer [5].

Immobilization of α-chymotrypsin and lipase into PVA aldehyde-bearing cryogel beads

Beaded PVA cryogel beads (1 mm) were prepared using a cryogranulating set-up "CryoMat" (BioChemMac Co., Moscow, Russia) as described earlier [6]. The obtained beads were treated with 7 % glutaric aldehyde solution at pH 1.0 for 1 h at room temperature. The activated gel was rinsed with water to remove excess of dialdehyde, and the aldehyde-bearing beads (1 g of wet weight) were stirred for 5 h with 5 ml of α-chymotrypsin solution (1 mg/ml) in 0.1 M Na-phosphate buffer (pH 7.4) at room

temperature, 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 beads were washed again with 0.1 M Na-phosphate buffer (pH 7.4) and used as such in further experiments. The enzyme amount bound to the carrier (1.95 mg protein/1 g wet beads) was determined spectrophotometrically at 280 nm (Hitachi UV-VIS Spectrophotometer 557, Japan).

Lipase was covalently attached to aldehyde-bearing PVA cryogel beads using the above procedure described for chymotrypsin, except that the beads were mixed with 5 ml of PPL solution (2.5 mg/ml) instead of α-chymotrypsin solution.

Measurement of enzyme activities

Spectrophotometric method

The amidase activity of α-chymotrypsin was determined spectrophotometrically at 405 nm with BTNA as the substrate at 25 °C. The final concentrations of BTNA and α-chymotrypsin were 1×10^{-4} and 1.8×10^{-6} M, respectively, in 80 mM tris-HCl buffer with 0.1 M $CaCl_2$ (pH 7.8). To estimate the activity of the entrapped enzyme, the substrate solution (1.5 ml) was added to 0.1 ml PVCL-CaAlg bead aliquots. In the case of PVA cryogel, the bead aliquot containing 0.2 mg of α-chymotrypsin was suspended in 3 ml of 0.05 M tris-HCl buffer solution, pH 8.0. Then, 0.01 ml of 1 mM BTNA solution in MeCN was added, and α-chymotrypsin activity was determined spectrophotometrically at 405 nm at 22 °C (a molar absorptivity for *p*-nitroaniline released was taken equal to 9620) [13]. The same procedure was used to study BTNA hydrolysis in MeCN/water solution. The beads were soaked overnight with an aqueous buffer solution, then equilibrated for 1 h with MeCN (2 ml/0.1 g beads) and thereafter were used as such in further experiments.

In the case of PVA cryogel, the amount of lipase bound to the beads (4.9 mg of protein per 1 g of the wet beads) was determined as the difference in the absorbance of the initial enzyme solution at 280 nm and that of combined washings using Hitachi UV-VIS Spectrophotometer 557 (Hitachi, Japan). The activity of lipase was determined spectrophotometrically using two substrates, namely, pNPP and *Z*-Gly-pNP as described earlier [14].

Potentiometric method

The esterase activity of native and entrapped α-chymotrypsin (in PVCL-CaAlg bead aliquots of 0.1 ml) was calculated from the initial rate of ATEE hydrolysis in the presence of 0.1 M $CaCl_2$ (20 °C) using 0.0167 M KOH on a TTT60 Radiometer pH-stat (Radiometer, Denmark). The concentrations of substrate and enzyme in the reaction mixture were 1×10^{-3} and 2×10^{-7} M, respectively. In the case of PVA cryogel beads, DEAMA was used as the substrate and standard titrimetric procedure was employed [15].

Stability of α-chymotrypsin immobilized in PVCL-CaAlg and PVA cryogel beads in an acetonitrile/water medium

To determine the stability of α-chymotrypsin [16,17] entrapped in PVCL-CaAlg beads in an acetonitrile/water system, 1 ml of wet beads was equilibrated with 1 ml of acetonitrile, filtered, and then a fresh portion of the solvent (3 ml) was added (final water content of 20 %). The bead suspension was incubated with stirring at 25 °C for 12 h, and then the activity of the enzyme was determined potentiometrically. To alter the final water content in the incubation mixture, the same procedure was performed with 1 and 3 ml (8.9 %) and 3 and 9 ml (0.5 %) of acetonitrile instead of 1 and 3 ml, respectively. The water content in the acetonitrile/water system was monitored by Karl Fischer's procedure [18]. To estimate the operational stability of the PVCL-CaAlg gel-entrapped α-chymotrypsin, 1.3 ml of the beads were mixed with 9.6 ml of acetonitrile and 0.4 ml of water (final water content of 12.3 %), and the suspension was incubated at 25 °C for 3 cycles (each of 90 h). After each cycle, bead aliquots (100 μl) were withdrawn, and α-chymotrypsin amidase and esterase activities were determined.

To determine the stability of α-chymotrypsin immobilized in PVA cryogel in the organic media, the beads were stored in MeCN for appropriate time. The wet beads (1 g) were equilibrated with 1 ml MeCN (water content < 0.1 %), filtered and transferred to 3 ml of fresh portion of the solvent. The bead suspension was incubated at stirring for various times at 25 °C. The water content in the MeCN/water mixture thus obtained was ca. 20 %. The aliquots of these beads (100 μl) were taken periodically to determine the enzyme activity.

Enzymatic hydrolysis using gel-entrapped enzymes

Schiff's base of SBPH was prepared as described earlier [19]. SBPH was dissolved in an acetonitrile/water system and then added to a suspension of the gel-immobilized α-chymotrypsin or lipase (beads/SBPH ratio was 0.2 w/w) in the same medium (5–20 % of water). The reaction mixture was stirred at room temperature for a certain period of time. When the water concentration was higher than 10 %, 5 ml of dry MeCN (water content < 0.1 %) was added. The sediment containing precipitated L-Phe and beads with the entrapped enzyme was filtered and washed twice with acetonitrile, then twice with 1 % aqueous ammonia, and filtered. The filtrate was evaporated in vacuo, and the residue 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. For D-Phe recovery, combined acetonitrile 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 a DOWEX-50W(H^+) column and analyzed. The chemical purity of the thus obtained Phe enantiomers was examined by ^{1}H NMR spectroscopy and TLC.

Enantiomeric excess analysis

Gas chromatographic analysis was performed on a chiral glass capillary column (l = 41 m, i.d. = 0.21 mm) of a diamide polysiloxane phase type "Chirasyl-Val" (synthesized at the Nesmeyanov Institute of Organoelement Compounds) using a flame ionization detector. The carrier gas was helium, temp. 147 °C; flow rate, 1 ml/min; inlet pressure, 1.6 bar. L-Phe and D-Phe were analyzed as *N*-trifluoroacetyl derivatives of their isopropyl esters. The chemical purity of Phe enatiomers was examined by ^{1}H NMR spectroscopy and TLC.

RESULTS AND DISCUSSION

Characterization of immobilized enzymes in acetonitrile/water media

Chymotrypsin was immobilized into the composite PVCL-CaAlg hydrogel by the earlier described method based on the property of the temperature-sensitive PVCL to form a hydrogel upon a temperature increase from 20 to 40 °C with simultaneous enzyme entrapment inside the polymer coils. To reach an additional stability of the PVCL-enzyme complex and to avoid the enzyme release at room temperature, the sulfonate-containing aromatic polyamide POLAR was added. POLAR is suggested to form an interpolymer complex with PVCL, the complex being insoluble at temperatures below 40 °C. Analogous polyvinylpyrrolidone-POLAR complex was described earlier [12]. The entrapment procedure is very simple and mild and has several advantages over commonly used entrapment methods. First, entrapment is carried out at physiological pH and temperature (37–40 °C) values, thus ensuring the minimal loss in the enzyme activity. Second, PVCL is able to form enzyme–polymer complexes, in which the entrapped enzyme is retained inside the polymer coils due to hydrophobic interactions without covalent binding. Finally, introduction of PVCL into the calcium alginate network provides a macroporous structure of the beads [20], which considerably decreases diffusional limitations. Earlier, the PVCL-CaAlg hydrogel was successfully used for immobilization of various proteases (trypsin, carboxypeptidase B, thrombin), which could work as good biocatalysts in water

medium [5,20]. Therefore, we decided using PVCL-CaAlg hydrogel for entrapment of α-chymotrypsin. The entrapment method provided retention of significant catalytic activity of the entrapped enzyme and its high storage stability without noticeable losses for at least 12 months (data not shown). Since we planned to use the entrapped biocatalyst in water/organic media, we studied the behavior of the gel-entrapped α-chymotrypsin in acetonitrile/water system. It is known that the yields of such reactions depend on the water content in the reaction medium. In our case, the lower is the water content in the system, the higher is L-Phe yield, since the decrease in the water content shifts the reaction equilibrium toward L-Phe formation [19]. On the other hand, the activity of the entrapped α-chymotrypsin may decrease with the increase in the organic solvent concentration. In view of this, we first studied the dependence of the esterase activity of the immobilized α-chymotrypsin on the water content in the reaction medium (Fig. 1).

The enzyme activity was measured at the water contents of 20, 8.9, and 0.5 % (as determined by Karl Fischer's method). It appeared that the decrease in the water content in acetonitrile did not result in a significant loss in the enzyme activity. As Fig. 1 shows, a decrease in the water content to 8.9 % hardly affected the enzyme activity. Even when the water content was decreased to 0.5 %, α-chymotrypsin retained 40 % of its initial activity.

The possibility of repeated usage of the immobilized α-chymotrypsin in an acetonitrile/water medium in a cyclic process was demonstrated (Fig. 2). The beads with the immobilized enzyme were transferred to an acetonitrile/water medium (water content, 12.3 %) and then incubated by stirring at room temperature for three cycles, each of 90 h. After each cycle, the activity of α-chymotrypsin was determined. The enzyme exhibited high operational stability and remained rather active even after three cycles (270 h in total).

As is seen in Fig. 2, even after incubation for 270 h in an acetonitrile/water system, the esterase and amidase activities of the entrapped enzyme were approximately 30 and 47 %, respectively. It seems interesting that the decrease in the esterase activity was more significant than that in the amidase activity. As is known, the limiting step during the hydrolysis of an amide substrate is the formation of the acyl–enzyme complex, whereas for hydrolysis of an ester substrate, this step is a breakdown of the acyl–enzyme complex [21,22]. We suppose that, in our case, the organic solvent affects to a higher extent the stage of the acyl–enzyme complex breakdown to a greater extent.

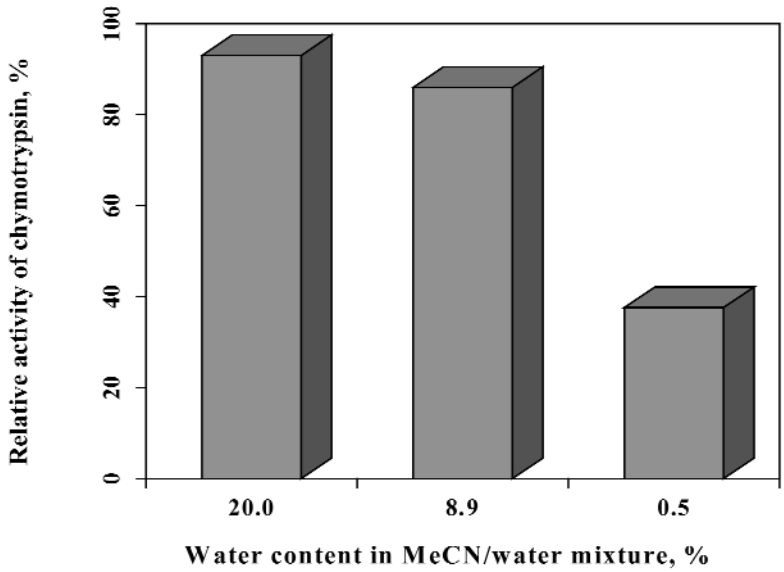

Fig. 1 Dependence of esterase activity of α-chymotrypsin entrapped in PVCL-CaAlg beads on the water content in an acetonitrile/water medium. Enzyme activity in water was taken as 100 %.

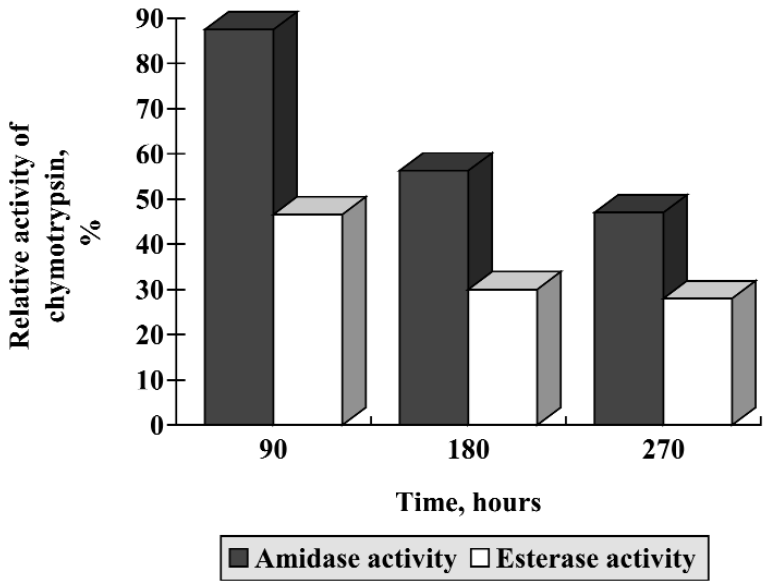

Fig. 2 Operational stability of α-chymotrypsin entrapped in PVCL-CaAlg beads in an acetonitrile/water medium (water content 12.3 %). Enzyme activity in water was taken as 100 %.

The stability of α-chymotrypsin covalently attached to PVA cryogel beads in acetonitrile/water mixtures (95/5, w/w) was also tested within the range of 1–912 h. It was shown that the bead storage up to 38 days in water-poor media had a slight effect on the initial rate of BTNA hydrolysis [23].

Enantioselective hydrolysis of a Schiff's base of SBPH using gel-immobilized enzymes

Recently, enantioselective hydrolysis of a set of Schiff's bases of amino acids esters, including SBPH, by powdered enzymes (lipase and α-chymotrypsin) in water-poor media has been demonstrated [19]. Here, to obtain optically pure L-Phe by hydrolysis of the Schiff base of SBPH, we used three bead-type biocatalysts, two based on entrapped α-chymotrypsin, namely, PVCL-CaAlg beads and PVA cryogel beads, and third one based on lipase immobilized in PVA cryogel beads. The racemic SBPH was synthesized from the ethyl ester of racemic phenylalanine and *p*-chlorobenzaldehyde as described earlier [19]. 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 by racemization (Fig. 3).

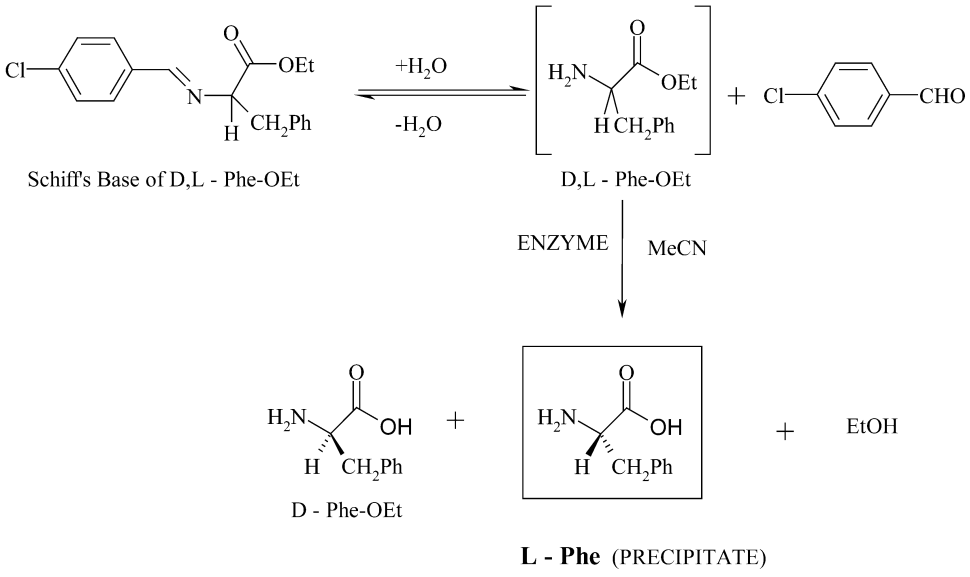

Fig. 3 Scheme of enantioselective hydrolysis of SBPH in an acetonitrile/water medium catalyzed by gel-entrapped α-chymotrypsin.

The results of enantioselective hydrolysis of SBPH in acetonitrile/water medium catalyzed by α-chymotrypsin entrapped in PVCL-CaAlg beads are summarized in Table 1.

Table 1 Enantioselective hydrolysis of Schiff's base of SBPH catalyzed by α-chymotrypsin entrapped in PVCL-CaAlg hydrogel beads.

Cycle	Amount of Schiff's base (mg)	Cycle time (h)	L-Phe yield (%)	L-Phe ee (%)	D-Phe ee (%)
I	100	144	40	80.9	40.6
II	75	168	90	87.8	87.3

Note: The quantity of α-chymotrypsin was 0.35 mg per 1 g of beads. Water content in the acetonitrile/water system was 20 %. The beads from the reaction mixture were reused in the next cycles.

Since SBPH is water-insoluble, the reaction was performed in an acetonitrile/water medium with water content of 20 % (v/v). Such a water concentration provided both high SBPH solubility and low product solubility, which considerably simplified separation of the reaction products. The reaction proceeded in three cycles (144, 168, 192 h, data for the third cycle are not shown) at room temperature and was terminated by adding a large excess of dry acetonitrile. The L-Phe precipitated out and remained in the system as either "free" solid or solid matter in the inner areas of the biocatalyst beads. To recover the amino acid, the sediment was filtered and washed with ammonia solution to dissolve L-Phe. The beads with the gel-entrapped α-chymotrypsin were used repeatedly after having been washed with water. As can be seen, PVCL-CaAlg gel-entrapped α-chymotrypsin efficiently and selectively hydrolyzed the L-form of SBPH with the formation of L-Phe. The D-form of SBPH remained in the reaction mixture and was then converted into D-Phe. The enantiomeric purities of L-Phe and D-Phe achieved were 87.8 and 87.3 %, respectively. The maximum yield of L-Phe obtained was 90 %.

As shown in Table 2, α-chymotrypsin immobilized in PVA cryogel beads was also effective in hydrolysis of the racemic SBPH ester, in acetonitrile/water mixture. This immobilized biocatalyst was active for a month and could be successfully used after another 4-month storage at +10 °C.

Table 2 Enantioselective hydrolysis of D,L-SBPH in acetonitrile/water medium catalyzed by α-chymotrypsin immobilized in PVA-cryogel beads.

Hydrolysis conditions			Hydrolysis products		
Cycle	Amount of D,L-SBPH (mg)	Cycle time (h)	L-Phe yield (%)	L-Phe ee (%)	D-Phe ee (%)
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

Note: The quantity of α-chymotrypsin was 1.5 mg of α-chymotrypsin per 1 g of PVA cryogel beads; 0.7 g of the water saturated beads, 1.5 ml of acetonitrile solution of SBPH were used. The water content in acetonitrile/water medium was 5 %. The beads recovered from the reaction mixture were reused in the next cycles.

The enantiomeric purity of L-Phe depended on the water concentration in the reaction medium. We have studied the influence of water content in MeCN on the enantioselectivity of L-Phe obtained by SBPH hydrolysis catalyzed by enzyme immobilized in PVA beads.

As seen from Table 3, increasing water content in the reaction medium from 6.7 to 20.5 % resulted in the decrease of enantioselectivity from 95.86 to 88.2 %. A drop in enantioselectivity might be a result of an increase in the rate of spontaneous hydrolysis of ester group yielding the racemic Phe.

The catalytic activity of the immobilized PPL measured in the aqueous buffer using *Z*-Gly-pNP as chromogenic substrate reached 70–75 % of the native enzyme activity at identical enzyme/substrate weight ratio. Definite loss of the biocatalytic activity of the immobilized enzyme as compared to soluble lipase could, obviously, be attributed to some chemical modification of the enzyme as a consequence of the immobilization procedure. Nonetheless, the residual esterolytic activity of cryoPVA-immobilized PPL in aqueous medium in respect to the water-soluble substrates was rather high.

Table 3 The effect of water content in acetonitrile/water system on enantioselectivity of D,L-SBPH hydrolysis catalyzed by α-chymotrypsin immobilized on PVA cryogel beads.

Hydrolysis conditions			Hydrolysis products		
Water content	Amount of D,L-SBPH (mg)	Cycle time (h)	L-Phe yield (%)	L-Phe ee (%)	D-Phe ee (%)
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

The results on L-Phe production using lipase immobilized in PVA cryogel beads are summarized in Table 4, it was found that the enantiomeric purity of L-Phe in acetonitrile/water medium is comparable to that obtained by using powdered lipase (Table 4).

Table 4 Enantioselective hydrolysis of D,L-SBPH in acetonitrile/water medium catalyzed by lipase immobilized in PVA beads and powdered lipase[a].

Carrier	Hydrolysis conditions		Hydrolysis products	
	Cycles[c]	Time (h)	L-Phe ee (%)	D-Phe ee (%)
PVA beads	1	144	83.0	6.5
	2	168	78.8	9.3
PE[b]	1	70	92.0	14.0

[a]The water content in acetonitrile/water system was 5 %.
[b]PE = powdered enzyme [19].
[c]The beads recovered from the reaction mixture were reused in the subsequent cycle after the 6-month storage at 4 °C.

However, the values of the enantiomeric excess (ee) reached were somewhat lower than in the case of the powdered enzyme (PE). Also, in the latter case, ee of 92 % has been reached in a shorter time. In principle, this could be due to several reasons. Firstly, the initial enzyme/substrate ratio earlier used was 1:1 (w/w), whereas in our case, this ratio was 1:7.65 (w/w). Hence, longer time was required for the relatively higher amount of substrate hydrolysis catalyzed by the immobilized lipase. Secondly, lower ee value of the final L-Phe produced in this case may be associated with a partial nonspecific spontaneous hydrolysis of SBPH occurring in the water-bearing beads. Since such hydrolysis of the SBPH took place for the longer time than that one induced by PE, the contribution of spontaneous nonenzymatic hydrolysis could be greater in the former case. It was also found that ee values of L-Phe produced by powdered and immobilized PPL were lower than those obtained in the case of powdered and PVA cryogel-immobilized α-chymotrypsin, where ee was at the level of 95–99 %, thus testifying to the better catalytic activity of the α-chymotrypsin in this reaction as compared to PPL. However, lipase had a very high stability: the immobilized biocatalyst did not loose its catalytic activity at least, for 6 months (the second run in Table 4).

In conclusion, gel-immobilized α-chymotrypsin and lipase can be proposed as effective biocatalysts for repeated usage in enantioselective hydrolysis of a Schiff's base of SBPH yielding individual enantiomers of Phe. The developed procedure of enzyme immobilization provided promising biocatalysts functioning in water-poor media for months.

ACKNOWLEDGMENTS

The research work has been carried out in the frame of Indo-Russian ILTP program. Financial support from the Russian Academy of Sciences (Moscow) and the Department of Science and Technology (DST, New Delhi) is gratefully acknowledged.

REFERENCES

1. F. Theil. *Chem. Rev.* **95**, 2203–2227 (1995).
2. B. Morgan, D. R. Dodds, A. Zaks, D. R. Andrews, R. Klesse. *J. Org. Chem.* **62**, 7736–7743 (1997).
3. K.-E. Jaeger and M. T. Reetz. *Trends Biotechnol.* **16**, 396–403 (1998).
4. S. Chattopadhyay, G. Sivalingam, G. Madras. *J. Supercrit. Fluids* **27**, 55–64 (2003).
5. E. A. Markvicheva, A. S. Bronin, N. E. Kudryavtseva, L. D. Rumsh, Y. E. Kirsh, V. P. Zubov. *Biotechnol. Tech.* **8**, 143–148 (1994).
6. V. I. Lozinsky and A. L. Zubov. Russ. Patent #2036095 (1992).

7. Y. E. Kirsh, T. A. Sus, T. M. Karaputadze. *Vysokomol. Soedin.* **A21**, 734–740 (1979) in Russian.
8. G. Li, L. Z. Min, E. Heiner. *Chin. Chem. Lett.* **14**, 167–168 (2003).
9. S. Sharma, S. Teotia, M. N. Gupta. *Enzyme Microb. Technol.* **32**, 337–339 (2003).
10. N. L. Eremeev and N. F. Kazanskaya. *Russ. Chem. Bull.* (Transl. of *Izvestiya Akademii Nauk, Seriya Khimicheskaya*) **50**, 1891–1895 (2001).
11. K. Laszlo, A. Szava, M. L. Simon. *J. Mol. Catal.* **16**, 141–146 (2001).
12. E. V. Anufrieva, T. N. Nekrasova, V. B. Lushik, Y. A. Fedotov, Y. E. Kirsh, M. G. Krakovyak. *Vysokomol. Soedin.* **33**, 31–34 (1992) in Russian.
13. J. Travis. *Biochem. Biophys. Res. Commun.* **29**, 294–297 (1967).
14. A. Bastida, P. Sabuquillo, P. Armisen, R. Fernandes-Lafuente, J. Huguet, J. M. Guisan. *Biotechnol. Bioeng.* **58**, 486–493 (1998).
15. *Worthington Enzyme Manual*, Freehold, New Jersey, p. 129 (1972).
16. K. Martinek, A. M Klibanov, V. S Gol'dmakher, I. V. Berezin. *Biochim. Biophys. Acta* **485**, 1–12 (1977).
17. K. Martinek, A. M. Klibanov, V. S. Gol'dmakher, A. V. Chernysheva, V. V. Mozhaev, I. V. Berezin, B. O. Glotov. *Biochim. Biophys. Acta* **485**, 13–28 (1977).
18. K. Fujinawa, T. Komatsu, M. Hozawa, N. Imaishi, H. Ino. *Kagaku Kogaku Ronbunshu* **10**, 226–232 (1984).
19. V. S. Parmar, A. Singh, K. S. Bisht, N. Kumar, Y. N. Belokon, K. A. Kochetkov, N. S. Ikonnikov, S. A. Orlova, V. I. Tararov, T. F. Saveleva. *J. Org. Chem.* **61**, 1223–1227 (1996).
20. E. A. Markvicheva, S. V. Kuptsova, T. Yu. Mareeva, A. A.Vikhrov, T. N. Dugina, S. M. Strukova, Y. N. Belokon, K. A. Kochetkov, E. N. Baranova, D. Poncelet, V. S. Parmar, R. Kumar, V. P. Zubov, L. D. Rumsh. *Appl. Biochem. Biotechnol.* **88** (1–3), 145–157 (2000).
21. M. L. Bender, F. J. Kezdy, C. R. Gunter. *J. Am. Chem. Soc.* **86**, 3714–3718 (1964).
22. B. Zerner, R. P. M. Bond, M. L. Bender. *J. Am. Chem. Soc.* **86**, 3674–3678 (1964).
23. Yu. N. Belokon, K. A. Kochetkov, F. M. Plieva, N. S. Ikonnikov, V. I. Maleev, V. S. Parmar, R. Kumar, V. I. Lozinsky. *Appl. Biochem. Biotechnol.* **88** (1–3), 97–106 (2000).