0273-2289/96/6101-02—0075$07.50

Stabilization of Proteases by Entrapment in a New Composite Hydrogel

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ABSTRACT

A new one-step procedure for entrapping proteases into a polymeric composite calcium alginate-poly(*N*-vinyl caprolactam) hydrogel was developed that provided 75–90% retention of the activity of entrapped enzymes compared to soluble ones. Properties of entrapped carboxypeptidase B, trypsin, and thrombin were investigated. The immobilized enzymes were active within a wide pH range. The temperature optima of entrapped trypsin and carboxypeptidase B were approx 25°C higher than that of the soluble enzymes, and the resistance to heating was also increased. The effects of various polar and nonpolar organic solvents on the entrapped proteases were investigated. The immobilized enzymes retained their activity within a wide concentration range (up to 90%) of organic solvents. Gel-entrapped trypsin and carboxypeptidase (CPB) were successfully used for obtaining human insulin from recombinant proinsulin. The developed stabilization method can be used to catalyze various reactions proceeding within wide pH and temperature ranges.

Index Entries: Poly(*N*-vinyl caprolactam) hydrogel; thrombin; trypsin; carboxypeptidase B; enzyme entrapment; stabilization of proteases.

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INTRODUCTION

The resistance of enzymes to increased temperatures and various denaturing agents is of particular theoretical and practical importance. Immobilization is the most promising and effective approach for stabilizing enzymes (*1*). Although many immobilization methods have been developed, no one can be regarded as universal. In each case, it is necessary to find a simple and inexpensive procedure that provides a product with high activity and operational stability. To stabilize proteases (trypsin, carboxypeptidase B [CPB], and thrombin), we chose the gel entrapment method in order to avoid partial inactivation of the enzymes owing to involvement of the active sites in immobilization. Immobilization under mild conditions is especially important for unstable and expensive thrombin and CPB. Despite wide practical application of CPB, only a few techniques were developed for successful CPB immobilization (*2*). The immobilization of thrombin and succinylthrombin on the Eupergit polyacrylamide-type support provided only 5–10% activity compared to the soluble enzyme (*3*).

Earlier, we developed a simple technique for entrapping hybridoma cells (*4*) and microorganisms (*5*) in thermally reversible hydrogels, in particular, poly(*N*-vinyl caprolactam) (PVCL). The method is based on the PVCL property to form a gel with a simultaneous cell entrapment on temperature increase from 20 to 37–40°C. PVCL was also used to obtain thermally precipitating conjugates with soybean trypsin inhibitor (*6*) and enzymes (penicillin amidase and chymotrypsin) (*7*). Recently, we proposed a new one-step procedure for entrapping enzymes into a composite PVCL-based hydrogel (*8*).

In this study, we investigated some properties of proteases (thrombin, trypsin, and CPB) that were entrapped into composite PVCL-calcium alginate (PVCL-CaAlg) hydrogel beads by the above-mentioned method. We studied the effects of temperature, pH, and various organic solvents on the enzyme activity. In order to demonstrate that the entrapment of the enzymes into a polymer matrix protects them from heat and various denaturing agents, we used native CPB and trypsin as well as their conjugates with poly(*N*-vinylpyrrolidone-acrolein) (*9*).

MATERIALS AND METHODS

Chemicals

PVCL (mol wt 900,000) was obtained by polymerization of *N*-vinyl caprolactam as described (*10*). Sodium alginate was from Bellco, USA. Aromatic polyamide POLAR (mol wt 25,000) was obtained by copolymerization of isophthalic acid and 4,4′-diaminodiphenyl-2,2′-disulfonic acid

(*11*). CPB (EC 3.4.17.2; 100 U/mg) and bovine trypsin (EC 3.4.23.1; 22 U/mg) were from Biolar, Latvia. *N*-Benzoyl-L-arginine ethyl ester (BAEE) was from Reanal, Hungary. Hippuryl-L-arginine (Hipp-Arg) and *N-p*-tosyl-Gly-Pro-Arg *p*-nitroanilide (Chromozym TH) were from Boehringer Mannheim. Conjugates of trypsin (11 U/mg, mol wt 100,000) and CPB (25 U/mg, mol wt 100,000) covalently bound to poly(*N*-vinylpyrrolidone-acrolein) were obtained from the Russian Institute of Antibiotics and Enzyme Preparation (St. Petersburg). Bovine thrombin (EC 3.4.21.5; 2500 NIH U/mg) was obtained by purification of a commercial preparation (Kaunas, Lithuania) as described elsewhere (*12*). Acetonitrile, *N,N*-dimethylformamide (DMF), and glycerol were from Sigma, USA. Recombinant proinsulin was obtained from the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry.

Entrapment of Enzymes in PVCL-CaAlg Beads

Enzymes were entrapped as described elsewhere (*8*). An enzyme solution (1.0 mL) was mixed with 2.0 mL of 10% (w/v) PVCL and 0.5 mL of 1% POLAR. Sodium alginate 5 mL of 2% (w/v) was then added. The mixture was added dropwise into 100 mL of 2% (w/v) $CaCl_2$ at 37–40°C. Beads (0.2–0.8 mm) were obtained using a special sprayer.

Measurement of Enzyme Activity

Spectrophotometric Method

The activities of soluble trypsin and CPB were determined using BAEE (*13*) and Hipp-Arg (*14*) as substrates, respectively. The clotting activity of thrombin was assayed using fibrinogen (*15*). Chromozyme TH was used as a substrate for determining the amidase activity of thrombin (*16*). To estimate activities of the entrapped enzymes, a 0.1-mL aliquot of the bead suspension was added to 10 mL of $10^{-3}M$ substrate solution in a Tris-HCl buffer (pH 7.5). The reaction mixture was stirred at room temperature for 5 min. The suspension was then filtered, and the filtrate was assayed as mentioned above. The activity of the immobilized enzymes was expressed as a percent of that of free enzymes. The durability of the alginate-containing beads was increased by adding 1% (w/v) $CaCl_2$ directly to the substrate solution.

Titrimetric Method

To determine the trypsin activity, BAEE (12.5 m*M*) was used as a substrate. The activity of native and entrapped trypsin was calculated from the initial rate of the BAEE hydrolysis at pH 7.5 and 20°C in the presence of 0.1*M* $CaCl_2$ using a TTT60 Radiometer pH-stat (Radiometer, Denmark). The final concentration of soluble trypsin in the reaction mixture was $4.2 \times 10^{-7}M$. To estimate the activity of entrapped trypsin, aliquots of the beads with the total activity equal to that of soluble trypsin were used.

The Effect of pH and Temperature on the Enzyme Activity

The activities of trypsin and CPB were determined by spectrophotometry. The effect of pH on the enzyme activity was evaluated by incubating the soluble enzymes or the beads at different pHs at room temperature for 5 min. The temperature dependencies of the activities of the soluble and entrapped enzymes were studied under the optimum pH in the temperature range 20–85°C. After adding substrate, the beads were incubated for 10 min at an appropriate temperature. To determine the heat stability, the soluble and gel-entrapped enzymes were incubated at an appropriate temperature for 1 h. After cooling, the enzyme solution or the bead suspension was assayed for the enzymatic activity spectrophotometrically as described above.

The Effect of Organic Solvents on the Enzyme Activity

To determine the effects of organic solvents on the activity of trypsin, DMF–, acetonitrile–, and glycerol–water mixtures were used. The concentration of organic solvent varied from 10–90%. The activity of trypsin was determined by titrimetric method.

Hydrolysis of Proinsulin by Immobilized Trypsin and CPB

The beads with entrapped trypsin and CPB were mixed and a column was packed with the slurry. The ratio of the total CPB and trypsin activities was approx 2:1. A solution of recombinant proinsulin (0.6 mg/mL) in 50 m*M* Tris-HCl buffer (pH 7.5) containing 1% (w/v) $CaCl_2$ was recirculated through the column at a flow rate of 1.0 mL/min at 37°C. The reaction products were monitored by HPLC as described (*8*).

Lyophilization of Thrombin Samples

A solution of thrombin (0.25 mL; amidase activity 3.62 U/mL) was mixed with 16% (w/v) PVCL, and Tris-HCl buffer (pH 6.9) was added to the final volume of 1 mL. The samples were then lyophilized. The final PVCL concentration in the samples was 2 or 4% (w/v). After lyophilization, the samples obtained were dissolved in 1 mL of Tris-HCl buffer (pH 6.9), and the amidase and clotting activities were determined as described above.

RESULTS AND DISCUSSION

Reducing the mobility of enzymes polypeptide chains (*17*) by their covalent binding to a support is a common approach for enzyme stabilization. However, it often results in a significant decrease in the catalytic activity, especially, in the case of unstable enzymes. The gel entrapment provides more or less loose attachment of enzymes to a polymeric matrix

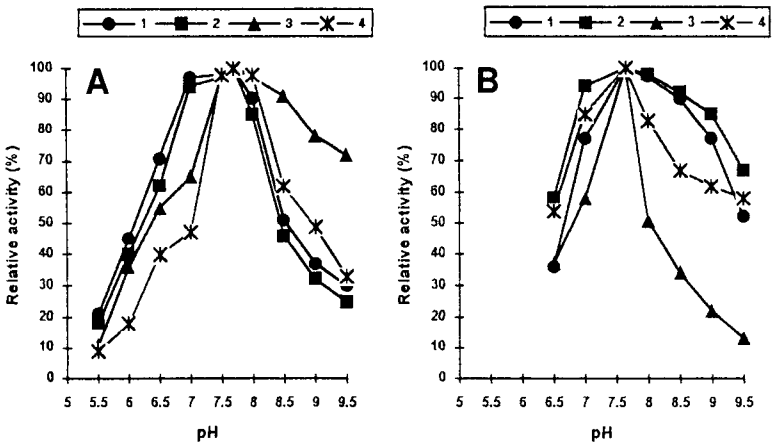

Fig. 1. The effect of pH on the activity of soluble and entrapped trypsin (**A**) and CPB (**B**). Soluble enzymes: 1—native enzyme; 2—enzyme-PVP conjugate. Entrapped enzymes: 3—native enzyme; 4—enzyme-PVP conjugate. Both enzyme-PVP conjugates were obtained by covalent binding to poly(vinylpyrrolidone-acrolein). The maximum enzyme activity was taken as 100%.

without a rigid fixation of a protein molecule and, therefore, seems to be promising for stabilizing unstable enzymes, in particular, thrombin and CPB. Earlier, we developed a method based on unique PVCL properties that has at least two advantages compared to other methods (*8*). First, the entrapment is performed at physiological pH and temperatures (37–40°C) allowing a minimal decrease in the activity. Second, PVCL is able to form water-soluble enzyme-polymer complexes at room temperature and to entrap enzyme into a polymeric gel formed on the temperature increase from 20 to 40°C. We also considered that the polymer matrix could protect entrapped proteases from heat and denaturing agents. To study the effect of pH on the activity of immobilized trypsin and CPB, the native enzymes and the enzymes covalently bound to poly(*N*-vinylpyrrolidone-acrolein) (trypsin–PVP and CPB–PVP conjugates, respectively) were used. As demonstrated in Fig. 1, the entrapment did not affect the pH optimum of the enzymes. Both entrapped trypsin and CPB were active in a broad pH range.

Figure 2 shows the effect of increased temperatures on the trypsin and CPB activities. The retained activity of both conjugates was higher than that of the native enzymes. Thus, the activities of native trypsin and trypsin–PVP conjugate at 55°C were 30 and 50%, respectively. The entrapment resulted in a significant increase in the retention of activity within a broad temperature range (up to 80–85°C) both for trypsin and trypsin–PVP conjugate. The trypsin–PVP conjugate retained 95% of its activity at 85°C. Similar results were obtained for CPB. Both gel-entrapped CPB forms retained their activity at temperatures above 75°C. As shown in Fig. 2B, the activity of native CPB was 38% at 75°C, and the activity of CPB–PVP conjugate was 45% at 85°C. Nonentrapped native CPB was

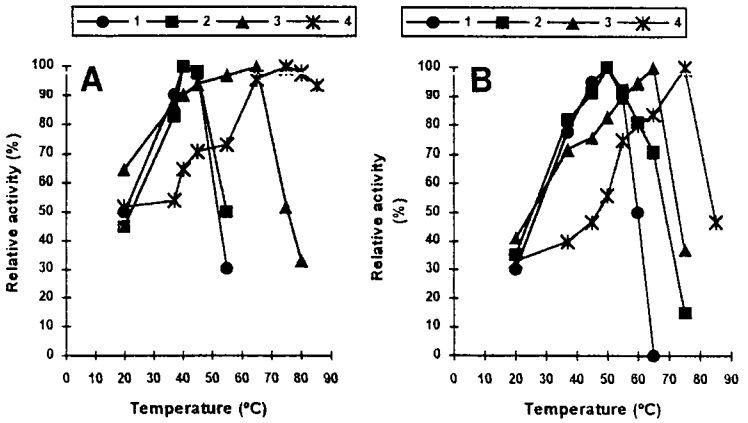

Fig. 2. The effect of temperature on the activity of soluble and entrapped trypsin (**A**) and CPB (**B**). Soluble enzymes: 1—native enzyme; 2—enzyme-PVP conjugate. Entrapped enzymes: 3—native enzyme; 4—enzyme-PVP conjugate. The enzyme conjugates were obtained by covalent binding to poly(vinylpyrrolidone-acrolein). The maximum enzyme activity was taken as 100%.

completely inactivated at 65°C, and PVP-CPB conjugate displayed only 15% activity at 75°C. Note that the temperature optimum of CPB covalently bound to a polyacrylamide-type support was 55°C (*2*). Hence, the entrapment allowed us to stabilize the activity at temperatures 75–85°C both for native enzymes and PVP-enzyme conjugates. The data on the heat stabilities of native and gel-entrapped trypsin and CPB are shown in Fig. 3. The thermostabilization achieved was quite impressive: the activity of entrapped trypsin was retained up to 65°C and comprised approx 20% at 85°C. At the same time, native trypsin was completely inactivated at 50°C. Entrapped CPB retained 60% activity even at 80°C. Native CPB was completely inactivated at temperatures above 50°C.

Enzyme catalysis in organic solvent is widely used for various applications, including peptide synthesis (*18*). A direct interaction of an enzyme molecule with organic solvents results in the alteration of its catalytically active conformation (*19*). Gel entrapment is considered to be one of the most promising methods to maintain the enzyme activity at low water contents (*20*). To study a behavior of gel-entrapped trypsin in water-organic mixtures, we used glycerol, DMF, and acetonitrile. As shown in Fig. 4, the activities of both native and entrapped trypsin were completely retained within a wide range of glycerol concentration. The most interesting results were obtained for native and entrapped trypsin when DMF and acetonitrile were used. First, the activation of the native enzyme (up to 125–130%) was observed at the solvent content of 25–30%. These results correlated well with those obtained for acid phosphatase (*21*). The retained activity of gel-entrapped trypsin in a DMF- or acetonitrile-water systems (90:10, v/v) was about 30 and 70%, respectively. Native trypsin completely lost its activity at DMF concentrations over 50%, and retained only 20% of

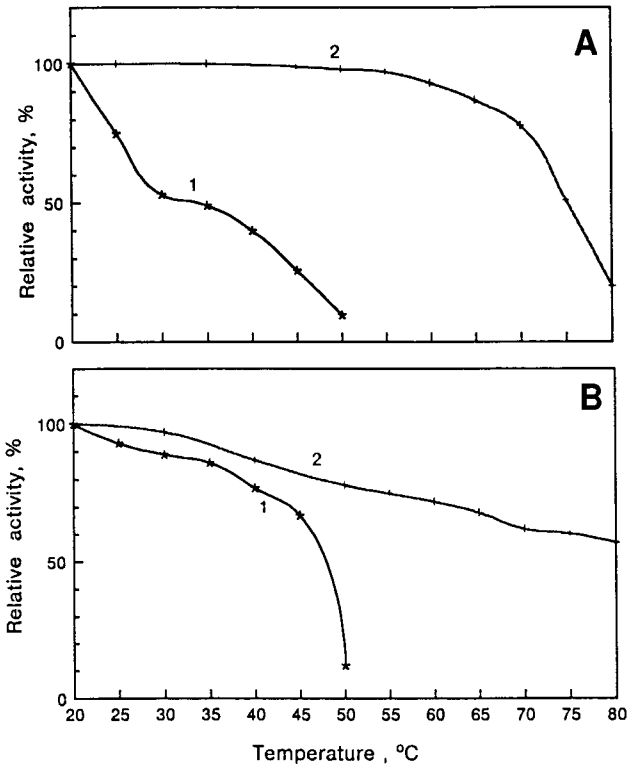

Fig. 3. The effect of heat treatment on the activity of trypsin (**A**) and CPB (**B**): 1—native enzyme; 2—entrapped enzyme. The treatment was carried out for 1 h, pH 7.5. The maximum enzyme activity was taken as 100%.

its initial activity in 90% acetonitrile. Hence, the thermostabilization was accompanied by the stabilization toward other denaturing agents, namely, nonaqueous solvents. A similar correlation was observed by Arnold (22), who suggested that "concepts that have proved useful in engineering protein thermostability may be applied to accomplish the goal of rational enzyme stabilization in non-aqueous solvents." The stabilization is known to be provided by keeping a local water layer around a protein molecule. We suggested that the enzyme stabilization could be explained by specific properties of PVCL matrix. First, the entrapment in PVCL gel as well as in other gels decreases the conformational mobility of enzymes. Second, PVCL macromolecules contain about 15 H_2O molecules/chain link (23) and promote the local water content around the protein. Note that PVCL concentration exceeded considerably that of enzyme (22 mg enzyme/g PVCL). Although water molecules are driven out of the polymer coils on the temperature increase, the residual water molecules are able to provide the catalytic activity of enzymes.

We assumed that the above-mentioned PVCL properties could be used to stabilize thrombin. Since thrombin is one of the most expensive proteases, its stabilization is of particular importance. Stabilized thrombin

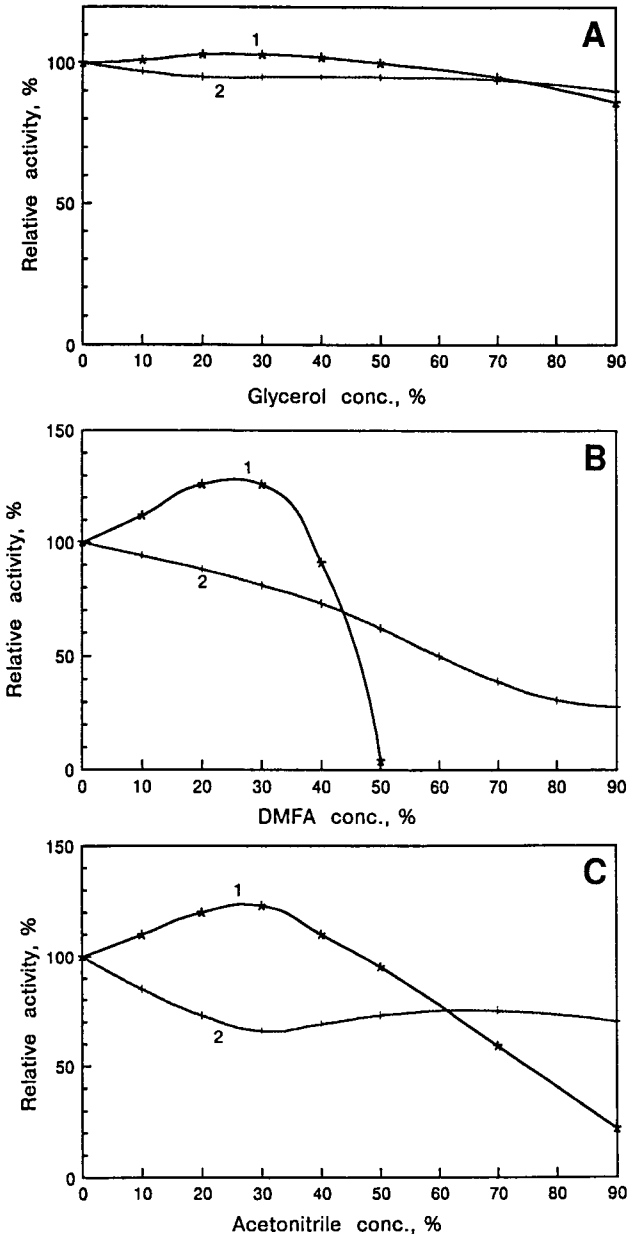

Fig. 4. The effect of organic solvents on the activity of native (1) and entrapped (2) trypsin: (**A**) glycerol, (**B**) DMFA, and (**C**) acetonitrile. The trypsin activity in the absence of organic solvent was taken as 100%.

is widely used in clinics for controlling heparin therapy and molecular disorders of fibrinogen. Additionally, thrombin is able to stimulate cell proliferation and to decrease inflammation. Therefore, it represents a promising agent for initiating tissue repair (*24*). Thus, entrapped thrombin could be used to accelerate wound healing. The introduction of certain hydrat-

Table 1
Stability of Thrombin Preparations

N	Sample	BSA conc., %	PVCL conc., %	Relative amidase activity, % After lyophilization	After 8 d storage at 20°C	After 8 d storage at 4°C
1	Thrombin	—	—	11	0	0
2	Thrombin-BSA	0.02	—	23	10	12
3	Thrombin-PVCL	—	2.0	34	n.d.	n.d.
4	Thrombin-PVCL	—	4.0	38	n.d.	n.d.
5	Thrombin-BSA-PVCL	0.02	2.0	35	27	40
6	Thrombin-BSA-PVCL	0.02	4.0	45	26	32

n.d., not determined.

ing additives is known to stabilize lyophilized enzyme preparations. There are reports on thrombin stabilization, in particular, by adding BSA (*25*). The data in Table 1 demonstrate the effect of PVCL on the amidase activity of thrombin solutions and lyophilized preparations. The activity of PVCL-containing thrombin samples increased after lyophilization compared to that of native thrombin or thrombin in the presence of BSA. Moreover, an increase in the PVCL concentration enhanced the stabilization. An addition of PVCL to thrombin solutions also stabilized thrombin preparations on storage for 8 d at 4 and 20°C. When stored under the same conditions, the native thrombin solutions were completely inactivated. Adding PVCL to the thrombin solutions did not decrease the clotting activity of the lyophilized preparations.

High operational stabilities of entrapped trypsin and CPB were shown by repeated application for obtaining human insulin from recombinant proinsulin (*8*). To prevent nonspecific sorption of the reaction products, ovalbumin (2% w/v) was added. The immobilized enzymes retained their activity after 20 cycles of proinsulin cleavage. The stabilities of entrapped CPB and trypsin were unchanged on storage at room temperature for 45 d and 4 mo, respectively.

In conclusion, the entrapment of proteases into the composite PVCL-based hydrogel protects enzymes from the action of various denaturing agents. This stabilization method is especially advantageous for application of unstable enzymes in biotechnology and medicine.

ACKNOWLEDGMENT

This work was supported by a grant from the State Program "Novel Methods in Bioengineering: Engineering Enzymology" (no. 4-63).

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