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Immobilized Chymotrypsin on Reversibly Precipitable Polymerized Liposome

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ABSTRACT

A polymerized liposome (PLS) was prepared using a synthesized phosphatidylethanolamine with a diacetylene moiety that showed a reversibly precipitable property on addition and removal of salt. To prepare a soluble–insoluble immobilized enzyme, chymotrypsin was covalently immobilized on the outer surface of the PLS. The carbodiimide method was employed for the enzyme immobilization. Coupling was rapid and nearly complete at a weight ratio of enzyme to the PLS of < 0.12. The immobilized enzyme showed favorable activity yields for both low- and high-mol-wt substrates, i.e., 90 ± 9% for *N*-benzoyl-L-tyrosine ethyl ester and 59 ± 5% for casein up to an enzyme coupling density of 0.38 g/g-PLS. The immobilized enzyme was reusable and more stable at high temperature and long-term incubation than the native enzyme.

Index Entries: Chymotrypsin; immobilization; polymerized liposome; precipitation; stability.

INTRODUCTION

Reversibly soluble–insoluble immobilized enzymes involve enzyme reactions in their soluble state, and after reactions, the enzymes are insolubilized and recovered by subsequent centrifugation or filtration. Hence,

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the enzymes can be reused like normal immobilized enzymes. Moreover, the diffusional resistance encountered in insoluble immobilized enzymes may be minimized, especially for high-mol-wt or insoluble substrates. It is also expected that the stability of the enzymes may be improved, which is frequently associated with normal insoluble immobilized enzymes.

Several soluble-insoluble enzymes have been prepared by immobilizing enzymes on reversibly precipitable carriers, including alginic acid (*1*), acrolein-acrylic acid copolymer (*2*), polyelectrolytes (*3*), enteric coating polymers (*4,5*), α_{s1}-casein (*6*), and acrylamide-based copolymers (*7*). These materials are precipitable by decreasing pH (*1–5*) or increasing ionic strength (*3,7*), or by calcium ion addition (*6*). The properties of the soluble-insoluble enzymes and their carriers have been reported in these studies (*1–7*). Chymotrypsin has been used for peptide synthesis (*4*), whereas raw starch has been hydrolyzed using immobilized amylase (*5*). Recently, the authors prepared a polymerized liposome (PLS) using a synthesized phospholipid with a diacetylene moiety. The PLS exhibited a reversibly precipitable property on addition and removal of salt (*8*). Thus, it is considered that the PLS can be used as a carrier for preparing soluble-insoluble enzymes or a ligand carrier for affinity precipitation of proteins. The latter has been reported (*9*). In this work, we report the covalent attachment of chymotrypsin to the PLS. We have investigated the properties of the immobilized chymotrypsin as a soluble-insoluble immobilized enzyme.

MATERIALS AND METHODS

Materials

Chymotrypsin (type II), *N*-benzoyl-L-tyrosine ethyl ester (BTEE) and 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma Chemical Company (St. Louis, MO). Casein was from Tianjin Biochemicals Inc. PLS was prepared using a synthesized phosphatidylethanolamine with a diacetylene moiety, that is, $CH_3(CH_2)_{11}C{\equiv}CC{\equiv}C(CH_2)_9OPO_3HCH_2CH_2NH_2$. The PLS had a diameter distribution from 100 to 200 nm, as described previously (*8,9*). All other reagents were of analytical grade.

Methods

PLS Precipitation

The precipitation behavior of the PLS was investigated by adding different concentrations of NaCl or phosphate buffer (pH 7.5) to 2-mL aliquots containing 2 mg of PLS (dry wt). The final volumes of the solutions were all maintained at 3 mL after salt additions. After 2 min of salt additions, the aliquots were centrifuged at 5000*g* for 2 min, and the absorbances of the supernatants at 400 nm were determined. Complete precipitation occurred when the absorbance was not detected.

Enzyme Immobilization

Chymotrypsin was directly immobilized onto the PLS by the carbodiimide method (*10*). Typically, a predetermined amount of chymotrypsin was added to 10 mL of phosphate buffer (0.05*M*, pH 4.9) containing 10 mg of PLS and 2 mg of EDC. The reactions were performed at 4°C for 1–21 h. After each reaction, 2 mL of 1*M* NaCl were added to precipitate the PLS-coupled chymotrypsin (PLS-C). After centrifuging at 5000g for 5 min, the precipitated PLS-C was washed twice with 0.2*M* NaCl and recentrifuged. The supernatants were combined, and the chymotrypsin remaining in the solution was analyzed by the Lowry method (*11*) to estimate the enzyme-coupling density. The PLS-C was dispersed in Tris-HCl buffer (0.025*M*, pH 8.0), stored at 4°C, and used within 1 d.

Enzyme Assay

Assays of both native and immobilized chymotrypsin were performed with BTEE as the substrate, as reported by Powers et al. (*12*). The proteolytic activity of chymotrypsin for casein was similarly determined by the method of Arnon and Shapira (*13*). To 2 mL of 0.5% casein (in 0.05*M* Tris-HCl buffer, pH 8.0) was added 0.1 mL of the enzyme (native or immobilized) solution containing 10–50 μg of chymotrypsin. The mixture was incubated at 28°C for 15 min. Four milliliters of 10% trichloroacetic acid was added to stop the reaction and precipitate the proteins. After 30 min, the precipitate was removed by centrifugation, and the absorbance of the supernatant at 280 nm was measured. Control experiments were also performed for the native and immobilized enzymes by adding trichloroacetic acid solution prior to introducing enzymes. The absorbance was linear with enzyme concentration in the experimental range (data not shown). The proteolytic activity yield of the immobilized enzyme was described as the percentage of the absorbance for immobilized enzyme to that for the native enzyme at the same concentration. The latter was defined as 100%.

RESULTS AND DISCUSSION

Effect of Salt Concentration on PLS Precipitation

Different salts gave slightly different behaviors for the PLS precipitation (Fig. 1). By using NaCl, the PLS began to precipitate at a concentration of 0.13*M*, and complete precipitation occurred at concentrations ≥ 0.17*M*. In phosphate buffer, however, the precipitate appeared and was completely settled at 0.1 and 0.14*M*, respectively. At much lower salt concentrations (e.g., < 0.1*M* for NaCl, 0.08*M* for the phosphate buffer), precipitation was not observed even after several days.

It is considered that at higher salt concentrations, the PLS precipitation was caused by osmotic shock (*9,14*). The PLS was not destroyed by the osmotic shock, since it has been observed by electron microscopy that the

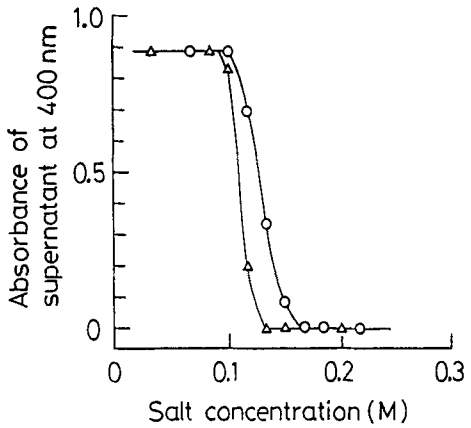

Fig. 1. Precipitation behavior of the PLS by NaCl (○) and phosphate buffer (△) additions.

Table 1
Effect of Reaction Time on Immobilized Chymotrypsin[a]

Reaction time, h	Enzyme coupled, %	Coupling density, g/g PLS	Activity yield, %
1	39	0.20	87
3	44	0.22	88
5	41	0.21	107
8	50	0.25	86
12	46	0.23	90
21	56	0.28	84
Statistical analysis	46 ± 6	0.23 ± 0.03	90 ± 9

[a]Five milligrams of Chymotrypsin and 10 mg of PLS were initially introduced.

redispersed PLS retained its size and spherical shape (*9*). Therefore, the PLS could be redispersed in water or buffers with lower ionic strength after salt removal by centrifugation. The same property of PLSs prepared with other diacetylene phospholipids was also noticed by Hub et al. (*14*).

Immobilized Chymotrypsin on the PLS

The coupled-enzyme percentage or enzyme-coupling density was little affected by the reaction time (Table 1). This agreed with that obtained for immobilizing chymotrypsinogen A on porous glass beads by the carbodiimide method, where the coupling density leveled off after several minutes of the immobilization reaction (*10*). Table 1 also shows the activity yield of the immobilized enzyme, as compared with the native enzyme with the same amount of protein, which activity was defined as 100%. The immobilized enzyme exhibited activity yields of 90 ± 9%. The values were somewhat higher than that of immobilized papain on enteric coating

Table 2
Effect of Enzyme Amount on Immobilized Chymotrypsin[a]

Enzyme amount, mg	Enzyme coupled, %	Coupling density, g/g PLS	Activity yield, %
0.9	89	0.08	83
1.2	93	0.11	86
1.6	85	0.14	90
2.3	63	0.15	91
5	41	0.21	107
10	36	0.36	83

[a]PLS, 10 mg; reaction time, 5 h.

Table 3
Proteolytic Activity Yield of Immobilized Chymotrypsin

Enzyme-coupling density, g/g PLS	Activity yield, %	
	BTEE	Casein
0.08	83	58
0.11	86	60
0.14	90	66
0.15	91	63
0.22	84	57
0.38	83	51
Statistical analysis	86 ± 4	59 ± 5

polymers (17–80%) (4) and immobilized alkaline phosphatase on acrylamide-based copolymers (68–80%) (7).

Table 2 shows the effect of the enzyme amount initially introduced in the reaction solution on the coupling density and the enzyme activity yield. About 90% of the initially introduced enzyme was coupled on the PLS at weight ratio of enzyme to PLS of < 0.12. The coupled-enzyme percentage decreased with increasing the enzyme introduced. Although the coupling density increased with the amount of enzyme addition, the activity yield was little affected for the low-mol-wt substrate, BTEE.

Six preparations of the immobilized chymotrypsin were used for the hydrolysis of casein. The results are listed in Table 3. The proteolytic activity yield was 59 ± 5% in the coupling density range of 0.08–0.38 g/g PLS. This value is similar to the activity yield reported for immobilized papain on methacrylic acid-methylacrylate-methylmethacrylate copolymer (4), but much higher than that for immobilized trypsin on acrolein-acrylic acid copolymer (2).

Papain has also been immobilized on an insoluble carrier, Sepharose (4). The activity yield was as high as 80% for a low-mol-wt substrate, BAPA (*N*-benzoyl-DL-arginine-*p*-nitroanilide), but only 20% for casein. The

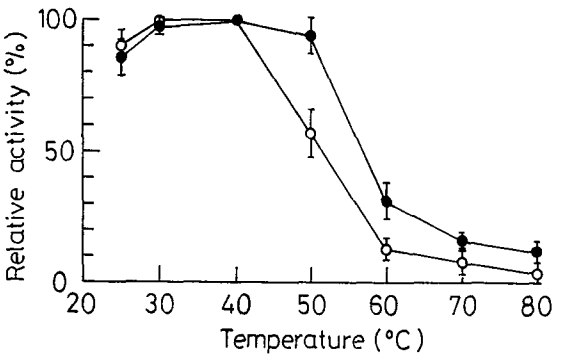

Fig. 2. Temperature profiles of native (○) and immobilized (●) chymotrypsin. The points are average values of three experiments. The error bars represent standard deviations for the three data.

results demonstrated the presence of severe diffusional resistance of the insoluble carrier for casein. Although in this work no experiment was carried out for the immobilization of chymotrypsin on insoluble carriers, the fact that the activity yield for casein was comparable to that of immobilized papain on enteric coating polymer (62%) (*4*) indicates the advantage of PLS-immobilized enzyme in minimizing diffusional resistance.

Several workers have immobilized enzymes or proteins onto various polymerized liposomes to functionalize them. Of enzyme immobilizations, the early report by Regen et al. (*15*) claimed that chymotrypsin was covalently coupled on an aldehyde-activated polymerized liposome with coupling densities of 0.3–0.5 g/g lipid, but with an activity yield of only 15% (substrate: BTEE). Kitano and coworkers immobilized trypsin on PLS composed of two kinds of phospholipids with dienoyl aliphatic acid (*16*). The coupling densities were 0.03–0.04 g/g lipid, with activity yields of 7.5–33%, depending on the components of the PLS and/or polymerization methods. It is considered that the lower activity yields in these studies were owing to the unsuitable enzyme-coupling methods and/or recovery method of the coupled enzymes (by gel filtration since the polymerized liposomes they used were not precipitable (*15,16*).

Figure 2 shows the effect of assay temperature on the activity of native and immobilized enzymes with BTEE as the substrate. The assay was performed for 3 min under each temperature. Compared with the native enzyme, the optimal assay temperature of the immobilized chymotrypsin had an extended range from 30 to 50°C. This indicated that the immobilized enzyme exhibited an improved stability at high temperature. This property has also been reported for immobilized papain on the enteric coating polymer (*4*).

The immobilized chymotrypsin had a similar pH profile to the native enzyme, as shown in Fig. 3, where phosphate buffers (pHs 4.7, 5.8, and 7.0), Tris-HCl buffer (pH 8.1), and carbonate buffers (pHs 9.1, 9.8, and 10.8) were used as the solvents of BTEE. This differed from immobilized

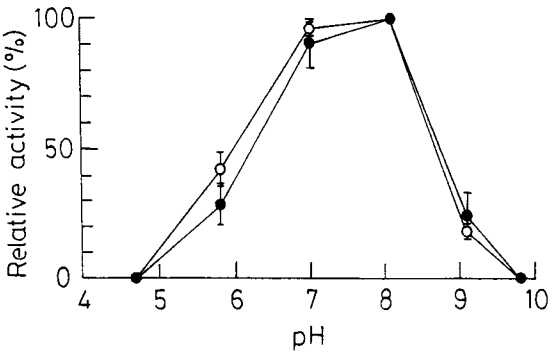

Fig. 3. pH profiles of native (○) and immobilized (●) chymotrypsin. The points are average values of three experiments. The error bars represent standard deviations for the three data.

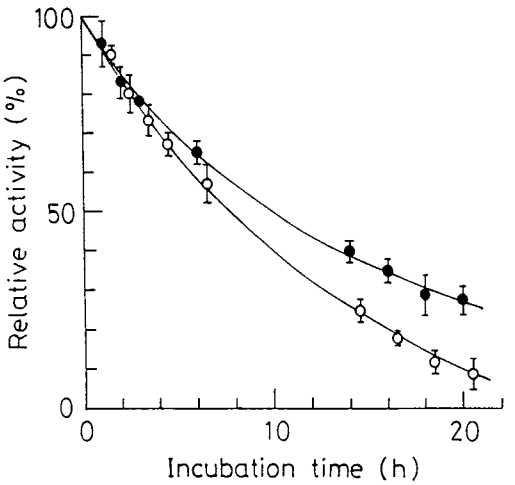

Fig. 4. Time stabilities of native (○) and immobilized (●) chymotrypsin under 30°C. The points are average values of three experiments. The error bars represent standard deviations for the three data.

papain, in which the optimal pH shifted about 0.7 pH units to the alkaline side compared with the native enzyme (4).

Both native and immobilized chymotrypsin were stable at 4°C for over 24 h in Tris-HCl buffer (0.05*M*, pH 8.1). At 30°C, however, significant inactivation was observed for both native and immobilized chymotrypsin, as shown in Fig. 4. It is encouraging that the immobilized enzyme exhibited more stable behavior than the native one for a long-term incubation. In the experiments, the initial activities of both native and immobilized enzymes were approximately the same.

Figure 5 shows the stability of the immobilized enzyme during repeated precipitations. The enzyme solutions were left at 28°C for 15 min between individual precipitations. The inactivation was promoted by precipitations, as compared with Fig. 4. It is notable that the stability was improved by

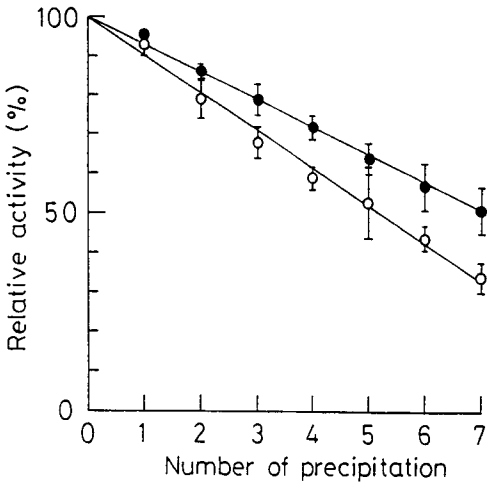

Fig. 5. Stability of immobilized chymotrypsin during precipitations in the presence (●) and absence (○) of casein under 28°C. The points are average values of three experiments. The error bars represent standard deviations for the three data.

the presence of casein, and 64 ± 4% of the initial activity remained after five precipitations. This was approximately the same as that for immobilized papain on enteric coating polymer (*4*), but smaller than those for immobilized lysozyme on alginic acid (*1*) and alkaline phosphatase on acrylamide-based copolymers (*7*). It is considered that the autolysis of the proteases (papain in ref. [*4*] and chymotrypsin in this work) might be the main reason for lower stability than the immobilized lysozyme (*1*) and phosphatase (*7*).

In summary, the PLS used in this study exhibited a favorable soluble-insoluble property on salt addition and removal. This supported its use as an enzyme-immobilized carrier, which allowed enzyme reactions to be performed in its soluble state and the enzyme to be recovered after the reactions. The amino groups occupied on the PLS surface made the enzyme immobilization simple, rapid, and nearly complete by the one-step carbodiimide method. The activity yields of the immobilized chymotrypsin were favorable for both low- and high-mol-wt substrate. By immobilization on the polymerized liposome, the enzyme stability was improved. The immobilized enzyme could be reused several times even for the protease, which is inherently unstable owing to autolysis.

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