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Journal of Molecular Catalysis B: Enzymatic 2 (1997) 233–241

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Preparations and properties of temperature-sensitive poly(N-isopropylacrylamide) –chymotrypsin conjugates

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Received 25 September 1996; revised 24 October 1996; accepted 24 October 1996

Abstract

A copolymer of *N*-isopropylacrylamide (NIPAAm) and *N*-acryloxysuccinimide (NAS) was synthesized and used as a support for the immobilization of α-chymotrypsin. The copolymer is temperature-sensitive with a lower critical solution temperature (LCST) of 33.5°C when synthesized with an initial molar ratio NIPAAm:NAS = 39. The immobilized enzyme exhibited a LCST of 35.5°C, precipitated and flocculated in aqueous solution above the LCST and redissolved when cooled below that temperature. The coupling yields and specific activity of the immobilized enzyme depended on the pH of the coupling buffer and the enzyme to polymer ratio. Specific activities of the immobilized enzyme were 83.5%, 67.1%, and 63.4% compared with those of free enzyme with Suc–Phe–4-NA, casein, and hemoglobin as the substrate, respectively. From kinetic analysis the K_m value and thus the affinity between enzyme and substrate changed after enzyme immobilization, and could be related to the hydrophobicity/hydrophilicity of the substrate and the copolymer. The immobilized enzyme showed enhanced thermal stability compared to free enzyme and had a similar activation energy. It could be recycled with repeated precipitation/dissolution cycles with high enzyme activity.

Keywords: *N*-Isopropylacrylamide; α-Chymotrypsin; Temperature sensitivity; LCST

1. Introduction

Enzyme immobilization has several advantages including ease of recovery, simplified downstream processing, easy adaptation for continuous operation, and sometimes enhanced stability. Conventionally, enzymes are immobilized on water-insoluble supports and such systems have been applied successfully in many industrial processes. However, limiting the diffusion of substrate into, and product out of, the solid support is a major problem with insoluble enzyme conjugates, especially when the substrate is macromolecular or insoluble. To overcome these problems, soluble polymer–enzyme conjugates can be prepared by immobilizing the enzyme on a reversibly soluble–insoluble polymer. The enzyme reaction can occur in solution, after which the enzyme can be recovered in an insoluble state by small changes of pH [1–3], temperature [4,5], or ionic strength [6,7]. To separate the enzyme from the product, to recover the product, and to recycle the enzyme, phase separation of a soluble polymer–enzyme

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PII S1381-1177(96)00032-X

conjugate by causing the polymer to precipitate is a more convenient way than dialysis, membrane ultrafiltration, or column chromatographic separation. The product can be recovered from the supernatant and the enzyme conjugate redissolved and recycled for further reaction with fresh substrate.

Poly(*N*-isopropylacrylamide) (PNIPAAm) is a water-soluble polymer exhibiting remarkable hydration–dehydration changes in aqueous media in response to changes in temperature, resulting in a lower critical solution temperature (LCST) [8]. The polymer precipitates when heated above the LCST (32–33°C), but is redissolved and becomes soluble when cooled below that temperature. This process is fully reversible. The reversible phase-separation behavior of PNIPAAm has been utilized by conjugating this polymer to an antibody for immuno-diagnostic assay [9], to protein A for separation of immunoglobulin by thermal affinity precipitation [10], and to amylase for hydrolysis of starch [11,12].

In the present work, we prepared copolymers containing NIPAAm and *N*-acryloxysuccinimide (NAS) for covalent immobilization of α-chymotrypsin. NAS has a high reactive ester group which reacts with amino groups in the enzyme while the copolymer shows reversibly soluble characteristics due to the properties of PNIPAAm. The immobilized enzyme was characterized by studying its properties and comparing to free α-chymotrypsin, and evaluating its reusability with repeated precipitation–dissolution cycles.

2. Materials and methods

2.1. Materials

N-isopropylacrylamide (NIPAAm, Aldrich Chemical Co., Milwaukee, WI, USA) was purified by recrystallization from hexane. *N*-acryloxysuccinimide (NAS, ACROS Organics, Geel, Belgium) was used without prior purification. 2,2″-Azobis(isobutyronitrile) (AIBN, TCI, Tokyo, Japan) was recrystallized from methanol. α-Chymotrypsin from bovine pancreas (Type II), *N*-succinyl-L-phenylalanine-4-nitroanilide (Suc–Phe–4-NA), casein, and hemoglobin were obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical or a higher grade.

2.2. Synthesis and characterization of NIPAAm–NAS copolymer

The copolymer was prepared by dissolving 7.42 g of NIPAAm and 0.58 g of NAS in 120 ml of 50/50 (v/v) of tetrahydrofuran (THF) and toluene mixture (molar ratio of NIPAAm to NAS is 39). After removing oxygen by nitrogen purging, 25 mg of AIBN was added as an initiator to start polymerization under nitrogen atmosphere. The polymerization process was continued for 24 h at 60°C, after which the mixture was transferred to a burette and added dropwise into 300 ml of petroleum ether to precipitate the copolymer. The precipitate was collected by filtration, dried in vacuum, and stored in a desiccator [10].

The yield of the copolymer was determined by drying the polymer solution at 90°C for 24 h. The copolymer was analyzed by FT-IR spectroscopy (Perkin Elmer 1725X spectrophotometer). Its molecular weight was determined by gel permeation chromatography (GPC) using THF as the mobile phase and polystyrene as standards. The active ester content in the copolymer was assayed by reaction of active ester with isopropylamine, which liberated *N*-hydroxysuccinimide to be determined at 259 nm [13]. Thermal analysis was done using a differential scanning calorimeter (DSC). The LCST was determined by measuring the solution turbidity at 600 nm as a function of temperature. The solution contained 0.2% (w/w) copolymer in water. The LCST was defined as the temperature at which 50% change in the absorbance occurs.

2.3. Immobilization of chymotrypsin to NIPAAm–NAS copolymer

0.4 g of copolymer was dissolved in 20 ml 0.05 M phosphate buffer containing a suitable amount of enzyme. The solution was mixed by stirring at low speed at room temperature for 2 h. To selectively separate the copolymer–conjugated enzyme from the solution, 2 g of NaCl was added, which lowers the LCST of the enzyme conjugate to below the room temperature and cause it to precipitate. The free enzyme remained in the supernatant and could be separated from the immobilized enzyme. The solution was warmed to 30°C and centrifuged at 9000 *g* for 40 min. The precipitate was collected and re-dissolved in 8 ml of 0.05 M Tris buffer, pH 7.8 for storage at 4°C.

The coupling yield of protein (enzyme activity) was defined as the amount of protein (enzyme activity) immobilized to that added initially during the immobilization step. The relative specific activity was defined as the specific activity of the immobilized enzyme to that of the free enzyme.

2.4. Analytical methods

Enzyme activity was measured with Suc–Phe–4-NA as the substrate at 25°C [14]. The reaction mixture contains 4 mM Suc–Phe–4-NA in 127 mM triethanolamine (TEA) buffer solution containing 12.6 mM Ca^{2+} (pH 7.8). The absorbance was monitored for 30 min at 405 nm and the enzyme activity was determined from the increase in absorbance due to the release of 4-nitroaniline (extinction coefficient = 960 M^{-1} mm^{-1}). One unit of enzyme activity is defined as the amount of enzyme which hydrolyzes 1 μmol substrate per min. For hydrolysis of macromolecular substrates, the reaction rate with casein or hemoglobin as the substrate was measured at pH 7.5 [15]. One unit of activity in this case is defined as the amount of enzyme which yields an absorbance at 278 nm equivalent to 1 μmol tyrosine per min. The protein concentration was determined by a BCA protein assay kit (Pierce Co., Rockford, IL, USA).

3. Results and discussion

3.1. Synthesis of copolymer

To introduce active ester groups which are highly reactive to primary amine groups in protein molecules in a temperature-sensitive copolymer, NAS was co-polymerized with NIPAAm. The LCST measured from solution turbidity is shown in Fig. 1, with three different compositions of the copolymer. The LCST shifted to a higher temperature and the solubility response of the polymer to the change in temperature was more sensitive at higher molar ratios of NIPAAm to NAS. In view of this property, the copolymer prepared with the highest molar ratio (i.e. 39), which has an LCST of 33.5°C was chosen for enzyme immobilization in this study. The peak in the DSC thermogram of this copolymer is 32.7°C. The results obtained from the DSC analysis correspond to the response of the solubility to temperature changes

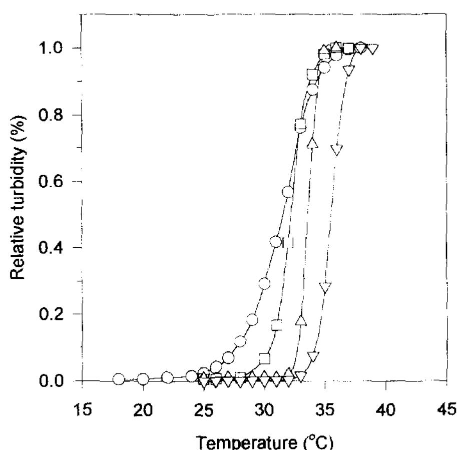

Fig. 1. Solubility response of temperature-sensitive NIPAAm/NAS copolymers and the immobilized enzyme to change in temperature. The turbidity was measured at 600 nm. The copolymers were prepared with different molar ratios of NIPAAm to NAS. (○) 9, (□) 19, (△) 39. (▽) Immobilized enzyme coupled to the copolymer was prepared with a molar ratio of 39.

estimated by measuring the turbidity with a spectrophotometer. The change in solution turbidity is fully reversible with a small temperature change between 33 and 35°C. The polymerization yield was about 83%. The copolymer contains succinimide groups as evidenced from a peak at 1737 nm from FT-IR spectroscopy, which is absent in pure PNIPAAm polymer. The molecular weight (M_n) of the copolymer was 27000 and its molar ratio of NIPAAm to NAS is 47.6. Thus, the copolymer on average contains 4.9 NAS groups per polymer chain.

3.2. Enzyme immobilization

The effect of solution pH during the immobilization step is shown in Fig. 2. When succinimide ester is employed for protein conjugation, it is generally recommended to use a buffer with a pH in the range 8.0–9.0 [16]. If the pH is too low, most of the amine groups are positively charged; the pK_a value of the lysine amine group is about 9.3. The positively charged amine

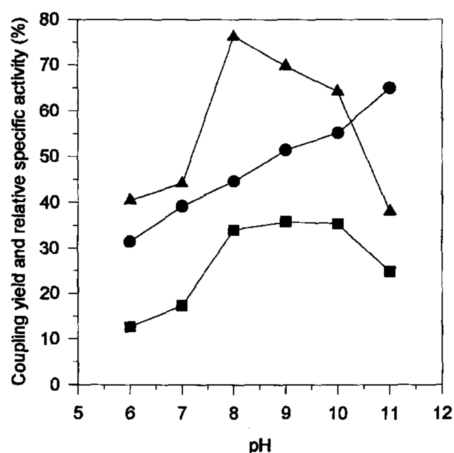

Fig. 2. The effect of immobilization buffer pH on the coupling yields of protein and enzyme activity for the immobilization of α-chymotrypsin on NIPAAm/NAS copolymer, and the relative specific activity of the immobilized enzyme with the specific activity of the free enzyme taken as 100%. The buffer used was 0.05 M phosphate buffer, 10 mg enzyme. (●) coupling yield of protein, (■) coupling yield of activity, (▲) relative specific activity. The activity was measured at 25°C with Suc–Phe–4-NA (4 mM) as substrate in 127 mM TEA buffer, pH 7.8.

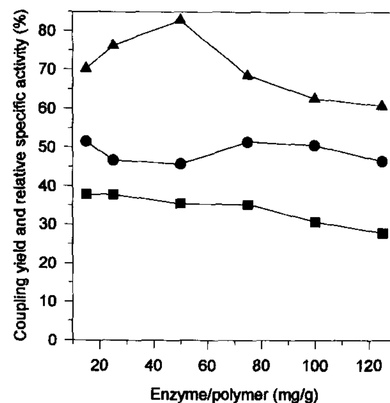

Fig. 3. The effect of enzyme/polymer ratio on the coupling yields of protein and enzyme activity for the immobilization of α-chymotrypsin on NIPAAm/NAS copolymer, and the relative specific activity of the immobilized enzyme with the specific activity of the free enzyme taken as 100%. The buffer used was 0.05 M phosphate buffer, pH 8. 0.4 g copolymer. The activity was measured at 25°C with Suc–Phe–4-NA (4 mM) as substrate in 127 mM TEA buffer, pH 7.8. (●) coupling yield of protein, (■) coupling yield of activity, (▲) relative specific activity.

has a very low reactivity with NAS ester to give a low immobilization yield. On the other hand, the ester is susceptible to hydrolysis at high pH. The pH stability of the enzyme also has to be considered. Selecting an optimum solution pH for enzyme immobilization is therefore important. It can be seen from Fig. 2 that the coupling yield of protein increased with pH while the coupling yield of enzyme activity showed a plateau between pH 8–10 with sharp decrease below or above those values. The highest relative specific activity (specific activity of free enzyme = 110.3 U/g) of the immobilized enzyme occurred at pH 8. Taken together, the immobilization step should be carried out in an alkaline solution with a pH close to 8.

The effects of the enzyme/polymer ratio (E/P ratio) on immobilization was studied next, and the results are shown in Fig. 3 with a constant copolymer weight of 0.4 g. The coupling yield of protein is rather insensitive to the E/P ratio, which remained at 50%. This indicates that the active ester groups of the copolymer were not saturated with enzyme molecules. In fact, considering the molecular weights of the copolymer and the enzyme, and the number of

active ester groups per copolymer chain, a maximum of 4.5 g enzyme could be coupled to one g of copolymer with 1:1 binding ratio. This value is expected to be reduced substantially since multi-point binding between enzyme and copolymer would occur and it could be as low as 0.32 g of enzyme per g of copolymer if all 14 lysine residues in α-chymotrypsin were accessible and used for coupling to the copolymer. Nonetheless, the coupling yield of protein did not decrease even at an E/P ratio of 120 mg/g and about half of the enzyme molecules were immobilized regardless of enzyme concentration in the concentration range studied.

However, the coupling yield of enzyme activity decreased with increasing E/P ratio. The reaction might be limited by mass transfer of the substrate after enzyme immobilization since free enzyme showed a constant activity within the enzyme concentration range studied. As the E/P ratio was increased, one may postulate that the molecular weight of the enzyme conjugate will increase, which changes the microenvironment viscosity and reduces the mass transfer rate of the substrate. The maximum relative specific activity of the immobilized enzyme occurred at an E/P of 50. Immobilized enzyme prepared in pH 8 buffer and with an E/P of 50 mg/g, which gave the highest relative specific activity of 83.5%, was used in the subsequent studies.

3.3. Properties of immobilized chymotrypsin

The immobilized enzyme could fully retain its activity in solution after 30 days if stored at 4°C. It showed a LCST of 35.5°C in Fig. 1, which is higher than the LCST of the copolymer from which it was prepared. As precipitation of the hydrophilic segment of the enzyme-conjugated copolymer (α-chymotrypsin) from the solution requires a high hydrophobic driving force, the increase in LCST may reflect the energy required for sufficient dehydration of the copolymer chain to precipitate the enzyme. Alternatively, the unbound activated esters in the copolymer may be hydrolyzed to generate carboxylate groups to make the copolymer more hydrophilic. The effect of pH of the reaction medium on the activity of chymotrypsin was investigated. The activities of both free and immobilized enzyme were sensitive to the pH of the reaction medium. The same activity–pH profiles were found in both cases and their optimum pH values remained the same at pH 7.8 (data not shown). This implies that copolymer chains may be coupled to the lysine groups of the enzyme away from the active site.

The effect of temperature on enzyme activity is shown in Fig. 4. The activity increased with increasing temperature until an optimum temperature was reached and then decreased due to thermal inactivation in both cases. The optimum temperature increased from 45 to 55°C after enzyme immobilization. This result was quite unexpected. It was hypothesized that the kinetic behavior of the immobilized enzyme will vary below and above the LCST of the copolymer due to expansion and collapse of the PNIPAAm chains on the enzyme surface. Increasing the temperature may result in a change in the microenvironment around the active site and/or

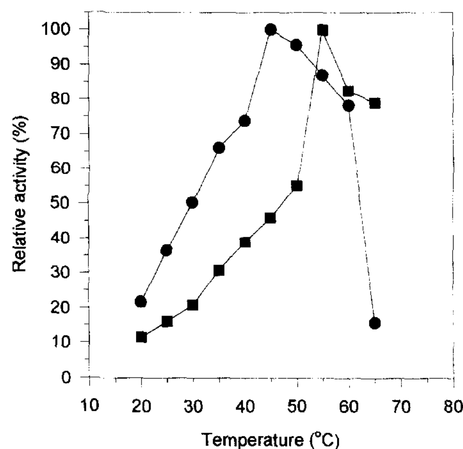

Fig. 4. The effect of temperature on the activity of α-chymotrypsin in pH 7.8 buffer. The relative activity of the enzyme at the optimum temperature is taken as 100%. 0.2 mg/ml free enzyme or 0.258 mg/ml immobilized enzyme. The activity was measured at 25°C with Suc–Phe–4-NA (4 mM) as substrate in 127 mM TEA buffer, pH 7.8. (●) free, (■) immobilized.

the flocculation of the immobilized enzyme, both of which should affect the enzyme catalytic activity. The reaction carried out by the immobilized enzyme above the LCST occurred heterogeneously with the precipitation of the polymer, and discontinuity in the activity–temperature profile was expected. However, no such discrete continuity was observed and the reaction rate increased with temperature smoothly above the LCST. As the temperature was increased above the LCST, it can be expected that the PNIPAAm chains will collapse and become hydrophobic, which may increase the partitioning of the substrate molecules near the active site. This may compensate for the activity decrease due to the collapse of PNIPAAm chains and resulted in continued increase of activity of the immobilized enzyme past the LCST. Another reason may be that the reaction occurs in low copolymer concentration and it does not form intermolecular aggregate above its LCST. In the case of a more concentrated PNIPAAm–lipase solution, the enzyme activity was dependent on its LCST [17], while a similar conjugate with trypsin but at a lower polymer concentration no dramatic difference of activity dependence on temperature from free enzyme was found [5]. Valuev et al. modified trypsin with PNIPAAm and reported that temperature dependence of enzyme activity changed little [18]. They also modified peroxidase with the same polymer, whose activity dramatically dropped at temperatures above its LCST [18]. Chen and Hoffman prepared PNIPAAm–β-galactosidase conjugates and observed no change in enzymatic activity–temperature profiles [19]. These results may suggest that some enzymes are sensitive to the hydrophobic environment while others are not.

Fig. 5 shows the effect of incubation at a variety of temperature on the residual activity of the enzyme. The immobilized enzyme is more stable at high temperature (> 40°C) than the free enzyme. For the time course of thermal inactivation of the enzyme at 40°C, the free enzyme retained only 35% of its original activity after 50 min, whereas the immobilized enzyme still retained 80% of its original activity (data not shown). We concluded that immobilization of chymotrypsin to the copolymer provides protection against thermal inactivation of the enzyme at temperatures well above the LCST of the polymer. It is known that an enzyme will exhibit enhanced thermal stability when covalently linked to an insoluble matrix by multi-point attachments, which may be explained by the reduction of mobility of the enzyme after immobilization on the support [20]. A similar reduction in the mobility of the precipitated conjugates above the LCST may also protect the immobilized enzyme against thermal inactivation. It is also well known that a soluble polymer such as PEG and polysaccharides have a stabilizing effect after conjugation to an enzyme by the introduction of free mobile functional polymer groups [21,22]. Collapse of NIPAAm above its LCST apparently does not induce a continuous strain in the conformation of the enzyme This indicates that multi-point attachments of a single polymer molecule to the enzyme, which is expected to induce destabilization, may not be prevalent in the immobilized

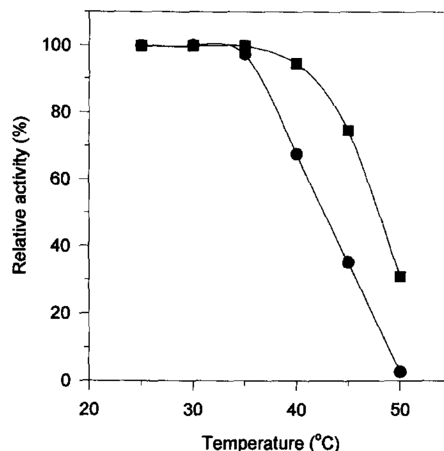

Fig. 5. The effect of temperature on the stability of α-chymotrypsin. The enzyme was incubated at the temperature indicated for 15 min in pH 7.8 buffer and assayed for the remaining activity. The retained activity at 25°C is taken as 100%. 0.2 mg/ml free enzyme or 0.258 mg/ml immobilized enzyme. The activity was measured at 25°C with Suc–Phe-4-NA (4 mM) as substrate in 127 mM TEA buffer, pH 7.8. (●) free, (■) immobilized.

Table 1
Kinetic constants for hydrolysis of Suc–Phe-4-NA by free and immobilized α-chymotrypsin at different temperature

Kinetic constant	Free			Immobilized		
	20°C	25°C	30°C	20°C	25°C	30°C
K_m (mM)	3.89	4.60	4.78	5.65	7.92	9.59
V_{max} (μmol/l/min)	6.07	9.99	14.33	11.12	19.68	27.52
k_{cat} (min^{-1})	0.73	1.20	1.72	0.74	1.30	1.83
r^2 [a]	0.9991	0.9996	0.9975	0.9994	0.9994	0.9989

Reaction conditions: 0.2 mg/ml free enzyme or 0.258 mg/ml immobilized enzyme at pH 7.8.
[a] Correlation coefficient.

enzyme. This is one of the advantages of using a low NAS to NIPAAm molar ratio during the synthesis of the copolymer.

The kinetic constants were determined from the Michaelis–Menten equation using Lineweaver–Burk plots at three temperatures. They are reported in Table 1. The K_m values increased after enzyme immobilization while the k_{cat} values changed slightly. Conjugation of chymotrypsin to the copolymer may cause a conformational change in the enzyme and subsequently changes in the affinity between enzyme and substrate. The increase in K_m indicates that the immobilized enzyme has somewhat lower affinity for the substrate than the free enzyme. This decreased affinity for the substrate could be due to an allosteric conformational change of the enzyme active site or change in the microenvironment of the immobilized enzyme, which causes unfavorable partitioning of the substrate near the active site. When amphiphilic polymers such as PEG and poly(*N*-vinylpyrrolidone) were conjugated to trypsin or α-chymotrypsin, the immobilized enzyme increased the local substrate concentration by interacting favorably with the hydrophobic substrate, resulting in a decrease in K_m values [23–25]. It is reasonable to suppose here that the decreased affinity toward the hydrophobic substrate originates from the reduced hydrophobicity of the protein surface, since below the LCST, polymer chains of PNIPAAm hydrate form an expanded structure in water which is very hydrophilic [8]. The activation energies calculated from the Arrhenius equation using the temperature dependence of k_{cat} values are 15.2 ($r^2 = 0.993$) and 14.3 ($r^2 = 0.981$) kcal/mol for the free and immobilized enzyme, respectively. The similarity in activation energy indicates that diffusion limitation is not important after enzyme immobilization.

The reusability of the enzyme conjugate is shown in Fig. 6 with temperature and precipitation cycles. The effect of temperature cycling between 4 and 40°C was studied, which crossed the LCST of the copolymer. The enzyme activity decreased to 80% of its original value after 9 cycles as shown in the insert of Fig. 6. If one considers the cumulative enzyme denaturation at 40°C in each 5 min cycle, the process of hydration and dehydration of the PNIPAAm chain apparently do not cause any change in the high-order structure of the immobilized enzyme. For the retained activity as a function of succes-

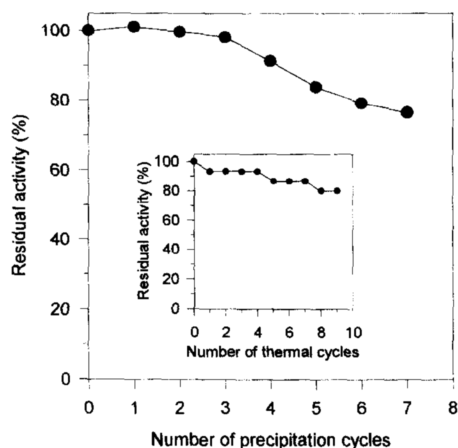

Fig. 6. The effect of successive thermal cycles and precipitation cycles on the residual activity of immobilized α-chymotrypsin in pH 7.8 buffer. For the thermal cycle in the insert, the temperature was raised to 40°C for 5 min and lowered to 4°C for 15 min, and then the enzyme was assayed for activity at 25°C. For the precipitation cycle, the enzyme was precipitated by adding NaCl to a final concentration of 10% (w/v) at 30°C. The precipitate was collected by centrifugation at 9000 g for 30 min, redissolved in pH 7.8 Tris buffer, and assayed for enzyme activity at 25°C. The activity before the cycling is taken as 100%. The activity was measured at 25°C with Suc–Phe-4-NA (4 mM) as substrate in 127 mM TEA buffer, pH 7.8.

sive precipitation cycles, the retained activity of the immobilized enzyme decreased after 7 cycles to 76.5% of its original activity. Since at 30°C α-chymotrypsin has fair thermal stability, and 10% NaCl does not affect enzyme activity, the loss of enzyme activity is not because of thermal deactivation but probably due to the incomplete precipitation, recovery, and recycling of the immobilized enzyme in each cycle.

3.4. Hydrolysis of macromolecular substrates

When α-chymotrypsin was immobilized to an insoluble carrier, its hydrolysis activity toward high molecular weight substrates such as casein and hemoglobin dropped significantly due to the low diffusion rate of the substrate and the steric hindrance from the support. O'Neil et al. reported a 7% relative specific activity with DEAE-cellulose as the support [26]; Hayashi and Ikada reported 4 to 22.5% retention of specific activity with polyacrolein microspheres as the support, depending on the length of the spacer [27]; Alves Da Silva et al. reported a maximum relative specific activity of 18.8% for chymotrypsin immobilized to PEG grafted hydroxyethyl methacrylate particles [28]. The specific activities of the immobilized enzyme prepared here were 63.4 and 67.1% for hemoglobin and casein, respectively, when compared to the free enzyme. Although casein forms micelles in solution in the size of 50 to 300 nm, diffusion is not restricting the action of the immobilized enzyme even when dealing with this substrate. The kinetics constants for the hydrolysis of casein are evaluated by Lineweaver–Burk plots as shown in Fig. 7. An upward curve was obtained for casein concentrations greater than 1% (w/v), indicating substrate inhibition above that substrate concentration in both cases. The results probably follow from the dependence of casein micelle size and structure on solution conditions. Because α-chymotrypsin preferentially cleaves polar amino acid residues, the percentage of available cleavage sites varies

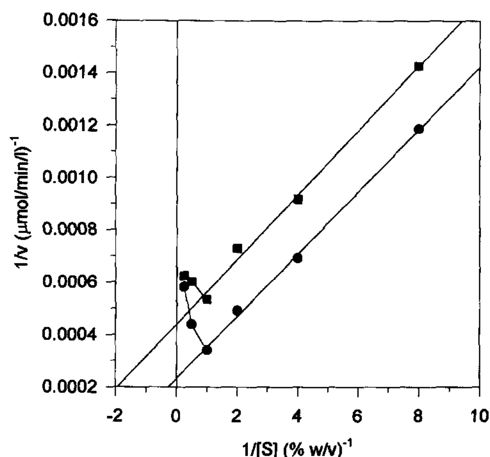

Fig. 7. Lineweaver–Burk plot of the hydrolysis of casein by α-chymotrypsin at 30°C in pH 7.5 buffer. The lines are best-fits of the data with substrate concentrations up to 1% (w/v) following Michaelis–Menten kinetics. 0.333 mg/ml free enzyme or 0.362 mg/ml immobilized enzyme. (●) free, (■) immobilized.

with casein micelle volume and structure. The ratio of average casein micelle volume to available cleavage sites could explain the dependence of activity on substrate concentration. The kinetic constants calculated from the data with substrate concentrations up to 1% (w/v) are reported in Table 2. The immobilized enzyme showed higher affinity toward casein as evidenced from the fact that the K_m value of the immobilized enzyme is about only half of that of the free enzyme. This change in K_m value after enzyme immobilization is opposite to that with a hydrophobic substrate (Suc–Phe–4-NA) and may be due to the hydrophilicity of the casein micelle surfaces.

Table 2
Kinetic constants for hydrolysis of casein by free and immobilized α-chymotrypsin

Kinetic constant	Free	Immobilized
K_m (% w/v)	0.512	0.281
V_{max} (μmol/l/min)	4310	2277
k_{cat} (min^{-1})	310.5	151.0
r^2 [a]	0.9980	0.9934

Reaction conditions: 0.333 mg/ml free enzyme or 0.363 mg/ml immobilized enzyme at 30, pH 7.5.
[a] Correlation coefficient.

Acknowledgements

Financial support from the National Science Council, Republic of China under grant NSC85-2321-B-182-001BA is highly appreciated.

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