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Immobilization of α-Chymotrypsin to a Temperature-Responsive Reversibly Soluble–Insoluble Oligomer Based on *N*-Isopropylacrylamide

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Abstract: A temperature-responsive *N*-isopropylacrylamide (NIPAAm) oligomer with an ester functional end group and a molecular weight of 3300 was prepared by chain-transfer polymerization using β-mercaptopropionic acid and subsequently activated by *N*-hydroxysuccinimide (NHS). This oligomer was coupled to α-chymotrypsin to yield a thermo-sensitive reversibly soluble–insoluble oligomer–enzyme conjugate, which is water-soluble at temperatures below 34°C and that precipitates above 36°C. The conjugated enzyme showed higher activity, and improved thermal stability compared with native enzyme. Kinetic properties and optimum conditions for activity were compared with those of native enzyme. More than 93% enzyme activity of the conjugate was recovered after eight cycles of thermal-induced precipitation. The oligomer–enzyme complex was used for repeated hydrolysis of casein; the biocatalyst was recovered between runs by thermal-induced precipitation and showed good stability. © 1998 Society of Chemical Industry

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Key words: *N*-isopropylacrylamide; α-chymotrypsin; immobilized enzyme

1 INTRODUCTION

Enzymes bound to solid phase carriers are generally not suitable for bioconversions involving macromolecular and particular substrates because of steric hindrance and diffusional limitations. To overcome such constraints support matrices can be made reversibly soluble and insoluble by changes of a particular environmental parameter such as pH, temperature, and presence of certain ions, to provide an alternative to insoluble matrices.[1] An enzyme conjugated to such a carrier may be used as a catalyst in its soluble form and then be recovered via the insoluble state. Phase separation of a soluble polymer–enzyme conjugate by precipitation is a simpler process for enzyme recycling and product separation than dialysis, membrane ultrafiltration, or column chromatographic separations. The recovered enzyme conjugate can readily be redissolved and recycled for further reaction with fresh substrate.

Poly(*N*-isopropylacrylamide) (PNIPAAm) is a well-known water-soluble polymer showing unique reversible hydration–dehydration changes in response to small temperature changes.[2] An aqueous solution of PNIPAAm shows phase separation and the polymer precipitates at the so-called lower critical solution temperature (LCST). The LCST of PNIPAAm is known to be 32°C. Copolymer containing NIPAAm and a co-monomer or co-monomers, bearing a reactive function group for covalent binding of enzyme molecules, can therefore be used to form a temperature-sensitive reversibly soluble–insoluble polymer–enzyme conjugate. Previous examples include α-amylase immobilized to copolymers of NIPAAm-MAA (methacrylic acid),[3] NIPAAm-GMA (glycidyl methacrylate),[4] and NIPAAm-NAS (*N*-acryloxysuccinimide);[5] isoamylase to

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NIPAAm-NAS;[6] α-chymotrypsin to NIPAAm-NAS;[7] alkaline phosphatase to NIPAAm-NAS;[8] thermolysin to NIPAAm-GMA-MAAm (methacrylamide).[9]

Random copolymers may have more than one reactive group in each polymer chain such that it may not be known if the enzyme is coupled to a polymer chain by single or multiple attachment. Also, it is unknown whether more than one enzyme is conjugated to one polymer chain and at what point along the chain the conjugation occurs. All of the above might influence significantly the activity and properties of the conjugate enzymes. Moreover, the conjugates may form protein aggregates with limited water-solubility by forming a cross-linked network of protein and polymer.

In order both to simplify and to define more precisely the structure of the conjugated enzymes, an NIPAAm oligomer having an end-capped carboxyl group has been synthesized and used for conjugation of enzyme. Oligomer molecular weight was controlled and synthetic routes to semitelechelic oligomers averaging one functional end group per oligomer chain were created by telomerization chemistry.[10] Thio-compounds such as β-mercaptopropionic acid having functional groups as telogens are known to be effective in introducing functional groups to the end of growing polymeric chains and regulating polymer molecular weight by radical telomerization via chain-transfer reaction.[11] These oligomers would be expected not only to maintain high enzyme activity by reduced hindrance from conjugated oligomers but also to impart high temperature sensitivity due to the inherent high mobile nature of oligomer free end groups. This method is controlled and well-defined for conjugation of polymers to proteins and is similar in principle to the previously reported method of conjugation of PEG to therapeutic enzymes.[12]

In this paper, the preparation of α-chymotrypsin conjugated with oligo-NIPAAm bearing carboxyl end groups is reported. This conjugate exhibits a temperature-induced phase separation attributed to the LCST of the oligomer and its response toward temperature change is both rapid and reversible due to the highly mobile free end groups remaining on the oligomers. The oligomer–α-chymotrypsin conjugate was found to retain its native enzyme activity and to be readily separable from the aqueous reaction system as a precipitate after being induced by a small temperature change.

2 EXPERIMENTAL

2.1 Materials

β-mercaptopropionic acid (MPA) and *N*-isopropylacrylamide (NIPAAm) were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). NIPAAm was purified by recrystallization from hexane and MPA was purified by distillation under reduced pressure. 2,2′-Azobis(isobutyronitrile) (AIBN, TCI, Tokyo, Japan) was recrystallized from methanol. *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), α-chymotrypsin from bovine pancreas (Type II), *N*-succinyl-L-phenylalanine-4-nitroanilide(Sue-Phe-4-NA), casein and haemoglobin were obtained from Sigma (St Louis, MO, USA). All other chemical and solvents were of analytical or higher grade.

2.2 Preparation of NHS-esterfied NIPAAm oligomer

NIPAAm oligomer terminated with a carboxyl group at one end was prepared by chain transfer polymerization using MPA as a chain transfer agent. Five grams of NIPAAm, 0·2 g of MPA, and 50 mg of AIBN as initiator were dissolved in 20 cm^3 methanol at room temperature. The solution was degassed by freezing and evacuating four times and then sealed. Polymerization was carried out at 60°C for 24 h. The polymer was collected by precipitation in 60 cm^3 diethyl ether and dried in vacuum. The molecular weight of the oligomer obtained was determined by titration with 0·01 mol dm^{-3} NaOH, which detects the carboxyl groups at the end of the polymer molecules. The LCST was determined by measuring the solution turbidity at 600 nm as a function of temperature. The solution contained 0·2% (w/w) oligomer. The LCST was defined as the temperature at which a 50% change in the absorbance occurred.

Samples (4 g) of the carboxylated NIPAAm oligomer were dissolved in 40 cm^3 of anhydrous methylene chloride and cooled to 4°C. Two mmol dm^{-3} each of NHS and DCC were slowly added during stirring. The stirring was continued for another 4 h and the temperature was raised to 20°C. After reaction the insoluble dicyclohexylurea was removed by filtration and the activated oligomer was obtained by precipitation into anhydrous diethyl ether and dried in vacuum. The yield of the oligomer was determined by drying the oligomer solution at 90°C for 24 h. The degree of NHS esterification was determined by the release of *N*-hydroxysuccinimide in alkaline solution with a spectrophotometer.

2.3 Conjugation of α-chymotrypsin with NHS-esterified NIPAAm oligomer

The NHS-esterified NIPAAm oligomer was dissolved in a solution of α-chymotrypsin in 0·05 mol dm^{-3} phosphate buffer. The amount of oligomer and enzyme, and the volume and pH of the buffer solution are summarized in Table 1. The solution was gently shaken at 4°C for 16 h, after which 0·5 cm^3 of saturated ammon-

TABLE 1
Effects of Reaction Conditions on the Conjugation of α-Chymotrypsin to NIPAAm Oligomer

Reaction conditions					*Experiment results*	
O (mg)[a]	*E (mg)*[a]	*O/E (w/w)*	*[O + E] (mg cm^{-3})*	*pH*	*Protein immobilized (mg E mg^{-1} polymer)*	*Relative specific activity (%)*[b]
40	10	4	6·3	8	17·4	82·1
40	10	4	12·5	8	19·1	82·3
40	10	4	25	8	22·3	88·9
40	10	4	50	8	27·2	105·1
33·3	16·7	2	50	8	25·3	92·4
28·6	21·4	1·3	50	8	16·7	91·8
25	25	1	50	8	13·8	81·9
40	10	4	50	7	24·0	102·0
40	10	4	50	9	30·4	99·0
40	10	4	50	10	35·4	98·2
40	10	4	50	11	40·4	97·9

[a] O = NIPAAm oligomer, E = α-chymotrypsin.
[b] The relative specific activity was defined as the specific activity of the conjugated enzyme to that of the free enzyme.

ium sulphate solution was added, the solution volume was brought to 8 cm^3 by adding the same phosphate buffer. Precipitate formed immediately by incubating the solution at 30°C, which was recovered by centrifugation at 9000 *g* for 30 min at 30°C. The collected precipitate was redissolved in 8 cm^3 of 0·05 mol dm^{-3} Tris buffer, pH 7·8 at 4°C. To this solution, 0·5 cm^3 of saturated ammonium sulphate solution was added and the mixture was warmed to 30°C. The resulting precipitate was recovered again by centrifugation. This procedure was repeated twice and the final precipitate was dissolved in 10 cm^3 of Tris buffer and stored at 4°C. The relative specific activity was defined as the ratio of the specific activity of the conjugated 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. The reaction mixture contained 4 mmol dm^{-3} Suc-Phe-4-NA in 127 mmol dm^{-3} triethanolamine (TEA) buffer solution containing 12·6 mmol dm^{-3} Ca^{2+} (pH 7·8). The absorbance at 405 nm was monitored for 30 min and enzyme activity determined from the increase in absorbance due to the release of 4-nitroaniline. One unit of enzyme activity is defined as the amount of enzyme which hydrolyses 1 μmol substrate per min. For hydrolysis of macromolecular substrates, the reaction rate with casein or haemoglobin as the substrate was measured at pH 7·5.[13] The unit of activity is defined in this case as the amount of enzyme which yields an absorbance at 278 nm equivalent to 1 μmol tyrosine per min. Protein concentration was determined using BCA Protein Assay Kits (Pierce Co., Rockford, IL, USA).

2.5 Cycling of conjugated enzyme and repeated batch hydrolysis of casein

Thermal and precipitation cycles of conjugated α-chymotrypsin were conducted in 10 cm^3 pH 7·8 Tris buffer. For the thermal cycle, temperature was raised to 40°C for 5 min and lowered to 4°C for 15 min, and the conjugate assayed for enzyme activity at 25°C. For the precipitation cycle, the enzyme was precipitated by adding 1 cm^3 saturated ammonium sulphate at 30°C. The precipitate was recovered by centrifuging at 9000 *g* for 30 min, redissolved in pH 7·8 Tris buffer, and assayed for enzyme activity. The activity before cycling was taken as 100%. The activity was measured at 25°C with Suc-Phe-4-NA as substrate in pH 7·8 TEA buffer.

Repeated batch hydrolysis of 2% (w/v) casein with conjugated α-chymotrypsin was conducted at 30°C in 50 cm^3 0·1 mol dm^{-3} pH 7·0 phosphate buffer using 0·2 g immobilized enzyme. Samples (2 cm^3) of solution were withdrawn at 5 min intervals and mixed with an equal amount of 0·3 mol dm^{-3} trichloro-acetic acid to inactivate the enzyme and to precipitate residual casein and high molecular weight peptides. This assay mixture was placed in a 30°C water bath for 30 min, and then centrifuged at 1000 *g* for 15 min. Samples of the supernatant were analysed spectrophotometrically at 275 nm to determined product concentrations. Hydrolysis products are reported in terms of absorbance units (275 nm), representing the total amount of low-molecular-weight peptides formed in the reaction. Every 30 min the immobilized enzyme was precipitated by adding 5 cm^3 saturated ammonium sulphate to the

mixture and the mixture was incubated at 40°C. The precipitate was collected by centrifuging at 9500 *g* for 5 min, then redissolved in 50 cm^3 pH 7·0 buffer containing 2% (w/v) casein to start the next cycle of reaction.

3 RESULTS AND DISCUSSION

3.1 Synthesis and characterization of oligomer

Polymerization was performed in four organic solvents, ethanol, methanol, *tert*-butanol, and 2-propanol, with oligomer yields ranging from 70% to 94% after 24 h. Methanol was chosen as the solvent for large synthesis because of its high solubility toward NIPAAm and ease of use. The oligomer synthesized in methanol was a white powder with a yield *c.* 88% and a molecular weight of 3300, by end-group titration. GPC data gave similar molecular weight values, indicating that there was one carboxyl end group per NIPAAm oligomer molecule. Aqueous solutions of carboxylated PNIPAAm had an LCST of 35·5°C, at which hydration–dehydration changes occurred abruptly. Phase transition behaviour was instantly and reversibly observed at the LCST.

Usually for coupling reactions involving end-capped carboxyl groups, the oligomer and enzyme were mixed together with the water-soluble activation reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC).[14] It is possible that in addition to the polymer–enzyme conjugates, enzyme–enzyme conjugates which could be either water-soluble or water-insoluble, might be formed during the coupling reaction since both carboxyl and amino groups are present in the enzyme. The enzymes might also be conjugated to each other along with the polymer to form polymer–enzyme–enzyme conjugates. This could also yield conjugates with poorly defined structures.

To overcome this problem, the carboxylated NIPAAm oligomer was first activated by esterification with NHS in methylene chloride in the presence of DCC to obtain the NIPAAm oligomer with a single functionally reactive NHS ester group. NHS esters are very reactive toward amino groups and have widely been used as a functional group for coupling reactions with proteins and ligands.[15] This NHS-activated end group on the oligomer can be coupled to the enzyme only, without forming enzyme–enzyme conjugates during the coupling reaction. The yield of activated oligomer was 89% and the conversion of carboxyl to the ester group was 84%.

3.2 Conjugation of α-chymotrypsin to NHS-esterified NIPAAm oligomer

Table 1 shows the effects of reaction conditions on the conjugation of α-chymotrypsin to the NIPAAm oligomer. In control experiments, enzyme was mixed with either the NIPAAm oligomer or the carboxylated NIPAAm oligomer and treated similarly as for enzyme conjugation to NHS-esterified oligomer. No protein or enzyme activity could be detected in these control precipitates, indicating that the enzyme in the precipitate was covalently conjugated with the oligomer, not physically entrapped within the oligomer. The amount of enzyme protein conjugated with the oligomer increased with increasing concentrations of enzyme and oligomer ([O + E]). The relative specific activity of the conjugated enzyme also increased with increasing [O + E]. Similarly, both the protein immobilized and the relative specific activity increased with increasing ratio of oligomer to enzyme (O/E).

The amount of protein bound increased with increased pH, probably as a result of more amino groups being available for conjugation. The pK_a value of the lysine amine group is about 9·3 and the positively charged amine has very low reactivity with succinimide ester. The relative specific activity of the immobilized enzyme was little affected by the pH of the coupling medium. The specific activity of some conjugated enzyme was higher than that of the native enzyme, possibly as a consequence of a more favourable microenvironment induced by conjugation. The conjugation of NIPAAm oligomer with the enzyme is similar to the way proteins have been conjugated with polyethylene glycol (PEG) and similar improvements in activity of PEG-modified enzyme compared with native enzyme have been reported.[16]

3.3 Properties of conjugated α-chymotrypsin

Conjugated α-chymotrypsin (prepared at pH 8, with O/E = 4, [O + E] = 50 mg cm^{-3}) was completely water-soluble at temperatures below 34°C and precipitated above 36°C, showing that conjugation did not alter the LCST of the oligomer. The activities of both native and conjugated enzyme were sensitive to the pH of the reaction medium. Native α-chymotrypsin, a mixture of native α-chymotrypsin with NIPAAm oligomer, and the conjugated enzyme all showed the same pH–activity profile and pH optima.

For each of the above three systems, enzyme activity increased with increase of temperature until the optimum reaction temperature (45°C) was reached and then decreased markedly, primarily because of thermal inactivation. Above the LCST of the NIPAAm oligomer (35·5°C), the conjugate precipitated, however the enzyme activity continued with gentle stirring of the reaction mixture. Despite this it was observed that the temperature dependence of the enzyme reaction was essentially the same regardless of enzyme state up to the optimum reaction temperature, above which the conju-

gate lost activity less rapidly than the native enzyme, possibly due to its superior thermal stability (see below). It might be expected that the activity–temperature profile of the conjugated enzyme above the LCST would show discontinuity with the precipitation of the oligomer and the reaction became heterogeneous. However, this was not observed and the reaction rate increased smoothly above the LCST of the NIPAAm oligomer. The low molecular weight of the substrate may facilitate diffusion to and from the active sites of the solid phase enzyme. Furthermore, the low molecular weight of NIPAAm oligomer may also minimize interference with the access of the substrate to the enzyme active site.

Figure 1 shows that conjugated enzyme was more stable at high temperature (>40°C) than the native enzyme. Free NIPAAm oligomer did not protect the enzyme from thermal inactivation (data not shown). Figure 2 shows that the native enzyme retained only 25% of its original activity following heat treatment at 40°C for 50 min, whereas the conjugated enzyme retained 61% of its original activity. The kinetics of inactivation followed first-order kinetics. The first-order inactivation rate constant calculated from the slopes of the linear regression lines were $2{\cdot}73 \times 10^{-2}$ ($r^2 = 0{\cdot}990$) and $1{\cdot}10 \times 10^{-2}$ min^{-1} ($r^2 = 0{\cdot}985$) of native and conjugated enzyme, respectively. The data showed that conjugation of α-chymotrypsin with NIPAAm oligomer provides the enzyme with better thermal stability, possibly as a result of reduction of enzyme mobility.[17] Reduced mobility of the precipitated conjugate above the LCST may also protect the conjugated enzyme against thermal inactivation. Collapse of NIPAAm oligomer above its LCST apparently did not affect the conformation of the enzyme.

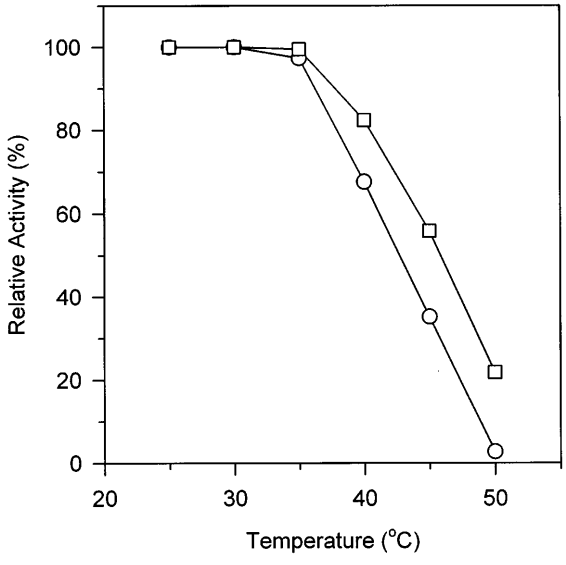

Fig. 1. Effects of incubation at various temperatures in pH 7·8 buffer for 15 min on the retained activity of native and conjugated α-chymotrypsin. The retained activity of the enzymes incubated at 25°C was taken as 100%. The activity was measured at 25°C with Suc-Phe-4-NA. (○) Native, (□) conjugated.

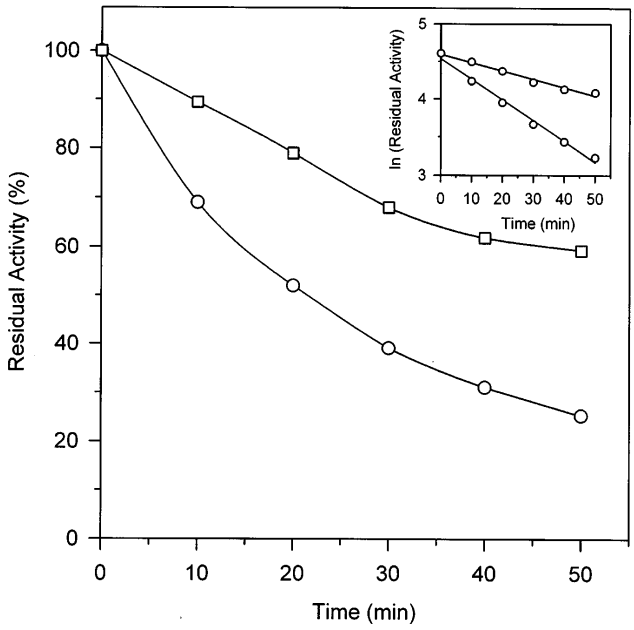

Fig. 2. Effects of incubation time at 40°C (pH 7·8) on the retained activity of native and conjugated α-chymotrypsin. The original enzyme activity without incubation was taken as 100%. The activity was measured at 25°C with Suc-Phe-4-NA. (○) Native, (□) conjugated.

Kinetic constants were determined from the Michaelis–Menten equation using Lineweaver–Burk plots at three temperatures (Table 2). K_m values increased after enzyme immobilization while the k_{cat} values changed only slightly. Conjugation of α-chymotrypsin to the oligomer may cause enzyme conformational changes that affected the affinity between enzyme and substrate. The increase in K_m indicates that the conjugated enzyme had somewhat lower affinity for the substrate than the native enzyme. Below its LCST, polymer chains of PNIPAAm hydrate to form an expanded hydrophilic structure in water.[2] The decreased affinity for the hydrophobic substrate (Suc-Phe-4-NA) suggests that conjugation with the oligomer resulted in reduced hydrophobicity of the protein surface. Using the temperature dependence of k_{cat} values, the activation energies calculated from the Arrhenius equation are 15·2 ($r^2 = 0{\cdot}993$) and 16·1 ($r^2 = 0{\cdot}991$) kcal mol^{-1} for the native and conjugated enzyme, respectively. The similarity in activation energy indicates that diffusion limitation was not important after enzyme immobilization.

The effect of temperature cycling between 4 and 40°C, which crossed the LCST of the oligomer, is shown in the insert of Fig. 3. The enzyme activity decreased to 81·5% of its original value after nine cycles. The cumulative effects of hydration and dehydration on enzyme denaturation at 40°C in each 5 min cycle (Fig. 2) apparently did not induce structured change of the conjugated enzyme. After eight successive precipitation cycles

TABLE 2
Kinetic Constants for Hydrolysis of Suc-Phe-4-NA by Native and Conjugated α-Chymotrypsin at Different Temperatures

Kinetic constant	*Native*			*Conjugated*		
	20°C	*25°C*	*30°C*	*20°C*	*25°C*	*30°C*
K_m (mmol dm^{-3})	3·89	4·60	4·78	6·55	9·29	10·1
V_{max} (μmmol dm^{-3} min^{-1})	6·07	9·99	14·3	8·27	13·4	18·6
k_{cat} (min^{-1})	0·73	1·20	1·72	0·77	1·24	1·73
r^2	0·9991	0·9996	0·9975	0·9996	0·9995	0·9998

r^2 = correlation coefficient.
Reaction conditions: 0·2 mg cm^{-3} native enzyme or 0·184 mg cm^{-3} conjugated enzyme at pH 7·8.

(Fig. 3), the immobilized enzyme retained 93·5% of its original activity. Since chymotrypsin is relatively thermally stable at 30°C, enzyme activity loss most probably resulted from the incomplete precipitation and recovery of the conjugate in each cycle.

3.4 Hydrolysis of macromolecular substrates

When α-chymotrypsin was immobilized on an insoluble carrier, its hydrolysis activity toward high molecular weight substrates such as casein and haemoglobin decreased significantly. Thus, immobilized α-chymotrypsin showed only 7% relative specific activity when immobilized to DEAE-cellulose,[18] 22·5% retention of specific activity with polyacroein microsphere as the support,[19] and a maximum relative specific activity of 18·8% with PEG-grafted hydroxyethyl methacrylate particles as the support.[20] In contrast, the relative specific activities of the conjugated enzyme prepared here were 77·5% and 72·9% for haemoglobin and casein (2% (w/v)), respectively. Although casein forms micelles in solution in the size of 50–300 nm, diffusion apparently did not restrict its interaction with the conjugated enzyme. The kinetics constants for hydrolysis of casein are obtained from the Lineweaver–Burk plots. An upward curve was obtained for casein concentration greater than 1% (w/v), indicating substrate inhibition. The K_m values calculated from the lower substrate concentrations are 0·509% and 0·202% (w/v) for native and conjugated enzymes, respectively, indicating higher affinity of the conjugated enzyme toward casein. This change in K_m value after enzyme conjugation is opposite to that when Suc-Phe-4-NA was used as the substrate (see Table 2), and may result from hydrophilicity of the casein micelle surface.

The progress of repeated batch hydrolysis of 2% (w/v) casein is shown in Fig. 4. Intermittent thermal pre-

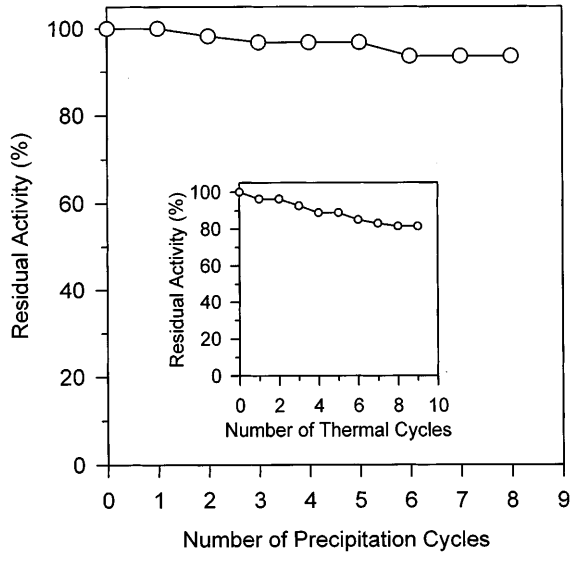

Fig. 3. Retained activity of conjugated α-chymotrypsin as a function of successive thermal and precipitation cycles. The activity before the cycling was taken as 100%. The activity was measured at 25°C with Suc-Phe-4-NA.

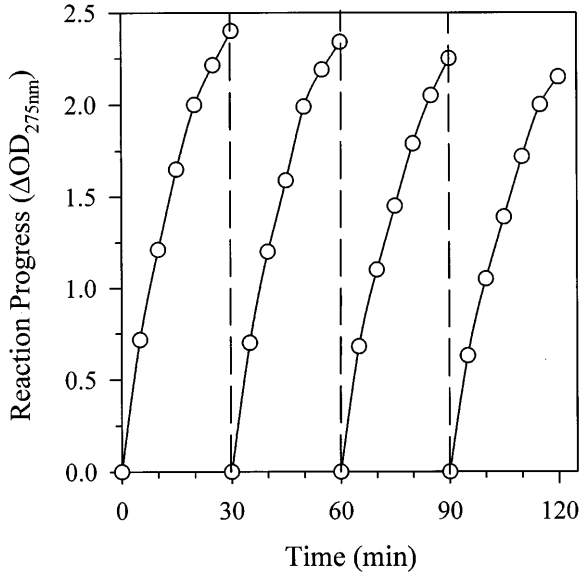

Fig. 4. Repeated batch hydrolysis of 2% (w/v) casein with conjugated α-chymotrypsin at 30°C in 50 cm^3 pH 7·0 buffer. The conjugated enzyme was precipitated by adding 5 cm^3 saturated ammonium sulphate at 40°C at 30 min intervals. The precipitate was collected by centrifugation and redissolved in 50 cm^3 pH 7·0 buffer containing 2% (w/v) casein to start the next batch of reaction.

cipitation between cycles efficiently recycled the conjugate and product formation rate decreased gradually in each successive cycle. The hydrolysis rate in the fourth cycle dropped to 87.5% of its initial value. It could be caused by loss of enzyme with incomplete precipitation or by enzyme denaturation. Also, adsorption of enzyme to residual casein micelles that was removed after each reaction may also result in enzyme being lost.

4 CONCLUSIONS

Conjugation of α-chymotrypsin to a reversibly soluble–insoluble temperature-sensitive NIPAAm oligomer by single point attachment could result in high specific activity of the conjugated enzyme, possibly exceeding that of the native enzyme. The conjugated enzyme had increased thermal stability and could undergo repeated precipitation–solution cycles with little loss of specific activity. For hydrolysis of macromolecular substrates such as casein and haemoglobin the retained activity of the conjugated enzyme was up to four times that of conventional immobilized enzymes using insoluble carriers. Repeated batch hydrolysis of casein was achieved by successive precipitation–dissolution cycles with conjugated α-chymotrypsin.

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