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Improvement in the behavior of bromelain coupled to pNIPAm polymers containing acrylamide or acrylic acid

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Abstract

Bromelain was coupled to N-isopropyl acrylamide (NIPAm) polymers, synthesized using NIPAm and various concentrations of acrylamide (Ac) or acrylic acid (AAc). Incorporation of Ac/AAc into the polymer increased the LCST (lower critical solution temperature) in a concentration dependent manner but AAc was more effective in this regard; the LCST rose to 40°C when 6 percent AAc was used. Incorporation of Ac/AAc increased the coupling of enzyme to the polymer and the η (effectiveness factor) of the coupled enzyme, moderately. Various studies indicate that such incorporation of hydrophilic monomers into the polymer does not impair its capacity to couple enzyme or expression of the activity of bound enzyme but seems to actually improve the stability of the enzyme against heat induced inactivation and alkaline pH.

Keywords: Bromelain, pNIPAm, Ac, AAc, LCST.

Introduction

Conjugates of enzymes with stimuli responsive polymers have received remarkable attention in recent years in view of the advantages they offer over enzymes immobilized on solid supports (Sheldon 2007). These polymers are responsive towards different stimuli like pH, temperature, electric field etc and various new trends are also being exploited (Roy et al. 2009). Numerous strategies have been developed for the conjugation of different bio molecules on these smart polymers and the resulting conjugates have a lot of applications in biotechnology, medicine and bio engineering fields (Jung and Theato 2013; Kumar et al. 2007; Hoffman and Stayton 2007). The enzyme polymer conjugates retain high catalytic efficiency comparable with that of free enzymes in solution but can readily be separated from the reaction mixture by inducing phase separation (Ding et al. 1998; Park and Hoffman 1990; Roy et al. 2004). Polymers of NIPAm are the most commonly used and have the sharpest phase transition among the class of thermosensitive N-alkyl acrylamide polymers. They exhibit a thermally reversible soluble-insoluble alteration in response to temperature changes across a lower critical solution temperature(LCST) in aqueous solution (Kanazawa et al. 2002; Aguilar et al. 2007). LCST is the temperature below which the polymer hydrates to form a soluble expanded structure and above which it collapses to compact structure by dehydration. This unique reversible hydration-dehydration change occurs extremely rapidly (Matsukata et al. 1996). Studies have shown that several enzymes including trypsin (Ding et al. 1998) lipase (Matsukata et al. 1994) and cellulase (Taniguchi et al. 1992) coupled to pNIPAm exhibit high stability against denaturants than to their respective soluble forms. Several enzymes conjugated to these smart polymers are being successfully used in various industries and biomedical fields (Roy et al. 2004; Podual et al. 2000). We have recently shown that bromelain coupled to pNIPAm was remarkably resistant against autolysis and acted effectively on protein substrates, both below and above LCST (Mahmood and Saleemuddin 2007).

The properties of the NIPAm polymers can be controlled to some extent by the selection of the monomers for co-polymerization. LCST of pNIPAm can be effectively controlled by co-polymerizing NIPAm with appropriate monomers (Uludag et al. 2001). LCST of pNIPAm can be increased or even made to disappear by the incorporation of monomers with charged groups while hydrophobic monomers lower it (Feil et al. 1993; Yoshida et al. 1994). Conjugates of enzyme with high LCST have the advantage of carrying out catalytic transformations at higher temperatures in soluble state. In an attempt to increase the LCST we have prepared polymer of NIPAm for enzyme conjugation by incorporating varying concentrations of neutral acrylamide (Ac) or negatively charged acrylic acid (AAc) molecules. It was observed that incorporation of the hydrophilic monomers enhanced the stability and accessibility of the enzyme for substrate.

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Materials and Methods

Chemicals

NIPAm, NAS (N-acrylosuccinimidyl ester) and stem bromelain (E.C. 3.4.22.32) were purchased from Sigma Chemical Company, St. Louis, USA. Other chemicals used were of analytical grade.

Coupling of bromelain to pNIPAm

The procedure of Zhu et al. (1994) was followed. 1340 U of bromelain was dissolved in 1.0 ml of 0.15 M NaCl containing 60 mM phospahate buffer, pH 8.0 (PBS) and vibrated with 3 ml of 0.1% NAS in a water bath at 37°C for 60 minutes in order to form the monomer-enzyme conjugate. This mixture was dialyzed thrice against the above PBS at 4°C, concentrated to 2.0 ml and poured into 8.0 ml of 60 mM phosphate buffer, pH 7.4. To this solution was added 300 mg of NIPAm and the polymerization was carried out with 400 µl of 1% APS and 20 µl of TEMED at 25°C for 2 hours. The solution was incubated at 37°C for 30 minutes, centrifuged for 10 minutes at 10,000 rpm to separate the insoluble pNIPAm-bromelain conjugate. The supernatant was analyzed for protein in order to calculate the amount of enzyme coupled to the polymer. The precipitate was again dissolved in 10 ml of phosphate buffer, pH 7.4 at 4°C and re-precipitated by incubation at 40°C. The procedure was repeated thrice to remove any adsorbed but uncoupled enzyme. Finally, the pNIPAm-bromelain conjugate was dissolved in 5.0 ml of 0.2M phosphate buffer, pH 7.0.

Coupling of bromelain to pNIPAm- acrylamide/acrylic acid

3 aliquots of Bromelain-NAS conjugate, prepared as described above, were mixed with 300 mg of NIPAm containing 2%, 4% or 6% (mol) of Ac/AAc. Polymerization and further proceedings were carried out as mentioned above.

Determination of enzyme activity and protein concentration

Proteolytic activity of bromelain was determined using casein as a substrate (Murachi et al. 1964). The standard incubation mixture contained enzyme in 0.25 ml of 0.2 M phosphate buffer, pH 7.0, 0.25 ml of 5 mM cysteine-HCl, 0.5 ml of 1% (w/v) casein, in a total volume of 1.0 ml. After incubation for 15 minutes, the reaction was stopped by the addition of 1.0 ml of 15%TCA. TCA soluble peptides were quantified by the procedure of Lowry et al. (1951). One unit of enzyme is the amount that brings about a change of 0.01 O.D. at 700 nm per minute. Values given in the tables and figures are the average of at least three independent experiments with variations not exceeding 5.0%. For kinetic studies, another substrate Na- Carboxybenzoxy-L-Lysine p- Nitrophenyl Ester (LNPE) was used. In the total reaction mixture there was 3.0 ml of 20 mM acetate buffer, pH 4.6 (100 mM potassium chloride, 1.0 mM cysteine-HCl) and 0.05 ml of 1.25 mM LNPE in 90% (v/v) acetonitrile. It was mixed and absorbance was measured at 326 nm after 5 minutes (Bajkowski and Frankfater 1975). Concentration of the protein was determined by the method of Lowry et al. (1951).

Determination of LCST

For the determination of LCST, samples of all the preparations were taken and incubated separately at different temperatures for at least 10 minutes in a thermostatic water-bath. O.D. at 500 nm was taken and transition temperature (LCST) was determined by plotting optical density against temperature, which fitted to a sigmoidal curve (Uludag et al. 2001; Chen and Hoffman 1992). All the determinations were done in triplicate and there was excellent agreement in the values.

Results and Discussions

Coupling of bromelain to pNIPAm containing acrylamide/acrylic acid

The procedure described by Zhu et al. (1994) was followed for the preparation of pNIPAm-bromelain conjugates, except that the preparations contained varying concentrations (2-6 mole percent) of acrylamide (Ac) or acrylic acid (AAc). As observed in the case of pNIPAm (Mahmood and Saleemuddin 2007), all the preparations containing Ac or AAc were also of large molecular weight as they excluded from Sepharose 4B column. Increase in the concentration of either Ac or AAc in the pNIPAm did not alter the quantity of bromelain coupled to the support significantly; although conjugation yield of the enzyme on the polymer containing lower concentrations of Ac or AAc appeared slightly higher (Table I). Similarly the additional incorporation of either Ac or AAc altered the η values, the ratio of actual to theoretical activity of the immobilized enzyme preparations (Jafri and Saleemuddin 1997), only marginally. The η value increased slightly with increase in the concentration of Ac or AAc. The incorporated Ac/AAc was expected to increase the hydrophilicity of the polymer and hence that of the microenvironment of the enzyme. The η was somewhat higher for AAc incorporated polymer preparations. Repulsion between the ionized carboxyl groups of AAc expands the polymer chains thus making the coupled enzyme more accessible for the action on substrate (Bulmus et al. 2000).

Table 1: Immobilization of bromelain on co-polymers of NIPAm and Ac/AAc

Additional monomer (mol %)	Enzyme activity (U/g)		Effectiveness factor (η) BA^{-1}
	Theoretical A	Actual B	
none	1733	381	0.22
2 Ac	1767	406	0.23
4 Ac	1738	435	0.25
6 Ac	1667	467	0.28
2 AAc	1800	414	0.23
4 AAc	1756	457	0.26
6 AAc	1700	527	0.31

*Each value is the average of at least three independent experiments with variation not exceeding 4.0 %.

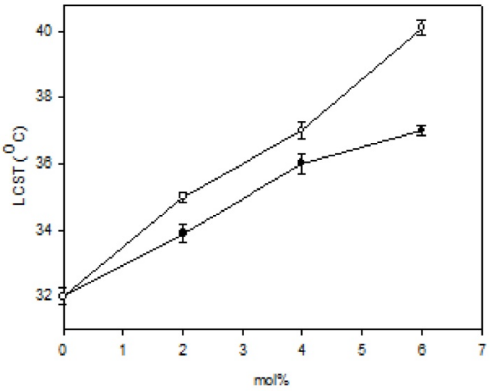

Figure 1: Effect of incorporation of Ac (●) and AAc (○) in pNIPAm on the LCST of the polymer. **Each value is the average of at least three independent experiments and S.D. shown with error bars.*

Effect of incorporated Ac/AAc on the LCST of the polymer

The LCST of pNIPAm and of that with conjugated enzyme remained at 32°C. Incorporation of either Ac or AAc resulted in a concentration dependent increase in the LCST values (Fig.1). This is in agreement with the earlier observation that incorporation of hydrophilic monomers increases the LCST of pNIPAm (Feil et al. 1993). The increase was more marked in case of the AAc incorporated polymers as compared to those containing Ac and the LCST was maximum for pNIPAm with 6% AAc. AAc is likely to contribute more towards the hydrophilicity of the copolymer than Ac because of the ionizable carboxyl groups; and repulsion between the groups may cause increase in LCST (Arasaratnam 2000).

Effect of pH

The effect of pH on the activity of pNIPAm coupled bromelain preparations was studied both at 28°C and 42°C (Fig. 2 A and 2 B). All the polymer preparations were soluble at 28°C but transformed into the insoluble form at 42°C. All polymer coupled preparations exhibited optimum activity at pH 10.0, both at 28°C and 42°C. The activity profiles of bromelain coupled to the polymer at 42°C were somewhat sharper than those coupled at 28°C. Arasaratnam et al. (2000) have shown that trypsin conjugated to the polymer is more accessible to the inhibitor in its soluble form than when the conjugate was in insoluble form. The pH-activity profiles

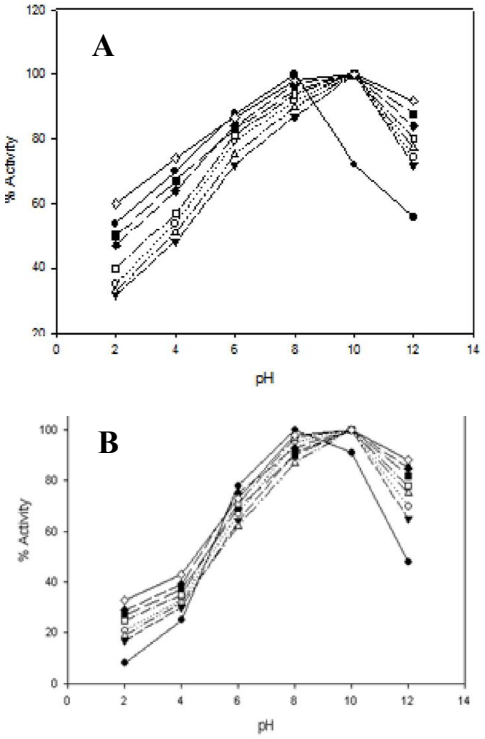

Figure 2: pH–activity profiles of native and pNIPAm coupled enzyme preparations at 28°C (A) or at 42°C (B).
(●) Native bromelain, (○) bromelain coupled to pNIPAm, (▼) bromelain coupled to pNIPAm containing 2.0% Ac, (Δ) 4.0% Ac, (■) 6.0% Ac, (□) 2.0% AAc, (♦) 4.0% AAc and (◊) 6.0% AAc were incubated with casein at various pH values and activity determined as described in the text.
**Each value is the average of at least three independent experiments with variation not exceeding 5 %.*

of all the polymer coupled enzyme preparations were broader than that of the native enzyme. The broadening of the activity profiles, increased with the increase in the concentration of Ac or AAc incorporated and was maximum for the preparation with 6% AAc. This may be related to the resistance of the polymer linked enzyme to autolysis or conformational alterations occurring at the pH values far removed from the optimum pH.
The shift in pH optimum towards alkalinity can be attributed to the alterations in the microenvironment of the enzyme. It is well recognized that concentration of positively charged substrate will increase in the vicinity of support matrices that have multiple negative charges in proportion to the charge density. The enzyme will consequently experience a pH below that of the bulk phase. This will evidently lead to the shift in pH optimum towards alkalinity.

Effect of temperature

Temperature activity profiles of polymer coupled enzyme preparations showed that the retained activities increased with the increase in temperature across the LCST (Fig. 3). There was no effect of thermal transition on the activity of polymer bound preparations (*Mahmood and Saleemuddin 2007; Chen and Hsu 1997*). Native bromelain exhibits optimum activity at 60°C, while all the pNIPAm linked preparations showed a shift in temperature optimum to 70°C. The preparations also retained greater fractions of maximum activity at higher temperatures. Preparation with 6% AAc at 40°C (where it is in soluble form) retained 65% activity, which is higher than that of other preparations and is almost comparable to that of free enzyme, which retained 68% of maximum activity. The

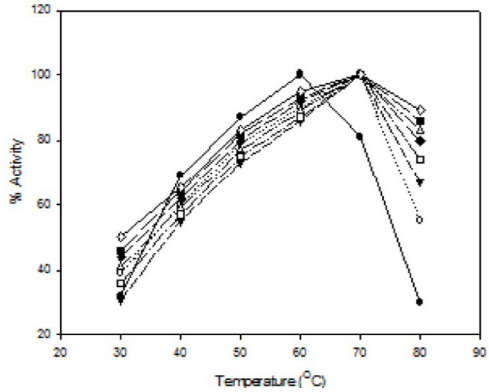

Figure 3: Effect of temperature on the activity of native and pNIPAm coupled bromelain preparations.
(●) Native bromelain, (○) bromelain coupled to pNIPAm, (▼) bromelain coupled to pNIPAm containing 2.0% Ac, (Δ) 4.0% Ac, (■) 6.0% Ac, (□) 2.0% AAc, (♦) 4.0% AAc and (◊) 6.0% AAc were incubated with casein at the indicated temperatures and caseinolytic activity was determined.
**Each value is the average of at least three independent experiments with variation not exceeding 4 %.*

preparation was also most stable with retention of nearly 80% of maximum activity at 80°C. The remarkable increase in the stability of bromelain preparations coupled to the polymer is also substantiated from the data shown in figure 4. All the pNIPAm linked preparations were more stable and retained higher fractions of activity than the native enzyme incubated at 60°C for up to 3 hours. The remarkable increase in the stability of polymer coupled enzyme preparations may be related to the multipoint attachment of the enzyme on the polymer and restriction in the mobility of enzyme due to the precipitated polymer at temperature above LCST (Ulbrich et al. 1986; Chen and Hoffman *1993*). The resistance to inactivation appears to increase slightly but consistently with the increasing concentration of Ac/AAc. pNIPAm preparation without incorporated Ac /AAc retained 45% of initial activity

while preparation with 6% AAc retained 83% activity at 60°C after 180 minutes of incubation.

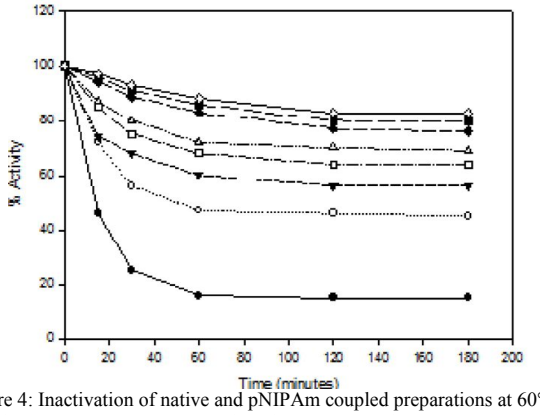

Figure 4: Inactivation of native and pNIPAm coupled preparations at 60°C. *All the preparations were incubated at various durations at 60°C and assayed under the standard conditions. The preparations used included (●) native bromelain, (○) bromelain coupled to pNIPAm, (▼) bromelain coupled to pNIPAm containing 2.0% Ac, (Δ) 4.0% Ac, (■) 6.0% Ac, (□) 2.0% AAc, (♦) 4.0% AAc and (◊) 6.0% AAc.*
**Each value is the average of at least three independent experiments with variation not exceeding 5 %.*

K_m values

K_m was determined for the polymer coupled preparations using LNPE as a substrate and the reaction was carried out at 25°C (Bajkowski and Frankfater 1975). The Lineweaver-Burk plot showed K_m for the native enzyme to be 2.86 mM which decreased to 1.54 mM on coupling of the enzyme to pNIPAm. This shows good access of the enzyme to the substrate, which was anticipated because the immobilized enzyme preparation was in soluble state at this temperature. The increase in K_m of the coupled enzyme for the substrate is difficult to explain and related to a conformational change of the enzyme. Alternatively, favorable partitioning of the substrate near the active site may result from the micro-environmental effect (Chen and Hoffman 1993). The decrease in K_m for the bromelain coupled to AAc containing polymers may be attributed to the attraction between the oppositely charged polymer matrix and the substrate. Lysine is a constituent of LNPE, used as a substrate in the determination of K_m values. The pK of ε-amino group of lysine is 10.2 and the group is expected to have positive charge at pH 4.6 used in the assay (Bajkowski and Frankfater 1975). The opposite charges of matrix and substrate contribute towards the apparent decrease in K_m. K_m values further decreased with the increasing concentration of incorporated Ac and AAc in the polymer and the decrease was more significant for the enzyme coupled to the polymer containing AAc (Table II). Increased hydrophilicity of the polymer may also facilitate the partitioning of substrate in the enzyme microenvironment which is also hydrophilic in nature.

Table 2: Determination of K_m values of native bromelain and of that coupled to pNIPAm containing Ac/AAc

Bromelain preparations	**K_m (mM)**
Native	2.86
Coupled to pNIPAm	1.54
Coupled to pNIPAm containing 2.0% Ac	1.05
Coupled to pNIPAm containing 4.0% Ac	0.95
Coupled to pNIPAm containing 6.0% Ac	0.83
Coupled to pNIPAm containing 2.0% AAc	0.67
Coupled to pNIPAm containing 4.0% AAc	0.59
Coupled to pNIPAm containing 6.0% AAc	0.54

**Each value is the average of at least three independent experiments with variation not exceeding 5 %.*

Digestion of hemoglobin

The activity of pNIPAm coupled bromelain preparations to digest denatured hemoglobin was investigated in order to examine possible alterations in proteolytic action of the enzyme. Usually enzyme immobilized on insoluble carriers show low activity towards macromolecular substrates due to low diffusion rate of the substrate or steric hindrance from the support but enzyme coupled to pNIPAm have shown better accessibility to these substrates like hemoglobin and casein (Chen and Hsu 1997). Digestion of hemoglobin observed in case of the polymer coupled preparations, as evident from the disappearance of the hemoglobin band, was fast, increasing significantly with increasing Ac/AAc content (Fig. 5).

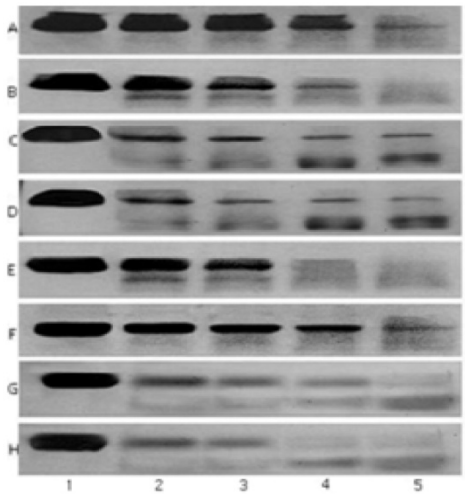

Figure 5: Degradation of hemoglobin Native bromelain (A) and that coupled to pNIPAm (B), co-polymers with 2.0% Ac (C), 4.0% Ac (D), 6.0% Ac (E), 2.0% AAc (F), 4.0% AAc (G) and 6.0% AAc (H). The enzyme preparations were incubated with hemoglobin as described in the text and subjected to SDS-PAGE. Lanes 1,2,3,4 and 5 contain samples incubated for 0, 2, 4, 8, and 12 hours respectively.

Hemoglobin was completely digested on incubation for 12 hours with the free enzyme. Some small molecular weight peptides generated during digestion, however, remained resistant to further digestion. This behavior was more marked in case of the bromelain preparations coupled to polymers containing higher concentrations of Ac/AAc. It is likely that the digestion resistant peptides may be relatively more hydrophobic or negatively charged and hence less accessible to action by the enzyme coupled to pNIPAm with higher concentrations of Ac/AAc.

Conclusion

These studies suggest that pNIPAm-enzyme conjugates with high LCST could be obtained by co-polymerization of NIPAm with hydrophilic Ac/AAc. The NIPAm polymers containing Ac/AAc were comparable with those prepared without the hydrophilic monomers. In fact, incorporation of Ac/AAc resulted in a moderate increase in the η; and decrease in the K_m value of the coupled bromelain, indicating improved accessibility of the coupled enzyme. Since the polymers containing Ac/AAc showed enhanced stability against temperature, induced inactivation and alkaline pH, they should be useful for protein digestion, also considering that protein substrates may be more susceptible at higher temperatures.

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