Results & Discussions
3.0 Results and Discussions

3.1 Immobilization of invertase on pNIPAm and con A coupled pNIPAm

Bioaffinity based procedures that usually result in favourably oriented immobilization of enzymes, have attracted remarkable attention for bioanalytical and other applications (Mattiasson, 1988; Gupta and Mattiasson, 1992). The favourable orientation of enzymes on the support offers several advantages as compared to the randomly oriented enzymes (Khare and Gupta, 1988). A number of studies have described a striking increase in the efficiency and stability of enzyme as a result of favourable uniform orientation (Saleemuddin and Hussain, 1991; Turkova, 1999; Liu et al., 2001). One of the affinity supports that had been exploited for bioaffinity-based immobilization of enzymes is the lectin, concanavalin A (con A). It has largely been used for the immobilization of glycoenzymes on a variety of supports (Saleemuddin and Hussain, 1991; Saleemuddin, 1999). In this study, invertase, an industrially useful glycoenzyme, was used as model and immobilized on pNIPAm by covalent coupling as well as affinity binding via con A.

A polymer for coupling of invertase was prepared by the polymerization of NIPAm containing 4% GMA. GMA provides epoxy groups in the polymer for covalent coupling of the proteins via amino groups (Hoshino et al., 1997). The polymer synthesized, thus exhibited a LCST of 32°C (Fig. 10). Preparations of immobilized invertase were obtained either by covalent coupling via amino groups to the pNIPAm or by the affinity binding of the enzyme to the polymer precoupled with con A. Con A was also linked to the polymer via amino groups using the procedure already described. Invertase, being a glycoenzyme, readily binds to the polymer precoupled with con A. Under the conditions used, 4.2 mg of con A was coupled per gram of pNIPAm. The LCST of pNIPAm remained unaffected as a result of coupling of invertase or con A.
Figure 10  Thermal transition of the NIPAm-GMA polymer

1% (w/v) of NIPAm-GMA polymer in 0.1 M phosphate buffer, pH 7.0 was taken and incubated separately at different temperatures for at least 10 min. O.D. at 500 nm was taken and LCST (T_m) was determined. All the determinations were done in triplicate with less than 2% of variation.
As compared to the amount of invertase that could be covalently linked to the pNIPAm, more units of enzyme were bound when con A-pNIPAm was incubated with the same amount of enzyme (Table 4). As shown in table 4, 71,250 units of invertase were covalently coupled to one gram of the polymer, while 1,78,125 units of invertase were bound to the same amount of polymer precoupled with con A. Also the fraction of bound catalytic activity expressed (η) of invertase was higher when the enzyme was bound on the con A support. The high η values suggest that the affinity-bound invertase preparation was more accessible for action on substrate than the enzyme covalently coupled to the polymer. The higher η value may result due to the high molecular weight con A, acting as spacer and facilitating favourable spatial orientation of the enzyme molecules. It is well known that spacers improve the accessibility of immobilized enzymes. Introduction of a spacer on the polymer matrix has been found to increase the expressed activity of trypsin (Nouimi et al., 2001). It is also likely that in the covalently linked invertase preparation, enzyme was coupled to the polymer by multiple linkages, resulting in unfavorable conformational changes in the enzyme molecules and in turn decreasing the catalytic activity. Also lack of spacer and attachment by multiple linkages may restrict access of active site by steric hindrance of polymer chains.

In order to purify the polymer with immobilized enzyme from the unbound polymer, the preparations were passed through a Sepharose 4B column (1.7cm×75cm, Vο= 46 ml) at 25°C (Fig. 11). At this temperature, which is significantly below the LCST, the free and enzyme linked polymers are completely soluble in buffer. The elution profile of the polymer measured at 226 nm, showed a single broad peak, while that of the polymer preparation with affinity-bound enzyme showed two peaks; one corresponding to the uncoupled polymer and the other in the void volume. This suggested that the enzyme bound polymer preparations had a very high molecular weight and is excluded from the Sepharose 4B column. Enzyme activity also co-eluted with the polymer in the void volume.
Table 4 Immobilization of invertase on pNIPAm and con A coupled pNIPAm

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Units added/g</th>
<th>Enzyme activity (U/g)</th>
<th>Effectiveness factor $\eta$ BA$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Theoretical A</td>
<td>Actual B</td>
</tr>
<tr>
<td>Covalently</td>
<td>2,50,000</td>
<td>71,250</td>
<td>14,965</td>
</tr>
<tr>
<td>coupled</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Affinity bound</td>
<td>2,50,000</td>
<td>1,78,125</td>
<td>58,780</td>
</tr>
</tbody>
</table>

Each value represents the mean of at least three independent experiments with variation not exceeding 3.0 %.
A column of 1.7 cm x 75 cm was used. Free pNIPAm (A) or that coupled to con A and bound with invertase (B) were passed through the column. The fractions were screened for the presence of enzyme activity (C). Con A (D) and invertase (E) were also passed separately through the column. The fractions were analysed for the polymer at 226 nm, protein content at 280 nm and invertase activity at 540 nm.
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The polymer with covalently coupled enzyme also showed comparable elution profile with two peaks corresponding to coupled and uncoupled polymer. These samples were collected and pooled for further investigations.

Figure 12 shows that the absorption spectra, of free pNIPAm and that with affinity bound invertase exhibit a peak at 226 nm. The latter however showed higher absorbance between 240 nm and 290 nm, apparently due to the coupled invertase. It is well known that most proteins absorb in the UV region with a maximum around 280 nm.

3.1.1 Behaviour of invertase immobilized on pNIPAm

3.1.1.1 Effect of Temperature

The pNIPAm preparations with invertase, either covalently coupled or affinity-bound via con A, exhibited temperature optima at 55°C, like the native enzyme (Fig. 13). The temperature-activity profiles of the immobilized enzyme preparations were also comparable with that of the native enzyme. There was also no significant effect of the thermal transition of the polymer, occurring at 32°C on the catalytic activity of the polymer bound enzyme. Above LCST, the reaction catalysed by the polymer coupled enzyme occurs, biphasingly and the collapse of the polymer chains is expected to change the microenvironment of the active site or cause the flocculation of the enzyme, both leading to the drop in enzyme activity (Chen and Hsu, 1997). However, no such effect of thermal transition on the enzyme activity was observed in this case. A possible explanation is the favourable partitioning of the substrate near the active site as the polymer turns insoluble. Such partition may compensate the activity loss due to the collapsed polymer chains. Alternatively, at low polymer concentrations there may be little intermolecular aggregates formation and hence no effect on enzyme activity. It was seen that enzyme activity of concentrated pNIPAm-lipase solution was dependent on LCST (Matsukata et al., 1994), while at lower concentrations, similar conjugate of trypsin was not LCST dependent (Matsukata et al., 1996).
Figure 12  Absorption spectra of free pNIPAm and invertase coupled to pNIPAm

1.0% (w/v) of free pNIPAm and invertase coupled pNIPAm in 0.1 M phosphate buffer, pH 7.0 were scanned at 25°C. (*) pNIPAm; (■) invertase bound on pNIPAm precoupled to con A.
Other studies on enzymes coupled to smart polymers suggest that the dependency of the enzyme activity on LCST is related to the sensitivity of the enzymes toward hydrophobic environment (Valuev et al., 1994). Lee and Park (1997) postulated that the lack of decrease in accessibility of the substrate to active site of the enzyme may result either by incomplete coverage of the active site by the collapsed pNIPAm chains or due to the conjugation of the polymer to regions of the enzyme far removed from the active site. As evident from the figure 13, the immobilized enzyme preparations showed greater fractions of maximum activity at higher temperatures than the native enzyme. This suggests that the precipitated form of the polymer provided stabilization against heat to the enzyme, presumably by changing its microenvironment while providing good access to the substrate. The broadening of the temperature-activity profiles was more marked in the case of affinity-bound enzyme.

Thermal inactivation of the native and immobilized enzyme preparations at 60°C (Fig. 14) also suggested a marked increase in stability of the latter. After incubation for three hours at 60°C only 23% activity remained of the native enzyme, while 68% and 74% of activities were retained by the covalently coupled and affinity-bound enzyme preparations, respectively. A number of glycoenzymes which have been immobilized on supports via the glycosyl chains exhibit markedly higher stability than those linked via side chain amino groups (Hsiao and Royer, 1979; Woodward and Zachry, 1982; Iqbal and Saleemuddin, 1983; Husain et al., 1992). The impressive increase in thermal stability of some glycoenzymes immobilized through carbohydrate moiety, especially on con A supports (Saleemuddin and Hussain, 1991; Vrabel et al., 1997) could also be explained by the observation that regions close to the glycosylation sites might be important in the unfolding of several glycoenzymes (Hofman et al., 1993; Younus et al., 2001). According to the authors, enzymes have labile regions where unfolding begins and modification/binding of these regions may restrict unfolding of the molecule. Labile regions have shown to be present in a number of enzymes.
Figure 13  Effect of temperature on native invertase and pNIPAm immobilized invertase preparations

Temperature activity profiles of native (○), covalently coupled (■) and affinity bound invertase (▲) were determined by performing enzyme assays at indicated temperatures under standard conditions. Each value represents the mean of three different experiments carried out in duplicate.

Figure 14  Thermal inactivation of native invertase and pNIPAm immobilized invertase preparations

Native (○), covalently coupled (■) and affinity bound invertase (▲) were incubated for various durations at 60°C, cooled at 0°C and assayed under the standard conditions. Each value is the average of at least three independent experiments carried out in duplicate.
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**Graph 1:**
- **Y-axis:** % Maximum activity
- **X-axis:** Temperature (°C)

**Graph 2:**
- **Y-axis:** % Maximum activity
- **X-axis:** Time (min)
(Hofman et al., 1993). Binding of con A linked pNIPAm to the glycosyl chains may therefore restrict unfolding of the enzyme.

3.1.1.2 Effect of pH
Activity profiles of native and pNIPAm linked invertase preparations were investigated in buffers of various pH at 25°C (Fig. 15). The optimum pH for the native as well as immobilized invertase preparations was 5.0. The fractions of activity retained by the immobilized preparations were higher both at low and high pH as compared to those of the native enzyme. The covalently coupled invertase preparation showed relatively lower loss of activity than that of the affinity-bound enzyme at acidic pH while in the alkaline pH range there was little difference in the activity profiles of the immobilized preparations. Since the covalent coupling of invertase to pNIPAm may involve multipoint attachment to the polymer resulting in more rigid conformation, it may unfold less readily at the extremes of pH.

3.2 Immobilization of β galactosidase on con A coupled pNIPAm differing in molecular weights
To substantiate the usefulness of affinity immobilization of enzymes on pNIPAm precoupled with con A, additional studies were carried out on β-galactosidase which is also a glycoenzyme. In this study the affect of the chain length of the pNIPAm was investigated, on the behaviour of immobilized enzyme. The polymer when passed through a Sepharose 4B column emerged with a broad elution profile, suggesting molecular weight heterogeneity of the preparation as observed earlier (Fig. 11). The polymer was further fractioned to high, medium and low molecular weight preparations. Fractions 18-26, 31-39 and 42-47 were collected, pooled, concentrated and again passed through Sepharose 4B column which emerged as three distinct peaks (Fig. 16). It may however be noted that molecular
Figure 15  Effect of pH on native invertase and pNIPAM immobilized invertase preparations

pH activity profiles of native (●), covalently coupled (■) and affinity bound invertase (▲) were obtained by incubating the preparations in buffers at various pH; 0.2 M glycine–HCl (pH 2.0, 3.0), 0.2 M acetate (pH 4.0, 5.0) and 0.2 M phosphate (pH 6.0, 7.0) and assayed for enzyme activity under standard conditions. Each value represents the mean of three different experiments carried out in duplicate.
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weights of the polymer could not be determined by gel filtration since no suitable standards were available.

The high, medium and low molecular weight polymers exhibited only slightly different LCSTs (Fig. 17). The apparent marginally high LCST of the lower molecular weight polymer is difficult to explain since one would expect the LCST of the larger molecular weight polymer to be higher. The polymer of different molecular weights were coupled with con A and it was observed that the amount of con A coupled to the polymer increased with the molecular weight of the polymer, being maximum for the HMW polymer. Under the conditions used, 6.5 mg, 4.6 mg and 2.8 mg of con A were coupled to HMW, MMW and LMW polymers, respectively. The HMW polymer, because of the greater GMA content than its lower molecular weight counterpart, will have more reactive groups and hence may facilitate greater coupling. The con A coupled polymers were allowed to bind to β-galactosidase. Binding of the enzyme was, as anticipated indicated a dependence on the amount of con A coupled; maximum in the case of HMW polymer followed by medium and low molecular weight polymers (Table 5). Predictably, the activity of the bound enzyme was also higher for the preparation precoupled with greater amount of con A. The η values, of all the preparations were however comparable.

3.2.1 Properties of β-galactosidase immobilized on different molecular weight pNIPAm

3.2.1.1 Effect of temperature

The temperature-activity profiles of all pNIPAm coupled enzyme preparations, showed optimum temperature of 60°C, which was also the optimum temperature of the native enzyme (Fig. 18). Also, no effect of thermal transition on the activity of the enzyme bound to the polymers was observed. The pattern of the activity profiles of the polymer bound enzyme across the transition temperature were somewhat similar to that of the native enzyme as observed in the case of invertase
Figure 16  Elution profiles of different molecular weight pNIPAm

The pNIPAm synthesized as described in the text was passed through a Sepharose 4B column (1.7 cmX75 cm). Fraction No. 18-26, 31-39 and 42-47 were pooled and rechromatographed on the column at 25°C. The fractions were respectively named as (●) high molecular weight (HMW), (■) medium molecular weight (MMW) and (▲) low molecular weight (LMW) polymer.

Figure 17  Thermal transitions of the various molecular weight pNIPAm

1.0% (w/v) of all the polymers in 0.1 M phosphate buffer, pH 7.0 was pre-incubated at various temperatures for at least 10 min. prior to recording of O.D. at 500 nm of HMW (●), MMW (■) and LMW fractions (x). All the determinations were conducted in triplicate with less than 2.0% of variation.
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[Graph 1: Elution volume (ml) vs. $\epsilon_{226\text{nm}}$ (% maximum)]

[Graph 2: Temperature (°C) vs. $\epsilon_{600\text{nm}}$ (% maximum)]

$T_{m1}$, $T_{m2}$, $T_{m3}$
<table>
<thead>
<tr>
<th>pNIPAm type</th>
<th>ConA coupled (mg/g)</th>
<th>Enzyme added (U/g)</th>
<th>Bound enzyme activity (U/g)</th>
<th>Effectiveness factor ( \eta ) BA^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMW</td>
<td>6.5</td>
<td>3030</td>
<td>1869 598</td>
<td>0.32</td>
</tr>
<tr>
<td>MMW</td>
<td>4.6</td>
<td>3030</td>
<td>1515 545</td>
<td>0.36</td>
</tr>
<tr>
<td>LMW</td>
<td>2.8</td>
<td>3030</td>
<td>990 307</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Each value represents the mean of at least three independent experiments with variation not exceeding 4.0 %. 

Table 5  Immobilization of \( \beta \) galactosidase on pNIPAm differing in molecular weights
coupled preparations, discussed earlier. Chen and Hoffman (1994) also found no effect of thermal transition across LCST on the activity of β-galactosidase directly coupled to pNIPAm.

Thermal inactivation of the native as well as polymer bound enzyme preparations were investigated at 60°C (Fig. 19) and the latter were far more stable than the native enzyme. Reduction in mobility of the three dimensional structure of enzyme after immobilization on a support is expected to enhance the thermal stability of the enzyme (Ulbrich et al., 1986). A similar effect is shown by the polymer coupled enzyme above LCST where the precipitated polymer reduces the mobility of the enzyme and hence improves thermal stability (Chen and Hsu, 1997). After four hours of incubation at 60°C, the remaining activity of the enzyme were 20%, 70%, 75% and 61% for native, HMW, MMW and LMW polymer bound preparations, respectively. This suggested that the HMW and the MMW polymer bound enzyme preparations were slightly more stable. Apparently the molecular weight of the polymer did not affect the thermal stability of the affinity-bound preparations markedly.

3.2.1.2 Effect of pH

pH-activity profiles for all the polymer bound enzyme preparations were investigated both at 25°C and 35°C. It was expected that the behaviour of the polymer coupled enzyme will be different below and above LCST of the polymer due to the expansion and collapse of the polymer chains on the enzyme surface. At 25°C, the profiles were somewhat broader to those at 35°C (Fig. 20). The optimum pH of the native enzyme was 7.0 and remained unaltered after immobilization on the different molecular weight polymers. The polymer bound β-galactosidase showed greater fraction of activity both at alkaline and acidic pH than the native enzyme, especially at 25°C. This might be due to the insolubilization of the polymer causing lower accessibility of the enzyme to the substrate at 35°C. Effect of precipitation of the polymer on conjugated enzyme activity has been studied by
Figure 18  Effect of temperature on the activity of native β-galactosidase and those immobilized on pNIPAm differing in molecular weights

Temperature-activity profiles of native (♦), HMW (■), MMW (▲) and LMW pNIPAm bound β-galactosidase (■) were obtained by performing enzyme assay at indicated temperatures under standard conditions. Each value represents the mean of three different experiments carried out in duplicate.

Figure 19  Thermal inactivation of native β-galactosidase and those immobilized on pNIPAm differing in molecular weights

Native (♦), HMW (■), MMW (▲) and LMW pNIPAm bound β-galactosidase (■) were incubated for various durations at 60°C and assayed under the standard conditions. Each value is the average of at least three independent experiments carried out in duplicate.
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![Graph showing temperature vs. maximum activity and time vs. maximum activity.]

- Temperature (°C) vs. % Maximum activity
- Time (min) vs. % Maximum activity
Figure 20  Effect of pH on the activity of native β-galactosidase and those immobilized on pNIPAm differing in molecular weight

pH-activity profiles at 25°C (A) and 35°C (B) of native (•), HMW (■), MMW (▲) and LMW NIPAm-GMA polymer bound β galactosidase (×) were obtained by incubating the preparations in the buffers of various pH; 0.2 M acetate (pH 4.0, 5.0), 0.2 M phosphate (pH 6.0, 7.0, 8.0) and 0.2 M bicarbonate (pH 9.0, 10.0) and assayed for enzyme activity under standard conditions. Each value represents the mean of three different experiments carried out in duplicate.
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A

% Maximum activity

pH

B

% Maximum activity

pH
Hoshino *et al.* (1997) by comparing the specific activity of polymer conjugated thermolysin at temperatures where the polymer exists in soluble and insoluble forms. It was shown that fraction of activity of polymer-coupled thermolysin, was more at a temperature where the polymer was in the soluble form than that in the insoluble form. Small differences were apparent in the fraction of activity retained by enzyme bound to pNIPAm of various molecular weights at high temperature (Fig. 20).

### 3.2.1.3 Determination of Km

The activity of native as well as immobilized enzyme preparations was measured as a function of substrate concentration at 25°C (Fig. 21). Km for all the preparations determined from their Lineweaver-Burk plots using ONPG as a substrate, were 12.5 mM, 5.9 mM, 5.3 mM and 7.4 mM for native, HMW, MMW and LMW polymer bound enzyme, respectively. This shows that the affinity of the enzyme for substrate did not decrease but actually showed a small increase by immobilization on the polymers. The increase was maximum for the MMW polymer bound enzyme. Affinity binding of the enzyme may bring about a favourable conformational change in the active site of the enzyme and hence increase the substrate affinity. It is also possible that the hydrophilic groups of the polymer may contribute to an increase in the local substrate concentration around the enzyme bound to the polymer and hence decrease the Km. Other reports also show that Km values of enzyme may decrease as a result of immobilization (Chen and Hoffman, 1993).

### 3.3 Immobilization of bromelain on pNIPAm

The third enzyme investigated for behavioral studies of enzymes coupled to pNIPAm, was bromelain. Bromelain is a plant thiol protease with broad specificity. It has a lone oligosaccharide chain which does not participate in catalytic function (Murachi *et al.*, 1967; Scocca and Lee, 1969) and earlier studies
Figure 21  Lineweaver-Burk plot for ONPG hydrolysis by native and pNIPAm immobilized β-galactosidase

Reciprocal concentrations of velocity were plotted as a function of reciprocal concentrations of substrate for native β-galactosidase (●), or the enzyme immobilized on HMW (■), MMW (▲) and LMW (●) NIPAm-GMA polymer. The enzyme assay was performed using ONPG as a substrate. Each value represents the average of three experiments carried out in triplicate with variations not exceeding 4%.
from this laboratory have utilized this unique feature to uniformly and favourably orient the enzyme on solid surfaces (Gupta and Saleemuddin, 2006).

3.3.1 Covalent coupling of bromelain to pNIPAm

Alternative strategies were used to prepare pNIPAm conjugates of bromelain in which bromelain was coupled first to NAS, either via the aminogroups (randomly coupled) or the lone carbohydrate chain (uniformly coupled) and then copolymerized with NIPAm using the procedure described by Zhu et al. (1998). When the succinimide ester is used for protein conjugation, the preferred pH range for reaction is around 8.0-9.0 (Brinmley, 1992). At low pH the aminogroups are positively charged and have low reactivity towards NAS ester groups while at high pH the ester groups are susceptible to hydrolysis (Chen and Hsu, 1997). The coupling yield of bromelain to the polymer via amino groups was investigated in buffers of various pH and it was maximum at pH 8.0 (Fig. 22). The polymer prepared and used for enzyme immobilization in the study was apparently of very high molecular weight, as it was excluded from a Sepharose 4B column.

Among the two preparations obtained, more bromelain was linked to the polymer, when coupling involved reaction between the amino groups of the enzyme and the polymer (Table 6). Thus, while 2267 U of bromelain were coupled per gram of polymer in case of the randomly coupled preparation, uniform coupling via the glycosyls resulted in association of only 910 U. Higher immobilization yield, that resulted in case of the randomly coupled preparation is apparently related to the reaction between the terminal amino group and/or one/more of the 15 side chain amino groups available in the bromelain molecule (Vanhoof and Cooreman, 1997). Stem bromelain on the other hand contains a single oligosaccharide chain (Murachi et al, 1967) and hence a lone linkage between support and the enzyme can be anticipated when it is coupled after oxidation of the oligosaccharide chain to the amino group bearing polymer. Coupling of bromelain through the oligosaccharide is therefore expected to result
Figure 22  Effect of pH on the coupling of bromelain to pNIPAm

17 mg of bromelain was modified with 3 ml of 0.1% NAS in buffers of various pH; 60 mM PBS, pH 6.0, 7.0, 8.0, bicarbonate buffer saline pH 9.0, 10.0 and further polymerized with 150 mg of NIPAm as described in methods section 2.2.1.4. Amount of enzyme coupled to the polymer was determined in all the samples. Each value represents the mean of three experiments.
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[Graph showing pH vs. % Coupled enzyme, with a peak around pH 8]
### Table 6  Coupling of bromelain to pNIPAm via amino groups and the oligosaccharide chain

<table>
<thead>
<tr>
<th>Nature of coupling</th>
<th>Enzyme added (U/g)</th>
<th>Bound enzyme activity (U/g)</th>
<th>Effectiveness Factor (η)</th>
<th>BA^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>via -NH₂ groups</td>
<td>7593</td>
<td>2167 628</td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td>via oligosaccharide chain</td>
<td>7593</td>
<td>910 200</td>
<td></td>
<td>0.22</td>
</tr>
</tbody>
</table>

Each value represents the mean of at least three independent experiments performed in duplicate.
in uniform orientation of the enzyme on the polymer. During peroxidation of the oligosaccharide chain of the enzyme, aldehyde groups are generated which react with the amino groups of the NAS pre-reacted with the diamine. The oxidation reaction was carried out in dilute solution of bromelain to avoid oligomer formation and this was evident from the SDS-PAGE of periodate oxidized bromelain that gave a single major band without evidence of the presence of large molecular weight aggregates.

Effectiveness factor, $\eta$ was 0.29, in case of the uniformly coupled bromelain, which was significantly higher than 0.22, that of the randomly coupled preparation. This suggested greater accessibility of the former for action on the substrate. Since a high molecular weight protein, casein was used as the substrate in these studies, steric hindrance in the approach of active site may be high hence, the observed $\eta$ values can be considered significant. Carbohydrates do not play any significant role in the catalytic function of bromelain (Murachi et al., 1967; Scocca and Lee, 1969), so coupling through the carbohydrate is not likely to inactivate the enzyme. In addition, the uniformly coupled preparation may have enzyme molecules in orientation that facilitates better accessibility of the active site. In contrast, the randomly coupled preparation is expected to be linked through one or more of the side chain amino groups, some of which may be positioned close to the active site or via those crucial for the maintenance of active conformation. It may however be mentioned that the observed enhancement in accessibility to protein substrate as a result of uniform orientation, was rather limited.

Wilchek and Miron (2003) have suggested that even during random immobilization, using group specific reactions, enzymes normally bind through one or two linkages and hence may not result in multiple types of orientations. Since under normal conditions only one or few side chain groups of enzyme react with reactive groups on support matrix, the “actual randomness” of the immobilization may be limited (Mateo et al., 2005, Mienglo et al., 2003). It is
therefore likely in case of enzymes that have the reactive side chain groups located in the proximity of active site, random coupling leads to the preparation exhibiting low activity. When such enzymes are favourably oriented, however the resulting preparation may exhibit far high activity. For the same reason, if the reactive side chain groups are located away from the active site, uniform orientation may not improve the activity of immobilized preparation significantly.

The moderate increase observed in the η value of the uniformly oriented bromelain may also be related to the lone glycosyl chain acting as a spacer between the support matrix and the enzyme (Hsiao and Royer, 1979; Woodward and Wiseman, 1978).

3.3.2 Properties of bromelain coupled to pNIPAm

3.3.2.1 Effect of pH

Native bromelain acts optimally on casein at pH 8.0 with a broad pH-activity profile at 25°C. The pH-activity profile was sharper at 35°C suggesting greater inactivation of the enzyme, particularly at extremes of pH. Bromelain randomly but not uniformly coupled to pNIPAm preparation, exhibited a shift in the pH optimum towards alkaline range at 25°C (Fig. 23 A). At 35°C however, both the immobilized preparations showed optimum activity at pH 10.0 and their activity profiles were broader than that of the native enzyme. The immobilized enzyme preparations retained far higher fraction of maximum activity, as compared to the native enzyme at extremes of pH (Fig. 23 B). This may be related to the resistance of the immobilized enzyme to rapid loss in activity due to conformational alteration/autolysis at the pH values far removed from optimum pH. The effect appeared more marked at 35°C. The shift in the pH-optimum of only the randomly coupled preparation at 25°C and that of both the immobilized preparations at 35°C is difficult to explain and may be related to specific micro-environmental effects and the preparations differing in proximity of the enzyme to the polymer chains.
3.3.2.2 Effect of Temperature

Stem bromelain shows a temperature optimum of 60°C that remained unaltered in case of the uniformly coupled preparation. The optimum temperature of the randomly coupled preparation however increased to 70°C (Fig. 24). Both the immobilized preparations retained far higher fractions of maximum activity than to that of native enzyme, both at 70°C and 80°C. The remarkable increase in the stability of bromelain preparations coupled to the polymer is also evident from figure 25. Native bromelain rapidly lost activity at 60°C and retained only about 10 percent of the initial activity after incubation for 180 minutes. Both the randomly and uniformly polymer coupled preparations were clearly more stable with retention of 35 percent and 24 percent activities respectively after 180 min incubation. The number of covalent/noncovalent linkages between enzyme and the support has been shown to influence the stability of enzymes to various forms of inactivation with stability increasing with the number of linkages (Iqbal and Saleemuddin, 1983). The randomly coupled preparation may therefore be more resistant to unfolding induced by heat. It is also well recognized that immobilized proteases are usually remarkably resistant to autolysis due to lowered contact between the enzyme molecules and the resistance appears very significant at higher temperatures (Lee and Park, 1998; Vlasov et al., 1981). Restricted autolysis may therefore contribute towards apparent stability exhibited by the bromelain preparation linked to pNIPAm.

In an attempt to further increase the stability of pNIPAm coupled bromelain, antibromelain antibodies were incubated both with randomly and uniformly coupled enzyme preparations. Polyclonal antibodies were raised in rabbits as detailed in the methods section 2.2.2.1 and the procedure resulted in high titre of antibodies in the immune serum revealed by direct binding ELISA. The preimmune serum did not show appreciable binding activity (Fig. 26). Immunodiffusion of bromelain against the antiserum showed single precipitin lines indicating homogeneity of the preparation (Fig. 27). The IgG fraction was
Figure 23  Effect of pH on the activity of native and pNIPAm coupled bromelain preparations

pH–activity profiles of native and pNIPAm coupled preparations were studied at 25°C (A) or at 35°C (B). Native bromelain (♦), that randomly (■) or uniformly coupled to pNIPAm (▲) were assayed in buffers of different pH; 0.2 M glycine-HCl (pH 2.0), 0.2 M acetate (pH 4.0), 0.2 M phosphate (pH 6.0, 8.0), 0.2 M bicarbonate (pH 10.0) and 0.2 M glycine-NaOH (pH 12.0). Each value is the average of at least three independent experiments.
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A

B

% Maximum activity

pH

% Maximum activity

pH
Figure 24  Effect of temperature on activity of native bromelain and pNIPAm coupled bromelain preparations

Temperature activity profiles of native bromelain (○), bromelain randomly (■) or uniformly coupled to pNIPAm (▲) were obtained by performing the enzyme assay at indicated temperatures under standard conditions of pH and substrate concentration. Each value represents the average of three different experiments carried out in duplicate.

Figure 25  Thermal denaturation of native bromelain and pNIPAm coupled bromelain preparations

Native bromelain and the pNIPAm preparations with coupled enzyme, preincubated with or without anti-bromelain antibodies, were incubated for various durations at 60°C and assayed under the standard conditions. (○) Native bromelain, (■) bromelain randomly coupled to p(NIPAm), (▲) bromelain uniformly coupled to the pNIPAm, (×) bromelain randomly coupled to pNIPAm and incubated with antibodies, (•) bromelain uniformly coupled to pNIPAm and incubated with the antibodies. Each value is the average of at least three independent experiments.
Results and Discussions

![Graph showing temperature vs. % maximum activity]

![Graph showing time vs. % maximum activity]

73
Figure 26  ELISA of bromelain antiseras

Direct binding ELISA of preimmunized (●) and bromelain immunized (■) rabbit sera were performed at various dilutions (1:100, 1:200, 1:1,000, 1:5,000, 1:10,000, 1:20,000, 1:50,000, 1:100,000, 1:200,000, 1:500,000, 1:1,000,000). Details are given in methods section 2.2.2.3.

Figure 27  Ouchterlony double immunodiffusion of bromelain against the bromelain specific antiserum

The central well contained 30 µg of bromelain, well 1, 2 and 3 were loaded with 20 µl, 10 µl and 5 µl of the appropriately diluted antiserum, respectively.
purified from the immunoserum as described in the methods section 2.2.2.4. Native and SDS-PAGE confirmed the purification of IgG (Fig. 28).

Fifteen mg of the purified IgG was incubated with 300 mg of the bromelain coupled pNIPAm preparations. 9.2 mg and 4.5 mg of IgG were found to be bound on randomly and uniformly coupled bromelain to pNIPAm, respectively. The thermostability of the polymer coupled bromelain was further improved remarkably on incubation with the antibodies (Fig. 25). About 70 percent and 58 percent of the initial activity were retained by the antibody-bound uniformly coupled and randomly coupled preparations respectively after 180 minutes of incubation at 60°C. Specific polyclonal/monoclonal antibodies have been successfully used to provide increased stability to the enzyme. Antibodies have been shown to confer stability to several enzymes by increasing resistance to unfolding and/or by physical shielding of the vulnerable sites on the surface of the enzyme (Shami et al., 1989; 1991; Saleemuddin, 1999).

The possible role of restricted autolysis in the inactivation of bromelain is supported by the data of figure 29. At 25°C not only are the polymer coupled preparations exposed to temperature below their LCST, but the temperature is relatively low for the induction of thermal inactivation. While it is true that autolysis rates are also likely to be low at lower temperatures, they may contribute significantly to the observed inactivation as compared to thermal denaturation. As shown in the figure 29, while native bromelain lost nearly all activity in 5 hours, the immobilized preparations retained more than 90 percent activity. While it is true that enzyme–enzyme interaction that may result in autolysis may be even more restricted above LCST in the pNIPAm coupled preparation (Ding et al., 1998), because of the large molecular dimensions of the polymer, autolysis may be quite low even at temperatures where the polymer is not insoluble. Also below LCST the polymer may form a protective colloid like hydrated surface layer that may restrict autolysis and/or unfolding (Ding et al., 1998).
IgG was purified from the immune serum as described in methods section 2.2.2.4.

(A) The electrophoretic pattern of anti-serum (lane 1), 20-40 % ammonium sulphate fraction (lane 2) and purified IgG (lane 3) are shown in 7.5 % non denaturing gel. 10-30 µg of each preparation was electrophoresed on the slab gel and coomassie brilliant blue staining was adopted.

(B) The electrophoretic pattern of purified IgG (lane 1), 20-40 % ammonium sulphate fraction (lane 2), anti-serum (lane 3) and molecular weight markers (lane 4) are shown in 12.0 % SDS-PAGE gel. 10-20 µg of each preparation was electrophoresed on the slab gel and silver staining was adopted.
Results and Discussions

A

1 2 3

B

1 2 3
Figure 29  Effect of incubation of native and pNIPAm coupled bromelain at 25°C for various durations on enzyme activity.

Native bromelain (●), or that randomly coupled (■) or uniformly coupled (▲) to pNIPAm preparations were incubated for various durations at 25°C and assayed under the standard conditions. Each value is the average of at least three independent experiments carried out in duplicate.
Results and Discussions

[Graph showing the percentage of maximum activity over time (hours).]
Coupling of bromelain to the pNIPAm did not significantly affect the LCST of the polymer that remained at 32°C. The LCST values were also only marginally affected by binding of the antibromelain antibodies to the bromelain linked to the polymer. These studies are in agreement with the earlier studies that coupling of proteins to the pNIPAm does not significantly alter the LCST (Chen and Hoffman, 1993; Fong et al., 2002).

3.3.2.3 Digestion of hemoglobin and IgG
The activity of pNIPAm coupled bromelain preparations to digest denatured and native protein was investigated in order to examine possible alterations in proteolytic activity. As shown in figure 30, hemoglobin was nearly completely digested both by the native bromelain and that coupled to pNIPAm via the amino groups, respectively to small molecular weight peptides. Similar results were obtained with the bromelain preparation coupled via the oligosaccharide chain.

Bromelain has been shown to be ineffective in complete digestion of human IgG (Vidal and Sasaki, 1975). This has been ascribed to the presence of IgG subclasses that are not susceptible to bromelain degradation. Figure 31 shows that extensive incubation either with free or polymer linked bromelain preparations resulted in only limited cleavage of the IgG. These studies suggest that coupling bromelain to pNIPAm does not result in marked alteration in its specificity and ability to degrade proteins.

3.4 Immobilization of bromelain on pNIPAm with altered LCST
pNIPAm normally exhibits a LCST value around 30°C that suits a number of applications (Park and Hoffman, 1993; Takei et al. 1994). Enzyme linked polymers with higher LCST may however be advantageous in carrying out catalytic transformations at higher temperatures in soluble state. Some studies have described strategies by which the LCST can be altered (Uludag et al., 2001). This
Figure 30  Digestion of hemoglobin by native and pNIPAm coupled bromelain

The native bromelain (A) and that randomly coupled to pNIPAm (B) were incubated with hemoglobin as described in the text and subjected to SDS-PAGE. Lane No. 1, 2, 3, 4 and 5 contain samples incubated for 0, 2, 4, 8, and 12 hours, respectively.
Results and Discussions
The rabbit IgG were incubated with native bromelain or randomly coupled to pNIPAm and subjected to SDS-PAGE. Lane 4 contains native IgG. Lanes 3, 2 and 1 contain IgG samples incubated with native bromelain for 4, 12 and 24 hours respectively. Lanes 5, 6 and 7 contain IgG samples incubated with bromelain coupled to pNIPAm via the amino groups for 4, 12 and 24 hours, respectively.
section describes a study in which LCST of pNIPAm was raised by incorporating acrylamide and acrylic acid and the effect of coupling of bromelain to the polymer was investigated.

3.4.1 Coupling of bromelain to pNIPAm containing acrylamide and acrylic acid

The procedure described by Zhu et al. (1998) was followed for the preparations of pNIPAm-bromelain conjugates except that the preparation contained varying 2-6 mole percent of acrylamide (Ac) or acrylic acid (AAc). As described earlier in case of the pNIPAm all the preparations containing Ac or AAc were of large molecular weight as they excluded from a 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 7). Similarly the additional incorporation of either Ac or AAc altered the η values only marginally. The η value increased slightly with increase in the concentration of Ac or AAc. The incorporated Ac/AAc is 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 may expand the polymer chains making the coupled enzyme more accessible for action on the substrate (Bulmus et al., 2000).

3.4.2 Properties of bromelain coupled to pNIPAm containing Ac/AAc

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

The LCST of pNIPAm and that with enzyme conjugated enzyme, as also shown earlier, remained at 32°C. Incorporation of either Ac or AAc resulted in a concentration dependent increase in the LCST values (Fig. 32). This is in agreement with the earlier observations that incorporation of hydrophilic monomer, increases the LCST of the pNIPAm (Feil et al., 1993; Yoshida et al.,
Table 7  Immobilization of bromelain on co-polymers of NIPAm and Ac or AAc

<table>
<thead>
<tr>
<th>Additional monomer (mol %)</th>
<th>Bound enzyme activity (U/g)</th>
<th>Effectiveness factor (η)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical A</td>
<td>Actual B</td>
</tr>
<tr>
<td>None</td>
<td>1733</td>
<td>381</td>
</tr>
<tr>
<td>Ac-2</td>
<td>1767</td>
<td>406</td>
</tr>
<tr>
<td>Ac-4</td>
<td>1738</td>
<td>435</td>
</tr>
<tr>
<td>Ac-6</td>
<td>1667</td>
<td>467</td>
</tr>
<tr>
<td>AAc-2</td>
<td>1800</td>
<td>414</td>
</tr>
<tr>
<td>AAc-4</td>
<td>1756</td>
<td>457</td>
</tr>
<tr>
<td>AAc-6</td>
<td>1700</td>
<td>527</td>
</tr>
</tbody>
</table>

Each value is the average of at least three independent experiments with variation not exceeding 4.0 %.
Figure 32  Effect of incorporation of Ac/AAc in pNIPAm on the LCST.

The LCST of the pNIPAm containing various concentrations of Ac (●) or AAc (●) was determined as described in the Methods. 2.2.1.4. Each value is the average of at least three independent experiments and S.D. shown with error bars.
1994). 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 to Ac because of the ionizable carboxyl groups and repulsion between the groups may cause increase in LCST (Bulmus et al., 2000).

3.4.2.2 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. 33). All the preparations were soluble at 28°C but transformed into the insoluble form at 42°C. All polymer coupled preparations exhibited activity optimum 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 at 28°C as also discussed earlier. Arasaratnam et al. (2000) have also shown that trypsin conjugated to the polymer is more accessible to the inhibitor in its soluble form than to when the conjugate is in insoluble form. The pH-activity profiles 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 concentrations of Ac or AAc incorporated and was maximum for the preparation with 6% AAc. This may be related, as also discussed earlier, to the resistance of the polymer linked enzyme to autolysis or conformational alterations occurring at the pH values far removed from 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 pH on the activity of bromelain coupled to pNIPAm containing Ac/AAc

pH–activity profiles of native and pNIPAm coupled enzyme preparations were determined at 28°C (A) or at 42°C (B). The bromelain preparations were incubated with casein at various pH values and activity determined as described in the text. Native bromelain (●), bromelain coupled to pNIPAm (○), pNIPAm containing 2.0% Ac (▼), AAc (▲); 4.0% Ac (△) AAc (◆), 6.0% Ac (■) AAc (▲). Each value is the average of at least three independent experiments.
Results and Discussions

A

B
3.4.2.3 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. 34). As also observed earlier, there was no effect of thermal transition on the activity of polymer bound preparations (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) showed 65% retained activity, higher than that of other preparations almost comparable to that of free enzyme which retained 68% of the maximum activity. The preparation was also most stable with retention of nearly 80% of maximum activity at 80°C.

The remarkable increase in stability of bromelain preparations coupled to the polymer is also substantiated from the data shown in figure 35. All the pNIPAm linked preparations were more stable and retained higher fractions of activity than the native enzyme 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 (Chen and Hsu, 1997; Ulbrich et al., 1986). The resistance to inactivation appears to increase slightly but consistently with the increasing concentration of Ac and AAc. Poly NIPAm preparation without incorporated Ac or AAc retained 45% of initial activity while preparation with 6% AAc retained 83% activity at 60°C after 180 minutes of incubation.

3.4.2.4 Km values

Km 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 Km for the native enzyme to be 2.86 mM
Figure 34  Effect of temperature on the activity of bromelain coupled to the pNIPAm containing Ac or AAc

Native bromelain (●); bromelain coupled to pNIPAm (○) and pNIPAm containing 2.0% Ac (▲), AAc (▼); 4.0% Ac (△), AAc (♦); 6.0% Ac (■), 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.

Figure 35  Thermal inactivation of pNIPAm coupled bromelain preparations with incorporated Ac/AAc

Native bromelain and the bromelain preparations coupled to pNIPAm were incubated at various durations at 60°C, cooled and assayed under the standard conditions. Native bromelain (●); bromelain coupled to pNIPAm (○); pNIPAm containing 2.0% Ac (▲), AAc (▼); 4.0% Ac (△), AAc (♦); 6.0% Ac (■), AAc (○). Each value is the average of at least three independent experiments.
Results and Discussions

% Maximum activity

Temperature (°C)

Time (minutes)

% Maximum activity

Time (minutes)
and it decreased to 1.54 mM on coupling of enzyme to pNIPAm (Fig. 36). This shows good access of the enzyme to the substrate which was anticipated because the polymer coupled enzyme preparation was soluble 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, favourable 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 $\varepsilon$-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 charge of matrix and substrate contributes toward 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 8). Increased hydrophilicity of the polymer may also facilitate the partitioning of substrate in the enzyme microenvironment which is also hydrophilic in nature.

3.4.2.5 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. 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 contents (Fig. 37).
Figure 36  Lineweaver-burk plots of bromelain coupled to pNIPAm incorporated with Ac/AAc

LNPE was used as a substrate under standard conditions and reciprocal concentrations of velocity were plotted as a function of reciprocal concentrations of substrate for native bromelain (●); bromelain coupled to pNIPAm (○) and pNIPAm containing 2.0% Ac (▼), AAc (□); 4.0% Ac (Δ), AAc (●); 6.0% Ac (■), AAc ( ). Each point represents the mean of three experiments carried out in triplicate with variations not exceeding 4.0%.
Results and Discussions
Table 8  Determination of Km values of native bromelain and bromelain coupled to pNIPAm containing Ac/AAc

<table>
<thead>
<tr>
<th>Bromelain preparations</th>
<th>Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>2.86</td>
</tr>
<tr>
<td>Coupled to pNIPAm</td>
<td>1.54</td>
</tr>
<tr>
<td>Coupled to pNIPAm containing 2.0% Ac</td>
<td>1.05</td>
</tr>
<tr>
<td>Coupled to pNIPAm containing 4.0% Ac</td>
<td>0.95</td>
</tr>
<tr>
<td>Coupled to pNIPAm containing 6.0% Ac</td>
<td>0.83</td>
</tr>
<tr>
<td>Coupled to pNIPAm containing 2.0% AAc</td>
<td>0.67</td>
</tr>
<tr>
<td>Coupled to pNIPAm containing 4.0% AAc</td>
<td>0.59</td>
</tr>
<tr>
<td>Coupled to pNIPAm containing 6.0% AAc</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Each value is the average of at least three independent experiments with variation not exceeding 5%.
Figure 37  Degradation of hemoglobin by bromelain coupled to pNIPAm containing Ac or AAc

The electrophoretic pattern of degradation of hemoglobin at 25°C by native bromelain (A) and that coupled to pNIPAm (B), co-polymers with 2.0% Ac (C), AAc (F); 4.0% Ac (D), AAc (G); 6.0% Ac (E), AAc (H) have been shown in 16.0 % SDS-PAGE. Lanes 1, 2, 3, 4 and 5 contain samples incubated for 0, 2, 4, 8, and 12 hours respectively.
Results and Discussions
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 behaviour was more marked in case of the bromelain preparations coupled to polymers containing higher concentrations of Ac or 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 p(NIPAm) with higher concentration of Ac or AAc.

3.5 Use of pNIPAm coupled bromelain in the purification of antibromelain antibodies

As shown in a previous section pNIPAm coupled bromelain (randomly oriented) was capable of binding specific antibromelain antibodies and experiments were performed to determine its potential as affinity matrix for the purification of antibodies. Antiserum was raised as described earlier and five ml appropriately diluted, was incubated with 150 mg of the pNIPAm coupled bromelain in buffers of various pH. About 4.9 mg serum proteins were bound at pH 8.0 per 150 mg of the polymer at 25°C. Antibody binding was significantly lower at 37°C (Fig. 38). At 37°C the enzyme linked polymer is in insoluble form and the low binding of the antibody may result from limited access of the antibodies toward the pNIPAm coupled bromelain due to steric hindrance.

Antibody was eluted at acidic pH by incubating the pNIPAm linked bromelain with bound antibody in 2.0 ml of 0.2 M glycine-HCl buffer, pH 3.0 for an hour at 25°C. Then the temperature was raised and the polymer was filtered. The eluate was immediately neutralized with 100 μl of 1.5 M Tris-HCl buffer, pH 9.0. SDS-PAGE of the protein loaded, unbound, washed and finally eluted samples, is shown in figure 39. The plasma contained multiple characteristic protein bands, while the eluted fraction revealed only two bands of 50 kDa and 25
Effect of pH on the binding of proteins from anti-serum to pNIPAm coupled bromelain

150 mg of pNIPAm coupled bromelain was incubated with anti-serum in buffers of various pH; 0.2 M phosphate buffer pH 6.0, 7.0, 8.0; 0.2 M bicarbonate, pH 9.0 at 25°C and 37°C separately for 12 hours. Protein bound was determined at 25°C (•) or 37°C (■). Each point represents the mean of three experiments carried out in duplicate.
Results and Discussions

![Graph showing the relationship between pH and bound protein (mg/g). The graph indicates a peak at pH 8, with the bound protein reaching its maximum at around 35 mg/g.]

- Bound protein (mg/g)
- pH

- 40
- 35
- 30
- 25
- 20
- 15
- 10
- 5
- 0

- 5
- 6
- 7
- 8
- 9
- 10
kDa corresponding to large and small molecular weight bands of IgG, respectively (Fig. 40). Recovery of the pure IgG was also superior when incubation of the polymer and antiserum was performed at pH 8.0 prior to the elution at pH 3.0 (Fig. 41). Specificity of the purified antibodies against bromelain was confirmed by the direct binding ELISA (Fig. 42). Absorbance at 450 nm for all the samples (1:2,00,000 dilution) also showed that maximum binding occurs at pH 8.0. Among conventional separation procedures, affinity chromatography continues to be a powerful strategy for various biomolecules. Unfortunately, most affinity supports are expensive, consequently affinity columns used for chromatography are generally small and purification of molecules from large volumes is problematic.

Affinity precipitation has been suggested as an alternative and may be more effective as the binding occurs in homogenous phase (Taniguchi et al., 1989). pNIPAm coupled antigen as shown in this study has remarkable potential in the large scale purification of antibodies from animal sera and/or hybridoma culture supernatants. Since pNIPAm is soluble below its LCST (32°C), binding of the antibody to pNIPAm-enzyme conjugate can be accomplished effectively in homogenous phase. This can be followed by raising the temperature slightly above the LCST to precipitate the pNIPAm-enzyme conjugate with bound antibody conveniently. Elution of the antibody from the insoluble pNIPAm-enzyme complex can then be performed in small volumes. The strategy may be useful in the isolation of both precipitating and non precipitating antibodies and hence applicable also to the purification of non-precipitating monoclonal antibodies from the hybridoma culture filtrates. Some earlier reports employed similar strategy for the purification of immunoglobulins (Nguyen and Luong, 1989) and some proteins (Mori et al., 1994; Hoshino et al., 1998; Teotia et al., 2001) at large scale. Kumar et al. (1998) have purified α-amylase inhibitor from the crude extract and wheat meal by the enzyme conjugated pNIPAm. Fong et al. (2002) conjugated pNIPAm to the IgG Fv fragment, the smallest fragment of the antibody that retains the antigenic affinity of the intact anti-lysozyme antibody for the successful
Figure 39  Purification of bromelain-specific antibodies from rabbit anti-serum

The SDS-PAGE of the antiserum (lane 1), unbound protein (lane 2), first, second, third and fourth washing of the polymer-antibody complex with buffer (lane No. 3, 4, 5 and 6 respectively) and sample eluted with 0.2 M glycine-HCl buffer, pH 3.0 (lane 7). 10 μg of serum and purified antibody and equal volumes of supernatant and washings were electrophoresed on the slab gel and coomassie brilliant blue staining was adopted.

Figure 40  Molecular weight determination of the purified antibody by SDS-PAGE.

A  Electrophoresis was performed in 12.0 percent gels. Lane 1 contained the standard molecular weight markers and lane 2 contained purified antibody (12 μg). Pattern of purified antibody (lane 2) with the standard molecular weight marker protein mixture (lane 1) are shown in 12.0% denaturing gel.

B  Semilogarithmic plot of the molecular mass against the distance of migration of the standard marker proteins. Protein markers used for calibration were phosphorylase b (97.4 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soyabean trypsin inhibitor (20 kDa) and lysozyme (14.3 kDa). Arrows 1 and 2 indicate the position of the small and large molecular weight peptides of the purified antibody, respectively.
Figure 41  Effect of pH on the binding of antibodies to pNIPAm coupled bromelain

The electrophoretic pattern of the antibody recovered from the 150 mg of pNIPAm coupled bromelain in buffers of various pH; 0.2 M acetate (pH 5.0); 0.2 M phosphate (pH 6.0, 7.0, 8.0) and 0.2 M bicarbonate buffer (pH 9.0) (lane 1, 2, 3, 4 and 5, respectively) incubated with same volume of serum for 12 hours are shown in 12.0% SDS-PAGE. Equal volumes of the eluate were electrophoresed on the slab gel and coomassie brilliant blue staining was adopted.

Figure 42  ELISA of loaded, unbound and eluted samples at different pH from pNIPAm coupled bromelain

150 mg of pNIPAm coupled bromelain in buffers of pH 5.0, 6.0, 7.0, 8.0 and 9.0 were incubated with same volume of serum for 12 hours at 25°C. Absorbance at 450 nm for added serum (●), unbound (■) and eluted (■) samples from pNIPAm coupled bromelain at 1:2,00,000 dilution The experiment was done in duplicate with variation not exceeding 4.0%.
Results and Discussions
purification of the enzyme. pNIPAm has also been conjugated to antibodies for immunodiagnostic assays, protein A for antibody purification, oligonucleotide for DNA purification at large scale (Costioli et al., 2003), trypsin to purify sporamin, a trypsin inhibitor from sweet potato juice (Lee and Chen, 2004). The work described above substantiates the observation and suggests the usefulness of the polymer-linked enzyme in antibody purification.

3.6 Entrapment of pNIPAm-bromelain conjugate in calcium alginate beads

The pNIPAm coupled bromelain was also investigated for possible temperature controlled release from porous calcium alginate beads. It is well recognized that calcium alginate beads are highly porous and readily release entrapped proteins and enzymes while retaining larger entities (Husain et al., 1985).

3.6.1 Effect of different factors on the entrapment yield of the polymer conjugated bromelain

Concentrations of alginate between 3-5% were taken to prepare calcium alginate beads for the entrapment of the pNIPAm-bromelain conjugate (randomly coupled). Beads were then dissolved in 1.0% (w/v) EDTA to estimate the amount of entrapped polymer conjugated bromelain. Entrapment of pNIPAm-bromelain (Fig. 43A) increased with the increase in alginate concentration up to 5.0% (w/v). Above this concentration, however the entrapment yield began to decrease. The preparations with 5.0% alginate were therefore used for the studies.

Effect of calcium chloride concentration on entrapment yield was also studied for 5.0% alginate beads (Fig. 43B). There was a slight increase in the entrapment yield at 2.0 M as compared to that at 1.0 M CaCl₂. Further increase in the CaCl₂ concentration did not affect the entrapment yield. Alginate beads were therefore prepared using 5.0% alginate and 2.0 M CaCl₂ concentration for further
Figure 43  **Effect of alginate and calcium chloride concentration on the entrapment yield of pNIPAm coupled bromelain in Ca-alginate beads**

200 U of bromelain coupled to pNIPAm was mixed with the indicated concentrations of sodium alginate and beads were prepared in 2M calcium chloride solution (A). The enzyme was mixed with 5% Na-alginate and beads prepared in solution containing the indicated concentration of CaCl₂ (B). The beads were removed by filtration, washed and dissolved with 1% (w/v) EDTA, for the estimation of enzyme entrapped.
Results and Discussions

A

% Entrapment yield

% Na-alginate (w/v)

B

% Entrapment yield

Calcium chloride conc (M)
experiments. It was also revealed from the experiments that as compared to the native bromelain (154 U), higher amount of bromelain coupled to the polymer (190 U) was entrapped in calcium alginate beads out of 200 U of enzyme added in each case.

Alginate entrapment of enzyme is widely used due to non-toxicity of the matrix and possibility of variation in bead size of the gel as well as high yields of immobilization. It has been used to immobilize cell organelles, micro-organisms, plant and animal cells successfully (Smidsrød and Gudmund, 1990; Kierstan and Bucke, 2000) but due to high porosity it has not proved to be an efficient system for enzyme immobilization (Blandino et al., 2000; Musthapa et al., 2004).

3.6.2 Leakage of free and pNIPAm coupled enzyme from Ca-alginate beads

Leakage profiles of both free and pNIPAm coupled bromelain were studied from Ca-alginate beads containing 3%, 4% or 5% alginate at 25°C (Fig. 44). The leakage was more significant in the case of free bromelain as compared to that of the pNIPAm bound enzyme from the beads containing various concentrations of alginate. The low leakage of the pNIPAm coupled bromelain may be related to the large molecular dimensions of the polymer coupled enzyme as compared to the native enzyme. As mentioned earlier, the polymer has large molecular weight and is excluded from Sepharose 4B. Release of enzyme decreased with increase in alginate concentration of the beads both for the free and polymer linked enzyme preparations and was least from the alginate beads prepared using 5% sodium alginate at 25°C. When the bromelain entrapped beads were incubated at 40°C, there was almost no leakage of the enzyme from the beads containing pNIPAm linked enzyme but release of free bromelain was comparable to that at 25°C. In an earlier study it was shown that glycoenzyme leakage from the calcium alginate beads can be remarkably decreased by complexing them with the lectin con A (Husain et al., 1985). Con A-glycoenzyme conjugates are very large and insoluble and hence retained in the polymeric network of calcium alginate.
Figure 44  Release of free and polymer coupled bromelain from Ca-alginate beads

Beads prepared using various concentrations of Na-alginate were entrapped with either free bromelain or that coupled to pNIPAm. The beads were removed by filtration, washed and incubated in sodium acetate buffer, pH 5.6 at 25°C. Appropriate volume of the supernatant was taken after various durations and was assayed under standard conditions. The alginate concentrations used were 3 % (●, ○), 4 % (■, □) and 5 % (▲, △). Hollow symbols indicate beads containing free enzyme and the filled symbols indicate those containing pNIPAm coupled enzyme. Each point represents the mean of three experiments.
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![Graph showing time (hours) vs. % release ](image)

- Time (hours) range from 0 to 20.
- % Release range from 0 to 50.

The graph illustrates the release profile over time, with different curves representing varying conditions or treatments.
With a view to investigate whether the release of pNIPAm coupled bromelain could be regulated by temperature alterations, two experiments were performed. In the first set, alginate beads containing the bromelain linked to pNIPAm were incubated at 25°C for 24 hours followed by raising the temperature to 40°C. In the second set of experiment, the beads were first incubated at 40°C followed by shifting them to water-bath at 25°C. As shown in figure 45, initial incubation at 25°C resulted in gradual leakage resulting in the release of about 15 percent of the entrapped polymer linked enzyme. When the temperature was raised to 40°C, however, the release was almost arrested with very little release of additional enzyme up to 48 hours of incubation.

In the preparation incubated initially at 40°C, the leakage of the polymer linked enzyme was only marginal but subsequent shift to 25°C resulted in rapid release of the enzyme. This clearly indicates that release of pNIPAm coupled enzyme can be regulated by altering the temperature. Since the LCST of pNIPAm is normally around 32°C, significantly below the body temperature of mammals, the alginate beads containing the polymer-linked enzyme are expected to release very little enzyme. However, by decreasing the local temperature below the LCST the enzyme can be made to release readily as shown in the figure 45. Several studies are available in which pharmacologically active compounds can be released from polymeric matrices etc by local hypothermia (You et al., 1994). The model here on the other hand suggests that decreasing the temperature in the surrounding of the bead can lead marked enhancement in the release of alginate entrapped pNIPAm coupled enzyme.

This difference in rate of release of free and polymer linked bromelain from the alginate beads was also studied by incubating them in 1% (w/v) casein (Fig. 46). SDS-PAGE of the filtrate showed that casein present in the incubation medium was more readily digested in the samples incubated with beads containing soluble enzyme as compared to those with entrapped polymer-linked enzyme at 25°C (Fig. 47). At 40°C pNIPAm-linked enzyme entrapped in alginate beads
Percent release of polymer coupled bromelain from 5.0 % Ca-alginate beads has been shown. The beads were incubated in 10 ml of sodium acetate buffer, pH 5.0 initially at 25°C up to 24 hours followed by the incubation at 40°C for another 24 hours (■). Alternatively, the beads were initially incubated at 40°C up to 24 hours followed by incubation at 25°C for 24 hours (●). Arrow indicates the time of switching of the incubation temperature. Each value represents the mean of three experiments carried out in duplicate.
Commercial casein was subjected to SDS-PAGE in 15.0% gel. Lane 1 contained the molecular weight markers and lane 2, casein (12 μg). The molecular weight markers were those used in experiments described in figure 40. Molecular weights of the two casein bands were 21.6 kDa and 19 kDa, respectively, determined by the semilogarithmic plot of the molecular mass against the distance of migration of the standard marker proteins.
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(kDa)
97.4
68
43
29
20
14.3
6.5
3.5

1 2
Figure 47 Digestion of casein by the enzyme released from Ca-alginate beads entrapped with free bromelain and that coupled to pNIPAm.

Free bromelain (A) or pNIPAm–coupled bromelain (B) was entrapped in 5.0% calcium alginate beads and were incubated with 1.0 % (w/v) casein at 25°C. Equal volumes of suspending medium at different durations were used for the measurement of hydrolysis of casein. Lane No. 1, 2, 3, 4, 5 and 6 contained casein samples incubated with the suspension incubated with the beads for 0, 2, 4, 8, 12 and 24 hours respectively.

Panel C contains casein samples from the suspension incubated for various durations with calcium alginate beads containing pNIPAm coupled bromelain at 40°C.
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showed almost no proteolysis of casein. The enhancement in the leakage of polymer bound enzyme by temperature change is an interesting feature in catalysis and could be employed for selective enhancement in catalysis.

3.7 Immuno-affinity layering of bromelain

Affinity immobilized enzymes have been found to be generally more active and stable than the enzymes immobilized by other methods (Saleemuddin, 1999). Also as discussed earlier, affinity bound invertase and β galactosidase preparations were generally superior to those containing covalently coupled enzyme, both in terms of η values and stability. Attempts were therefore made to improve the amount of enzyme bound to the polymer by building affinity layers of enzyme and antibody. It was shown in earlier studies that binding of affinity layers using antibodies or lectins was remarkably effective in enhancing the stability of the enzymes against various forms of inactivation (Farooqi et al., 1997; 1999).

3.7.1 Layering of bromelain and antibromelain antibodies on pNIPAm

Additional binding of bromelain on pNIPAm-antibody conjugate could be accomplished by alternately incubating the conjugate with bromelain and soluble antibody, as detailed in method section 2.2.2.6. As shown in the table 9, about ten-fold increase in the activity of bound bromelain to the polymer could be achieved after subjecting the polymer to four incubation cycles with enzyme and antibody. It is also evident from the table that the η value increased significantly up to the formation of four layers. The observed small increase in η after the fourth affinity layer formation may be due to the overcrowding of the enzyme, hence, restriction in the substrate accessibility to the active site. Figure 48 shows that with each incubation cycle the total amount of antibody bound to the polymer also increased significantly which in turn bound additional enzyme, leading to layer-by-layer increase in enzyme immobilization yield.
Table 9  Immuno-affinity layering of bromelain on pNIPAm coupled antibodies

<table>
<thead>
<tr>
<th>No. of Affinity layers</th>
<th>Bound enzyme activity (U/g)</th>
<th>Effectiveness factor (η)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical A</td>
<td>Actual B</td>
</tr>
<tr>
<td>1</td>
<td>1111</td>
<td>267</td>
</tr>
<tr>
<td>2</td>
<td>2445</td>
<td>782</td>
</tr>
<tr>
<td>3</td>
<td>4446</td>
<td>1690</td>
</tr>
<tr>
<td>4</td>
<td>6222</td>
<td>2551</td>
</tr>
</tbody>
</table>

Each value represents the mean of at least three independent experiments with variation not exceeding 5.0 %.
Figure 48  Effect of the number of immunoaffinity layers on the binding of antibody

300 mg of pNIPAm coupled antibody was incubated with bromelain and antibody alternately. Bound antibody was investigated for every immunoaffinity layer on pNIPAm. Each value represents the mean of three experiments.
Results and Discussions
3.7.2 Properties of immuno-affinity layered bromelain preparations

3.7.2.1 LCST
The LCST of the antibody conjugated polymer was 32°C in 0.1 M phosphate buffer. The LCST remained unaffected till the formation of two affinity layers, but decreased significantly after the formation of third and fourth layers (Table 10). The crowding of enzyme/antibody molecules on the polymer may contribute towards hydrophobicity and decrease in solubility, hence decrease in LCST.

3.7.2.2 Effect of pH
Binding of bromelain on polymer conjugated antibromelain IgG shifted the pH optimum of the enzyme towards alkaline range, at both 25°C and 35°C (Fig. 49). The pH optimum of native bromelain is pH 8.0, which shifted to pH 10.0 after binding to the pNIPAm precoupled with IgG. Further layering did not alter the pH optimum of the preparation but increased the retained fraction of activity of the enzyme both in alkaline and acidic pH range. The preparation with four affinity layers appeared most stable and exhibited greater fraction of maximum activity at extremes of pH. The activity profiles of the immuno-affinity layered preparations at 25°C and 35°C were comparable but those at 25°C were broader, suggesting loss of relatively small fraction of enzyme activity at the extremes of pH at this temperature. Apparently the insoluble form of the polymer at 35°C, causes restriction in mass transfer of the substrate and decrease of enzyme activity is observed at the temperature (Hoshino et al., 1997).

3.7.2.3 Effect of temperature
Figure 50 shows that the temperature optimum of the immobilized preparations changed to 70°C from 60°C, the temperature optimum of free bromelain. The immobilized preparations also retained greater fraction of maximum enzyme activity at 80°C and lower temperatures with the increasing number of affinity layers. The data suggests the affinity layering increases the resistance of the
Table 10  Effect of enzyme layering on LCST of the polymer

<table>
<thead>
<tr>
<th>No. of affinity layers</th>
<th>LCST (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
</tr>
</tbody>
</table>

Each value represents the mean of at least three independent experiments performed in triplicate with variation not exceeding 2.0 %. 
Figure 49  Effect of pH on the activity of bromelain assembled on pNIPAm by affinity layering

Appropriate quantities of native bromelain (♦) and those containing one (■), two (▲), three (●) or four layers (○) of anti-bromelain antibody/bromelain were incubated in buffers of various pH and were assayed at 25°C (A) and 40°C (B). Each value represents the mean of three different experiments carried out in duplicate.
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A

\[
\text{% Maximum activity vs pH}
\]

B

\[
\text{% Maximum activity vs pH}
\]
enzyme to inactivation at higher temperatures. The increase in stability is substantiated by the thermal inactivation performed at 60°C (Fig. 51). Affinity immobilized enzymes on supports with antibodies have shown enhanced resistance against various forms of inactivations (Solomon et al., 1987; Turkova, 1993; Jafri and Saleemuddin 1997). Native bromelain loses its activity rapidly whereas immobilized preparations showed increase in retained enzyme activity with increase in the number of enzyme layers. The preparation with fourth enzyme layer retained 94% of the maximum activity under the conditions in which the free enzyme retained only 20%.

Stable preparations with very high concentration of enzymes are valuable for enzyme-based biosensors where large amount of enzymes need to be attached on small area for maximum sensitivity (Alvarez-Kaza and Biletewski, 1993; Vandenbergs et al., 1994). Quantity of glycoenzymes immobilized on supports was effectively raised by building bioaffinity layers of glycoenzymes with the lectin con A (Farooqui et al., 1997; Sardar and Gupta, 2005). Layering and raising the amount of immobilized enzyme is also possible by specific antibodies. Immuno-affinity layering has markedly improved the detection limits of the sensors (Bourdillon et al., 1994; Farooqi et al., 1999).

Chen and Hoffman (1993) and Fong et al. (2002) as well as the work reported earlier in the thesis showed that coupling of protein does not alter the thermal transition of pNIPAm. It is interesting to note that the binding of alternate antibody/enzyme layers also did not affect the LCST behaviour of pNIPAm. As mentioned earlier, the pNIPAm used in this study is of very high molecular weight and it is apparent that the polymer chains in spite of the association of enzyme and antibody are still capable of interacting with each other, forming insoluble complexes above LCST. It is therefore likely that each chain is linked to one or few molecules of enzymes only leaving large free areas that interact with each other at temperatures above LCST. The stability increases with
Figure 50  Effect of temperature on the activity of bromelain assembled on pNIPAm by affinity layering

Temperature activity profiles of native bromelain (●) and that containing one (■), two (▲), three (○) or four layers ( ) of anti-bromelain antibody/bromelain were determined. Each value represents the mean of three different experiments carried out in duplicate.

Figure 51  Thermal inactivation of immunoaffinity layered bromelain preparations on pNIPAm

Thermal inactivation curves of native bromelain (●) and that containing one (■), two (▲), three (○) or four layers ( ) of anti-bromelain antibody/bromelain were obtained by incubating for various durations at 60°C prior to the enzyme assay. Each value represents the mean of three different experiments carried out in duplicate.
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Residual activity vs. Temperature (°C)

Residual activity vs. Time (minutes)
increase in the enzyme layers is comparable with the observation made by Farooqi
et al. (1999) using Sepharose supports. A highly active and stable preparation of
enzyme that can be reversibly precipitated by change in the temperature may find
several applications specially on the processing of large molecular weight/insoluble
substrates.