ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 3, pp. 307-312. © Pleiades Publishing, Ltd., 2007.
Published in Russian in Biokhimiya, 2007, Vol. 72, No. 3, pp. 375-382.

Additional Stabilization of Stem Bromelain Coupled to a Thermosensitive Polymer by Uniform Orientation and Using Polyclonal Antibodies

Rubab Mahmood[1] and M. Saleemuddin[1,2]*

[1]*Interdisciplinary Biotechnology Unit and* [2]*Department of Biochemistry, Aligarh Muslim University, Aligarh 202002, India; fax: (915) 712-721-776; E-mail: alg_btisamua@sancharnet.in; rubab1@rediffmail.com*

Received September 15, 2006
Revision received December 8, 2006

Abstract—Stem bromelain was covalently coupled to a thermosensitive polymer of N-isopropylacrylamide (p(NIPAm)) either through the amino groups of the enzyme (randomly coupled) or via the lone oligosaccharide chain (uniformly coupled). The enzyme coupled via the oligosaccharide chain exhibited better access to the substrate casein as compared to the preparation in which the amino groups formed the point of contact between the enzyme and the polymer. Native bromelain exhibited a pH optimum of 8.0 and a broad pH-activity profile. The polymer-coupled preparations exhibited broader pH-activity profiles and shifting of pH optimum to 10.0 at 35°C. At 25°C, the shifting of pH optimum was observed for the randomly coupled enzyme only. The temperature–activity profiles of bromelain coupled to p(NIPAm) also showed appreciable broadening and the preparations retained greater fraction of maximum activity above the temperature optimum. The optimum temperature of the uniformly oriented preparation also rose to 70°C. Inactivation rates of the polymer-coupled bromelain were remarkably low at 60°C as compared to the native protease, and binding of antibromelain antibodies improved the resistance to inactivation of the polymer-coupled preparations. The cleavage patterns of hemoglobin and IgG by the native bromelain and the polymer-coupled preparations were comparable.

DOI: 10.1134/S000629790703008X

Key words: poly(N-isopropylacrylamide), bromelain, thermosensitive polymer, oriented immobilization

Thermoresponsive polymers are being extensively studied as carriers for proteins and enzymes for a variety of applications in biotechnology including drug delivery, achieving useful biotransformations, etc. [1]. Among these, polymers of N-isopropylacrylamide (p(NIPAm)) are the most widely studied [2, 3]. p(NIPAm) exhibits low critical solution temperature (LCST) (i.e. temperature for soluble ↔ insoluble transition) of 30-32°C in water and above the LCST the hydrated polymer collapses into a globular state forming micelles [4]. LCST is believed to represent the temperature at which hydrophobic forces due to the interaction of $-N(CH_3)_2$ groups causing insolubility in an aqueous environment are balanced by hydrogen bonding between water and –NH- and/or –C=O groups that retain the polymer in solution. Strategies for favorably altering the LCST are also available and it can be effectively controlled by incorporating charged or hydrophobic units in the polymer [5, 6]. For coupling of proteins, NIPAm is generally copolymerized with acrylyl monomers containing reactive carboxylic [7], succinimidyl [8], or glycidyl groups [9]. While earlier studies involved the coupling of enzyme to multiple reactive sites along the polymer backbone, strategies in which the oligomer is linked to the enzyme by a single attachment are also available [10, 11].

Trypsin [11], lipase [12], cellulase [13], and β-D-glucosidase [8, 14] immobilized on p(NIPAm) polymers have been shown to be easily recoverable from reaction mixture and exhibit improved stability against some forms of inactivation. Some studies have also described strategies for site specific coupling of proteins to p(NIPAm) using proteins engineered by inserting specific amino acids like cysteine with its exclusive –SH group [15] or polylysine tag [16]. Stem bromelain is an endoprotease with a single oligosaccharide chain linked to the polypeptide, which is not involved in catalytic function [17, 18]. It therefore provides an opportunity for coupling to the polymer, through the lone oligosaccharide chain, thereby orienting it uniformly on the support. In a recent study, we demonstrated that bromelain can be uniformly and

Abbreviations: LCST) low critical solution temperature; p(NIPAm)) poly(N-isopropylacrylamide).

* To whom correspondence should be addressed.

favorably oriented on concanavalin A support to yield a useful immobilized preparation [19].

This study describes a comparison of the properties of stem bromelain coupled randomly via the amino groups or uniformly via the lone oligosaccharide moiety to p(NIPAm) polymer. Additional stabilization of the polymer-bound enzyme with the use of antibromelain antibodies raised in rabbits is also demonstrated.

MATERIALS AND METHODS

Materials. NIPAm, N-hydroxy-succinimidyl acrylate, and stem bromelain (EC 3.4.22.32) were purchased from Sigma (USA). Other chemicals used were of analytical grade.

Coupling of bromelain to p(NIPAm) via amino groups. According to the procedure of Zhu et al. [20], 34 mg of bromelain was dissolved in 1 ml of 0.15 M NaCl containing 60 mM phosphate buffer, pH 8.0 (PBS), and vibrated with 3 ml of 0.1% N-hydroxy-succinimidyl acrylate in a water bath at 37°C for 60 min in order to form the monomer–enzyme conjugate. This mixture was dialyzed thrice against the above PBS at 4°C, concentrated to 2 ml, and poured into 8 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 200 µl of 1% ammonium persulfate and 10 µl of TEMED at 25°C for 2 h. The solution was incubated at 37°C for 30 min and centrifuged for 10 min at 10,000*g* to separate the insoluble p(NIPAm)–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 37°C. The procedure was repeated thrice to remove any adsorbed but uncoupled enzyme. Finally, the p(NIPAm)–bromelain conjugate was dissolved in 5 ml of 0.2 M phosphate buffer, pH 7.0.

Coupling of bromelain to p(NIPAm) via the oligosaccharide chain. Bromelain (34 mg) was activated with 5 mM L-cysteine-HCl in 0.05 M phosphate buffer, pH 7.0, and after 10 min, 750 µl of 0.1 M sodium tetrathionate was added to block protein –SH groups. Then the sample was dialyzed extensively for 2-3 h. The volume of tetrathionate-treated enzyme was made up to 20 ml with the above phosphate buffer and then was oxidized with 5 ml of 20 mM sodium metaperiodate [21] by incubating the mixture with stirring in dark at 4°C. The reaction was stopped after 2 h by adding 5 ml of 95% (w/v) ethylene glycol, and the mixture was dialyzed at 4°C against 0.05 M phosphate buffer, pH 7.0. Extent of oxidation was confirmed by carbohydrate estimation by the procedure of Dubois et al. [22]. The dialyzed preparation was concentrated and its volume was made up to 5 ml with PBS, pH 8.0, then stirred overnight at 4°C with 3 ml of 0.1% N-hydroxy-succinimidyl acrylate preliminarily treated with excess of 0.1 M ethylenediamine. The mixture was dialyzed against PBS, pH 8.0. The final preparation was then allowed to co-polymerize with NIPAm as described above.

Protein concentration and enzyme activity. The concentration of protein was determined by the method of Lowry et al. [23]. Proteolytic activity of bromelain was determined using casein as a substrate [24]. 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, and 0.5 ml of 1% (w/v) casein in a total volume of 1 ml. After incubation, the reaction was stopped by the addition of 1 ml of 15% trichloroacetic acid. Trichloroacetic acid-soluble peptides were quantified by the procedure of Lowry et al. [23]. One unit of the enzyme is the amount that brings about a change of 0.01 OD at 700 nm per minute. Values given in the text and figures are the average of at least three independent experiments with variations not exceeding 5%.

Immunization of rabbits. Rabbits were subcutaneously injected with 150 µg of bromelain in 0.5 ml of 0.01 M phosphate buffer mixed with Freund's complete adjuvant (1 : 1). The animals were rested for 15 days, and booster doses, prepared by mixing 100 µg of bromelain/0.5 ml solution with equal volumes of Freund's incomplete adjuvant, were subcutaneously given at weekly intervals for 4 weeks. Blood was withdrawn through an ear vein and clot formation was allowed to take place at room temperature for 6 h. Serum was collected by centrifugation and stored at 0°C. Ouchterlony double immunodiffusion and ELISA were used for the detection of antibody formation and determination of the antibody titer.

Purification of IgG. IgG was purified from the serum by ammonium sulfate precipitation at 40% saturation followed by dialysis for 36 h. It was then applied to the DEAE-cellulose column (pH 7.2, 20 mM phosphate buffer) and was allowed to bind at 4°C for 12 h. Unbound fraction containing IgG was collected, and its purity was tested by SDS-PAGE [25].

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 min. Then OD at 500 nm was taken and transition temperature was determined by plotting optical density against temperature, which fitted to a sigmoidal curve [6, 7]. All the determinations were done in triplicate, and there was excellent agreement in the values.

RESULTS AND DISCUSSION

Bromelain was coupled to the p(NIPAm) either via the amino groups or the lone carbohydrate chain. The polymer used for the enzyme immobilization obtained using the procedure described by Zhu et al. [20] was apparently of high molecular weight, as it was excluded from a Sepharose-4B column (data not shown). More

enzyme was coupled to the polymer when coupling involved reaction between the enzyme amino groups and the polymer. Thus, while 2267 U were coupled per gram of polymer in case of the randomly coupled preparation, uniform coupling resulted in association of only 910 U per gram of polymer. This is apparently related to the possible coupling through one or more of the terminal or the 15 side chain amino groups available in the bromelain molecule [26] in case of the randomly coupled molecules. Stem bromelain, on the other hand, contains a single oligosaccharide chain and hence a lone linkage between support and the enzyme can be anticipated when bromelain is coupled by reaction of oxidized enzyme with the amino group-bearing polymer. SDS-PAGE suggested absence of formation of large molecular weight aggregates during the oxidation of the oligosaccharide chain. Coupling of bromelain through the oligosaccharide is therefore expected to result in uniform orientation of the enzyme on the polymer. The effectiveness factor (η), the ratio of bound to expressed activity [27], was 0.29, in case of the uniformly coupled preparation which was significantly higher than 0.22, that of the randomly coupled bromelain, suggesting greater accessibility of the bound enzyme for action on the substrate. Since high molecular weight casein was used as the substrate in these studies, the observed η values are significant.

Since carbohydrates do not seem to play any significant role in the catalytic function of bromelain [17, 18], the preparation may have enzyme molecules with relatively more accessible active site. In contrast, the randomly coupled preparation is expected to be linked through 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 be however mentioned that enhancement in accessibility to protein substrate because of uniform orientation is not very high. While a number of studies have described striking increase in efficiency of the enzyme because of favorable uniform orientation [28-30], the advantages were not so significant in others [31]. Wilchek and Miron [31] 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 the enzyme react with reactive groups on support matrix, randomness of the immobilization may be limited [32, 33]. It is therefore likely that in the case of enzymes that have the reactive side chain groups located in the proximity of active site, random coupling leads to preparation exhibiting low activity. When such enzymes are favorably oriented, the resulting preparation may exhibit far higher activity. For the same reason, if the reactive side chain groups are located away from the active site, uniform orientation may not improve activity of the immobilized preparation significantly. The moderate increase observed in the η value of the uniformly oriented preparation in this study may also be related to the coupling through the carbohydrate chain [34, 35]. The glycosyl chain may act as a spacer between the support matrix and enzyme, thereby contributing to better accessibility of the active site of immobilized enzyme towards substrate [28].

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 at this temperature at high pH. The enzyme randomly coupled to p(NIPAm) but not the uniformly coupled preparation exhibited a shift in the pH optimum towards alkaline pH at 25°C (Fig. 1a). At 35°C however, both of the immobilized prepara-

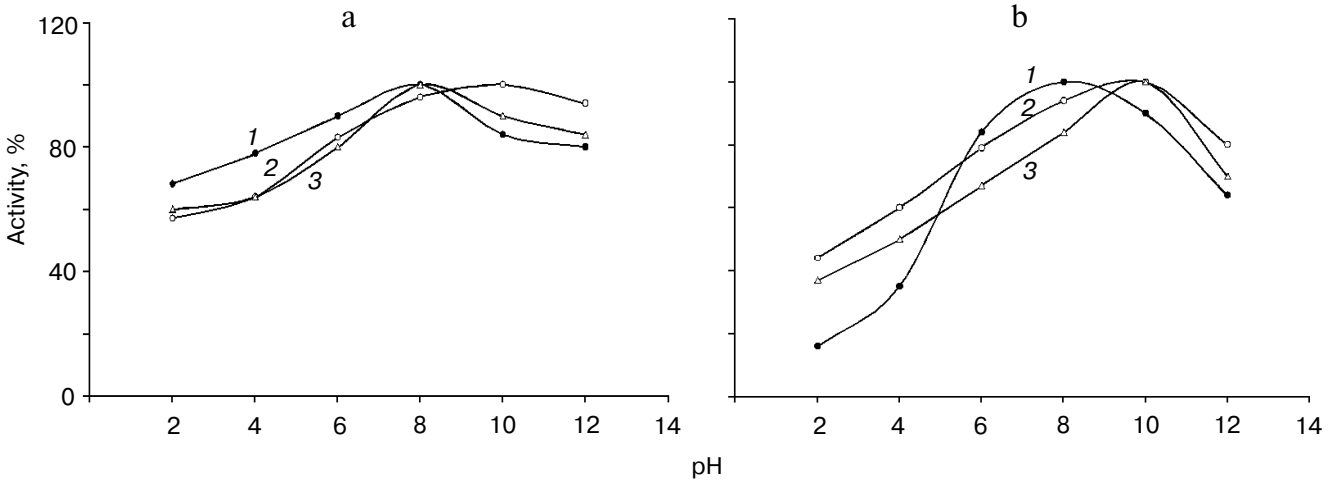

Fig. 1. pH–activity profiles of native and p(NIPAm)-coupled preparations at 25 (a) and 35°C (b). Native bromelain (*1*) and that randomly coupled to p(NIPAm) (*2*) or uniformly coupled (*3*) were assayed in buffers of different pH: 0.2 M glycine-HCl (pH 2.0), 0.2 M acetate (pH 4.0), 0.2 M phosphate (pH 6.8), 0.2 M bicarbonate (pH 10.0), 0.2 M glycine-NaOH (pH 12.0). Each value is the average of at least three independent experiments with variation not exceeding 4%.

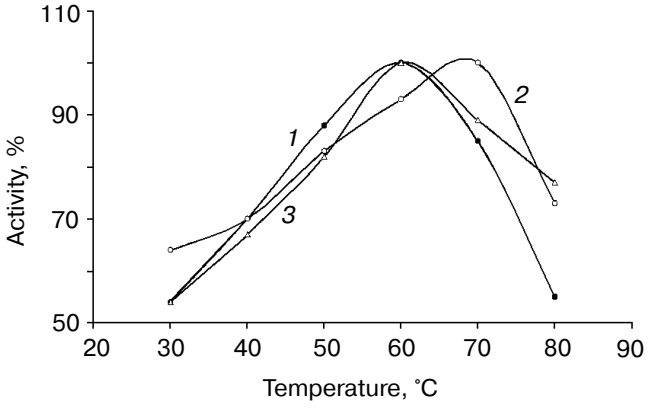

Fig. 2. Effect of temperature on the activity of native and p(NIPAm)-coupled bromelain. Native bromelain (*1*) or that randomly coupled to p(NIPAm) (*2*) or uniformly coupled (*3*) were incubated with casein at the indicated temperatures and activity measured described in the text. Each value is the average of at least three independent experiments with variation not exceeding 5%.

tions showed an optimum activity at pH 10.0, and their activity profiles were broader with the enzyme retaining far higher fraction of maximum activity as compared to the native enzyme at extremes of pH (Fig. 1b). This may be related the resistance to rapid loss in activity due to conformational alterations/autolysis at pH values far removed from the 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 of the immobilized preparations at 35°C is difficult to explain but may be related microenvironmental effects of the preparations differing in proximity to the polymer chains.

Effect of temperature. Stem bromelain has 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. 2). Both the immobilized preparations however retained far higher fraction of maximum activity on incubation at various time intervals both at 70 and 80°C. The remarkable increase in stability of bromelain preparations coupled to the polymer is also evident from Fig. 3. Native bromelain rapidly lost activity at 60°C and retained only about 10% of the initial activity after incubation for 180 min. Both randomly and uniformly polymer-coupled preparations were clearly more stable with retention of 44 and 35% of activity, 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 [36]. 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, which is increased significantly at higher temperature [37, 38].

Further, we allowed polyclonal antibromelain antibodies to bind on both the polymer-coupled enzyme preparations. The thermostability was further improved remarkably on incubation with the antibodies. About 70 and 58% of the initial activity was retained by the antibody-bound uniformly coupled and randomly coupled preparations, respectively, even after 180-min incubation at 60°C. Specific polyclonal/monoclonal antibodies have

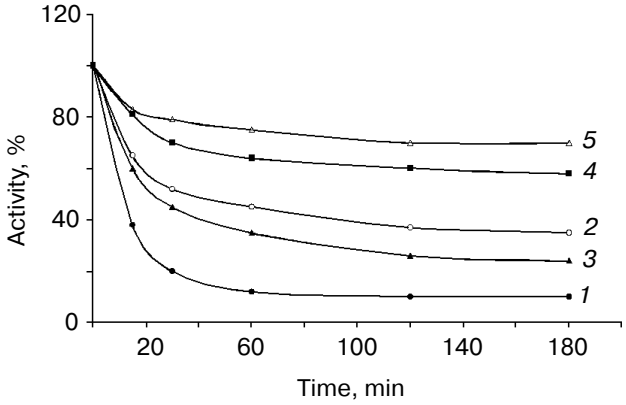

Fig. 3. Inactivation of native and p(NIPAm) coupled preparations at 60°C. Native bromelain and the preparations coupled to the polymer with or without incubation with antibromelain antibodies were incubated at various durations at 60°C and assayed under the standard conditions: *1*) native bromelain; *2*) bromelain randomly coupled to p(NIPAm); *3*) bromelain uniformly coupled to the p(NIPAm); *4*) bromelain randomly coupled to p(NIPAm) and incubated with antibodies; *5*) bromelain uniformly coupled to p(NIPAm) and incubated with the antibodies. Each value is the average of at least three independent experiments with variation not exceeding 4%.

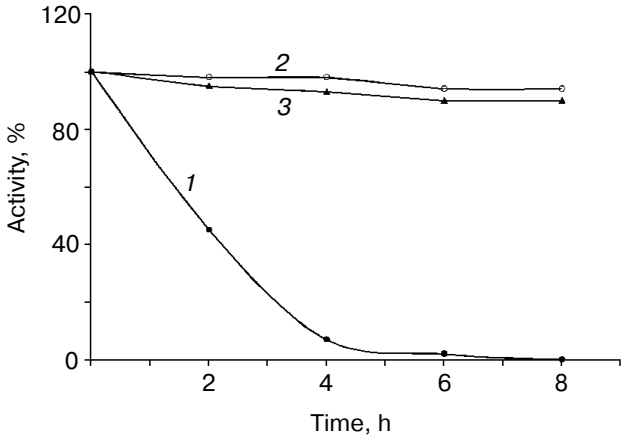

Fig. 4. Stability of activated native and p(NIPAm)-coupled preparations at 25°C. Native bromelain and the preparations coupled to the polymer were activated and incubated for various durations at 25°C and assayed under the standard conditions: *1*) native bromelain; *2*) bromelain randomly coupled to p(NIPAm); *3*) bromelain uniformly coupled to p(NIPAm). Each value is the average of at least three independent experiments with variation not exceeding 4%.

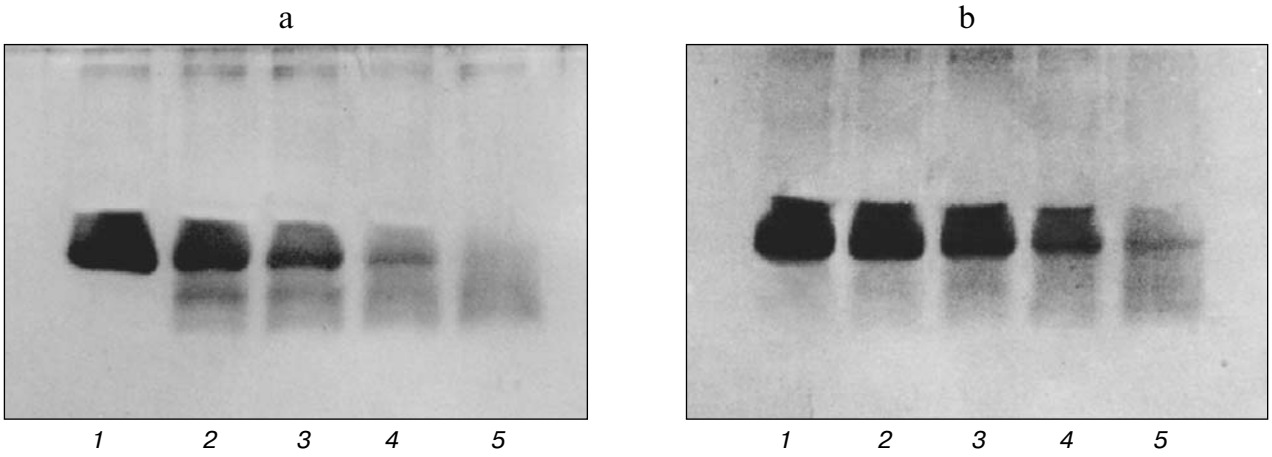

Fig. 5. Degradation of hemoglobin by native bromelain (a) and that randomly coupled to p(NIPAm) (b). The enzyme preparations were incubated with hemoglobin as described in the text and subjected to SDS-PAGE. Lanes *1-5* contain samples incubated for 0, 2, 4, 8, and 12 h, respectively.

been successfully used to provide increased stability of 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 [39-41].

The possible role of restricted autolysis in the inactivation of bromelain is supported by the data of Fig. 4. At 25°C, not only the polymer-coupled preparations are 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 denaturation. As shown in the figure, while native bromelain lost nearly all activity in 5 h, the immobilized preparations retained more than 90% activity. While it is true that enzyme–enzyme interaction resulting in autolysis in the p(NIPAm)-coupled preparation may be highly restricted above LCST [11], because of the large molecular nature of the polymer autolysis may be quite low even at temperatures where the polymer is not insoluble. Also, below the polymer LCST it may form a protective colloid-like hydrated surface layer that may restrict autolysis and or unfolding [11].

Coupling of bromelain to the p(NIPAm) did not significantly affect the LCST of the polymer, which was 30°C. The LCST values were also only marginally affected by binding of the antibromelain antibodies. Earlier studies have also revealed that coupling of proteins to p(NIPAm) does not alter the LCST significantly [8, 42].

Digestion of hemoglobin and IgG. The activity of p(NIPAm)-coupled bromelain preparations to digest denatured and native protein was investigated in order to examine possible alterations in its proteolytic activity. As shown in Figs. 5a and 5b, hemoglobin was nearly completely digested to small molecular weight peptides both by the soluble bromelain and that coupled to p(NIPAm) via the amino groups, respectively. Similar results were obtained with the preparation coupled via the oligosaccharide chain (data not shown). Bromelain has been shown to be ineffective in complete digestion of human IgG [43]. This has been ascribed to the presence of IgG subclasses that are not susceptible to bromelain cleavage. Figure 6 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 p(NIPAm) does not result in marked alteration in its specificity and ability to degrade proteins.

To conclude, these studies suggest that bromelain linked to p(NIPAm) either through amino groups or the

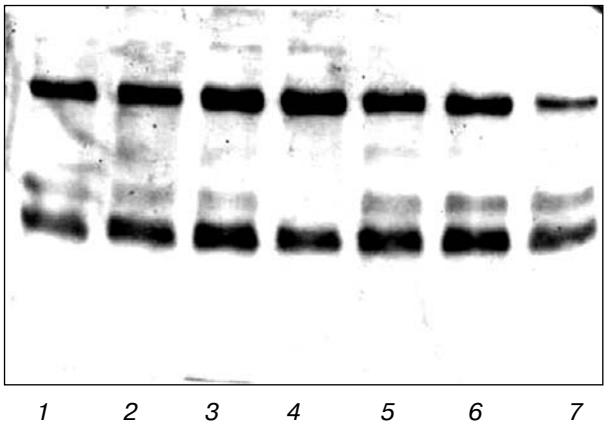

Fig. 6. Digestion of IgG by native and p(NIPAm)-coupled bromelain. The IgG were incubated with native or randomly coupled to p(NIPAm) bromelain and subjected to SDS-PAGE. Lanes: *1-3*) IgG incubated with soluble enzyme for 24, 12, and 4 h, respectively; *4*) IgG; *5-7*) IgG incubated with bromelain coupled to p(NIPAm) via the amino groups for 4, 12, and 24 h, respectively.

lone oligosaccharide chain remains accessible for action on protein substrates below and above LCST, although the later appeared more accessible. The enhanced stability of the polymer-coupled preparations against heat inactivation could be markedly improved by binding of specific antibody. We have used bromelain, which because of the presence of the lone oligosaccharide chain offers a unique opportunity for favorably oriented immobilization, without the need for chemical or genetic modification. Although the procedure used in the study for coupling via the oligosaccharide chain is expected to link the enzyme at more than one position on the polymer, it is however possible to restrict the coupling only through a terminal reactive group of p(NIPAm), such that each molecule is only at one end of the polymer [7]. These studies suggest that the advantages of favorable uniform orientation of enzyme on support [29] are also evident when the support is a reversibly soluble polymer like p(NIPAm).

The authors are grateful to the Department of Science and Technology, Government of India, for its support under the FIST program. R. M. is grateful to CSIR for the award of a fellowship.

REFERENCES

1. Galaev, I. Y., and Mattiasson, B. (1999) *TIBTECH*, **17**, 335-340.
2. Hoffman, A. S. (2000) *Clin. Chem.*, **46**, 1478-1486.
3. Hoffman, A. S., et al. (2000) *Founder's Award, Sixth Biomaterials Congress 2000*, Kamuela, HI, May 15-20.
4. Schild, H. G. (1991) in *Water-Soluble Polymers* (Shalaby, S. W., McCormic, C. L., and Butler, G. B., eds.) ACS Press, Washington, DC, pp. 249-260.
5. Feil, H., Bae, Y. H., Feijen, J., and Kim, S. W. (1993) *Macromolecules*, **26**, 2496-2500.
6. Uludag, H., Norvie, B., Kousinioris, N., and Gao, T. (2001) *Biotechnol. Bioeng.*, **73**, 510-521.
7. Chen, G., and Hoffman, A. S. (1992) *J. Biomaterial Sci. Polymer Edn.*, **5**, 371-382.
8. Chen, G., and Hoffman, A. S. (1993) *Bioconj. Chem.*, **4**, 509-514.
9. Mori, S., Nakata, Y., and Endo, H. (1994) *Protein Express. Purif.*, **5**, 153-156.
10. Ding, Z., Chen, G. H., and Hoffman, A. S. (1996) *Bioconj. Chem.*, **7**, 121-125.
11. Ding, Z., Chen, G., and Hoffman, A. S. (1998) *J. Biomed. Material Res.*, **39**, 498-505.
12. Matsukata, M., Takei, Y. G., Aloki, T., Sanul, K., Ogata, N., Sakurai, Y., and Okano, T. (1994) *J. Biochem.*, **116**, 682-686.
13. Taniguchi, M., Hoshino, K., Watanabe, K., Sugai, K., and Fujii, M. (1992) *Immunol. Bioeng.*, **39**, 287-292.
14. Park, T. G., and Hoffman, A. S. (1988) *J. Appl. Biochem. Biotechnol.*, **19**, 1-9.
15. Chilkoti, A., Chen, G. H., Stayton, P. S., and Hoffman, A. S. (1994) *Bioconj. Chem.*, **5**, 504-507.
16. Allard, L., Cheynet, V., Oriol, G., Veron, F., Merlier, G., Scremin, G., and Mallet, G. (2001) *Bioconj. Chem.*, **12**, 972-979.
17. Murachi, T., Suruki, A., and Takahashi, N. (1967) *Biochemistry*, **6**, 3730-3736.
18. Scocca, J., and Lee, Y. C. (1969) *J. Biol. Chem.*, **244**, 4852-4863.
19. Gupta, P., and Saleemuddin, M. (2007) *Biotechnol. Lett.*, in press.
20. Zhu, Q.-Z., Lin, F., Xu, J.-G., Su, W., and Hunan, J. (1998) *Analyst*, **123**, 1131-1134.
21. Murachi, T., Yasuda, Y., and Takahashi, N. (1971) *Biochemistry*, **10**, 2624-2630.
22. Dubois, M., Gilles, A. K., Hamilton, J. K., Rebes, P. A., and Smith, F. (1956) *Analyt. Chem.*, **28**, 350-356.
23. Lowry, O. H., Rosebrough, N. J., Farr, A. I., and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-267.
24. Murachi, T., Yasui, M., and Yasuda, Y. (1964) *Biochemistry*, **3**, 48.
25. Catty, D., and Raykundalia, C. (1988) *Antibodies – A Practical Approach* (Catty, D., ed.) Vol. 1, IRL Press, p. 50.
26. Vanhoof, G., and Cooreman, W. (1997) in *Pharmaceutical Enzymes* (Lawers, A., and Scharpe, S., eds.) Marcel Dekker Inc., New York, p. 131.
27. Jafri, F., and Saleemuddin, M. (1997) *Biotechnol. Bioeng.*, **55**, 605-609.
28. Saleemuddin, M., and Hussain, Q. (1991) *Enzyme Microb. Technol.*, **13**, 290-295.
29. Turkova, J. (1999) *Chromatography, B*, **722**, 11-31.
30. Liu, J., Wang, J., Bachas, L. G., and Bhattacharya, D. (2001) *Biotechnol. Progr.*, **17**, 866-871.
31. Wilchek, M., and Miron, T. (2003) *J. Biochem. Biophys. Meth.*, **55**, 67-70.
32. Mateo, C., Abian, O., Bernedo, M., Cuenca, E., Fuentes, M., Fernandez-Lorente, G., Palomo, J. M., Gazu, V., Pessela, B. C. C., Giacomini, C., Irazoqui, G., Villarino, A., Ovsejevi, A., Batista-Viera, F., Fernandez-Viera, F., Fernandez-Lafuente, R., and Guisan, J. M. (2005) *Enzyme Microb. Technol.*, **37**, 456-462.
33. Mienglo, I., Moreira, M. T., Palma, C., Guisan, J. M., Fernandez-Lafuente, R., Feijoo, G., and Lema, J. M. (2003) *Enzyme Microb. Technol.*, **32**, 769-775.
34. Hsiao, H., and Royer, G. P. (1979) *Arch. Biochem. Biophys.*, **198**, 379-385.
35. Woodward, J., and Wiseman, A. (1978) *Biochim. Biophys. Acta*, **527**, 8-16.
36. Iqbal, J., and Saleemuddin, M. (1983) *Biotechnol. Bioeng.*, **25**, 3191-3195.
37. Lee, H., and Park, T. G. (1998) *Biotechnol. Progr.*, **14**, 508-516.
38. Vlasov, G. P., Nikonava, I. N., and Illarionova, N. G. (1981) *Prikl. Biokhim. Mikrobiol.*, **17**, 494-499.
39. Shami, E. Y., Rothstein, A., and Ramjeesingh, M. (1989) *Trends Biotechnol.*, **7**, 186-190.
40. Shami, E. Y., Ramjeesingh, M., Rothstein, A., and Zywulko, M. (1991) *Enzyme Microb. Technol.*, **13**, 424-429.
41. Saleemuddin, M. (1999) *Adv. Biochem. Eng. Biotechnol.*, **6**, 203-206.
42. Fong, R. B., Ding, Z., Hoffman, A. S., and Stayton, P. S. (2002) *Biotechnol. Bioeng.*, **79**, 271-276.
43. Vidal, G. P., and Sasaki, M. (1973) *J. Biochem.*, **74**, 497-503.