Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 45 (2007) 78–83
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Oriented immobilization of stem bromelain via the lone histidine on a metal affinity support

Pawan Gupta [1], Tariq Maqbool [2], M. Saleemuddin *

Department of Biochemistry, Faculty of Life Sciences and Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India

Received 8 November 2006; received in revised form 13 December 2006; accepted 18 December 2006
Available online 23 December 2006

Abstract

Bromelain is a basic, 23.8 kDa thiol proteinase obtained from the stem of the pineapple plant (*Ananas comosus*) and is unique for it contains a single histidine residue (His-158) in the polypeptide. Based on the technology of protein separation with immobilized metal ion affinity chromatography (IMAC), a method for oriented immobilization of bromelain was selected. Bromelain was successfully immobilized on iminodiacetic acid carrier Sepharose 6B. Cu^{2+} complexed with iminodiacetate (IDA) was used as the chelating ligand to bind the lone histidine on bromelain. Simultaneously, preparation of a high affinity immobilized preparation was attempted using a soluble cross-linked preparation of bromelain on Cu-IDA-Sepharose. However this second method proved unsuccessful, possibly due to poor histidine accessibility in the cross-linked preparation. The immobilized preparation obtained using uncrosslinked bromelain was more resistant to thermal inactivation, as evidenced by retention of over enzyme 50% activity after incubation at 60 °C, as compared to 20% retained by the native enzyme. The immobilized preparation also exhibited a broader pH-activity profile in acidic range. The native, immobilized and soluble cross-linked bromelain showed apparent Michaelis constant (K_m) values of 1.08, 0.42, 1.56 mg/ml, respectively, using casein as the substrate. While the maximum velocity (V_{max}) values of the soluble and immobilized preparations were comparable, cross-linked preparation showed a 20% decrease, suggesting inactivation. The mild conditions used for predominantly oriented immobilization exploiting the unique property of single histidine, the high recovery of immobilized preparations, the stability, reusability and the regenerability of the matrix are the main features of the method reported here.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Metal affinity; Oriented immobilization; Stem bromelain; Histidine

1. Introduction

Traditional procedures of enzyme immobilization involve attachment of enzymes to supports via amino acid side chain groups, e.g., amino, carboxylic, –SH, etc. Since such groups occur in multiple numbers in most enzymes, immobilization often results in heterogeneous preparations bearing enzymes in a variety of orientations. The performance of immobilized enzymes can be improved by uniformly orienting them favorably on solid supports [1–3]. The strategies currently used for this purpose include the use of monoclonal antibody supports, immobilized metal ions or avidin (streptavidin) on metal complexes and binding or coupling through lone ligands present naturally or introduced chemically or genetically [4–7].

Bromelain is well studied due to its relatively non-specific action on proteins and antiedemic, anti-inflammatory and coagulation inhibitory potential and is an enzyme of commercial utility [8–10]. Stem bromelain, isolated from the stem of the pineapple plant, is a thiol proteinase with a molecular mass of 23.8 kDa and is unusual in having a single histidine residue at position 158 [11]. The lone histidine is not involved in the catalysis [12] and therefore provides a unique opportunity for favorable and uniform orientation of the enzyme without the need of any kind of modification. This paper describes a study in which bromelain is affinity-bound to IDA-Sepharose 6B matrix loaded with different metal ions. It also describes an attempt to obtain an oriented immobilized preparation of soluble cross-linked bromelain. The immobilization of native

* Corresponding author. Tel.: +91 571 272 0449; fax: +91 571 272 1776.
E-mail address: pawan_g75@hotmail.com (M. Saleemuddin).
[1] Current address: 6-120 Jackson Hall, 321 Church Street SE, Department of Pharmacology, Basic Science Biomedical Engineering, University of Minnesota, Minneapolis, MN 55455, USA.
[2] Current address: INSERM U. 384, Facult de Mdecine, 28 Place Henri Dunant, 63001 Clermont Ferrand, France.

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.12.002

bromelain was achieved, but the soluble cross-linked procedure proved untenable. The implications of this are discussed. The stability properties of the immobilized preparation are compared to those of native enzyme, as well as the soluble cross-linked preparation.

2. Materials and methods

Bromelain (EC 3.4.22.32) and IDA-Sepharose 6B were from Sigma. Other chemicals were of analytic grade and purchased from Sisco Research Laboratory, Bombay, India.

2.1. Preparation of cross-linked stem bromelain

Appropriate units of bromelain were incubated with 1% (w/v) glutaraldehyde in 0.2 M sodium phosphate buffer, pH 7.0 for 2 h at room temperature with constant stirring. The cross-linked preparation was treated with 1% (w/v) ethanolamine/glycine for 1 h to neutralize the residual aldehydic groups. The cross-linked preparation was then subjected to extensive dialysis to remove excess glutaraldehyde and ethanolamine/glycine.

2.2. Immobilization of soluble/cross-linked stem bromelain on immobilized metal ion support

IDA-Sepharose 6B was thoroughly washed with distilled water and loaded with various divalent metal ions by the procedure described in the Instruction Bulletin of Pharmacia Biotech, Uppsala, Sweden (1997). Briefly, 1.0 ml of chelating Sepharose was loaded with equal volumes of 0.1 M solutions of $CuCl_2$, $NiCl_2$ or $ZnCl_2$ and stirred at room temperature for a period of 30 min. The matrix was washed four times with equal volumes of distilled water and finally with 0.2 M sodium phosphate buffer, pH 7.0. The matrix was then mixed with an 11,550 units of soluble/cross-linked bromelain preparation and stirred at room temperature for 2 h. The matrix was centrifuged at 2000 rpm for 30 s and the supernatant collected. The matrix was finally washed repeatedly with equal volumes of 0.2 M sodium phosphate buffer, pH 7.0, until no detectable activity remained in the washings. Finally the matrix was suspended in the same buffer. The immobilized enzyme units were calculated by subtracting the remaining units in the supernatant and washings from those added to the chelating Sepharose matrix. Alternate attempt to immobilization; pre-treatment of the enzyme solution with Cu^{2+} ions and subsequent immobilization of bromelain-metal ion conjugate to the matrix could not be used with merit, as there was significant inactivation of enzymes.

2.3. Caseinolytic assay of stem bromelain

Proteolytic activity of bromelain was measured using casein as a substrate [13]. The standard incubation mixture contained 0.2 M sodium phosphate buffer, pH 7.5, 5.0 mM L-cysteine, 0.1 M KCl and appropriate quantities of bromelain in a final volume of 0.5 ml. Enzyme preparations were pre-incubated for 5 min at 37 °C and the reaction was initiated by addition of 0.5 ml of 1% (w/v) casein. The reaction was stopped with 1 ml of 10% (w/v) trichloroacetic acid. The resulting soluble peptides were quantitated by the procedure of Lowry. Activity of immobilized bromelain was determined in a similar manner except that the reaction mixture was continuously stirred during the reaction. Appropriate aliquots containing nearly equal numbers of enzyme units of native (free) and various immobilized preparations were taken for comparison of various stability parameters. One unit of bromelain activity was considered the amount that resulted in a change of 0.001 absorbance units (at 670 nm) per min. Activity assay were calibrated in adaptation to Kunitz [14].

2.4. Determination of protein concentrations

Protein concentrations were determined either spectrophotometrically at 280 nm or by the method of Lowry, using BSA as the standard. Protein concentrations of stem bromelain were determined using an extinction coefficient $\varepsilon^{1\%}_{1\,\mathrm{cm},280\,\mathrm{nm}} = 20.1$ [15]. The molecular mass of stem bromelain was taken as 23,800 Da [11].

2.5. Agitational effect, reusability and stability

Mechanical stability of Cu-IDA-Sepharose immobilized bromelain preparation was investigated on a rotary shaker by agitation at 2000 rpm for different time intervals. For reusability experiments standard incubation mixture was incubated with immobilized matrix as detailed in Section 2.3. Immobilized matrix was separated from soluble protein/digested peptides by centrifugation at 2000 rpm for 30 s. Supernatant was collected, precipitated with 10% TCA followed by estimation of soluble peptides. The matrix was reused after single wash with 0.2 M sodium phosphate buffer, pH 7.5. For shelf life, equal aliquots were incubated at 4 °C for different time intervals and then assayed for activity. Elution of bromelain bound to Cu-IDA-Sepharose was monitored in presence of 10 mM EDTA.

2.6. Effectiveness factor

The effectiveness factor (η) of the immobilized preparation represents the ratio of actual to theoretical activity of the immobilized enzyme [16]. The actual activity value of the enzyme was determined by assaying an appropriate aliquot of the immobilized preparation. The theoretical activities of enzyme preparations were calculated by subtracting the soluble enzyme units remaining (after immobilization) from that added for immobilization. The η of an immobilized enzyme is, among others, a measure of internal diffusion effects and reflects the efficiency of immobilized enzyme activity. Binding capacity on the other hand reflects the ratio of protein bound to that added. Binding capacity is a measure of the efficiency of the binding of the enzyme to its respective carrier.

Table 1
Immobilization of bromelain on IDA-Sepharose charged with different divalent metal ions[a]

Immobilization procedure	Units added/ml gel	Units in wash/ml gel	Bound bromelain (units/ml gel)		Effectiveness factor (η) (B/A)
			Theoretical (A)[a]	Actual (B)[b]	
Br-Cu^{2+}-IDA-Sepharose	11,550	1861	9689	6298	0.65
Br-Ni^{2+}-IDA-Sepharose	11,550	3046	8504	4082	0.48
Br-Zn^{2+}-IDA-Sepharose	11,550	3550	8000	3040	0.38

Each value represents the mean of three independent experiments performed in duplicate. Standard errors of mean were within limits of 8% to the average value. η values of affinity bound to covalently coupled preparations were statistically significant at $p < 0.05$.

[a] Determined by subtracting the number of units of enzyme remaining in the supernatant and washings after incubation from those added.

[b] Determined by assaying appropriate aliquots of the immobilized enzyme under standard assay conditions with continuous agitation.

3. Results and discussion

3.1. Immobilization of bromelain on IDA-Sepharose 6B charged with various divalent metal ions

Binding of bromelain was performed on IDA-Sepharose loaded with different divalent metal ions, i.e., Cu^{2+}, Ni^{2+}, and Zn^{2+}. Enzyme activity measurements revealed a high η for bromelain-Cu-IDA-Sepharose, followed by bromelain-Ni-IDA-Sepharose and bromelain-Zn-IDA-Sepharose, with values of 0.65, 0.48 and 0.38, respectively (Table 1). Further (Table 1), when equal amounts of enzyme units were added to IDA-Sepharose charged with various divalent metal ions, maximum immobilization yield/binding capacity was observed for bromelain-Cu-IDA-Sepharose (84%), followed by bromelain-Ni-IDA-Sepharose (74%) and bromelain-Zn-IDA-Sepharose (69%).

The high η obtained in the case of bromelain-Cu-IDA-Sepharose can be explained in light of the fact that although the histidine side chain dominates in binding to the chelated metal ion, free –SH groups of bromelain may also contribute to the binding. While imidazole has an affinity 15 times higher for Cu^{2+} [17,18] than other metal ions, IDA-Sepharose loaded with Cu^{2+} may predominantly bind with the single histidine at position 158 [11]. A fraction of IDA-Sepharose loaded with Ni^{2+} or Zn^{2+} may also bind bromelain via the lone free –SH group at the active site, causing some inactivation of the enzyme. The overall low η value obtained in immobilized preparations is explainable by the high exclusion limit of the support (10–4000 kDa), which results in the binding of bromelain protein moieties (23.8 kDa) not only at the surface but also in the interior of beads, thereby causing some minimal internal diffusional limitations [19]. Use of a macromolecular substrate may further contribute to the steric hindrance.

Maximal binding capacity in the case of bromelain-Cu-IDA-Sepharose can be well explained in light of the work of Porath [20], which states that the affinity of different metal ions complexed with IDA for protein follows the order Cu(II) > Ni(II) > Zn(II)–Co(II). Our observed order is in agreement with these works. This may be due to a maximum number of effective coordination sites available for interaction with proteins in the above order.

The above results indicate that Cu-IDA-Sepharose is the most suitable adsorbent for immobilization of bromelain. It has a higher effectiveness factor/immobilization yield for proteins than Ni-IDA-Sepharose or Zn-IDA-Sepharose. Moreover, by virtue of the single histidine in bromelain, it provides by default a predominantly oriented immobilized preparation. An absolute oriented preparation cannot be expected, as N-terminal amino groups as well as single free –SH groups may contribute insignificantly towards binding to chelated Cu^{2+}. However, histidine side chains may dominate in bromelain binding, due to a higher affinity of imidazole for Cu^{2+}. This study also suggests that, in bromelain, histidine is present at the surface in a manner that is available for binding to the IDA-support. Moreover, despite its presence in the vicinity of the active site [21], immobilization through this residue presumably does not interfere with catalytic activity, as has been suggested by Murachi et al. [12].

3.2. Immobilization of native and cross-linked bromelain preparations on Cu-IDA-Sepharose

Based on the above study, we chose Cu-IDA-Sepharose as an adsorbent matrix for the immobilization of native and cross-linked preparation of bromelain. Cross-linked preparations of bromelain were obtained as detailed in the Methods section. We hypothesized that use of a cross-linked immobilized preparation would bind strongly to the matrix due to multiple histidines present and provide a more useful preparation.

SDS-PAGE (reducing) of native and cross-linked (soluble) bromelain ascertained the near-homogeneity of the enzyme preparations and ensured that no autolysis had taken place (Fig. 1a). Bromelain migrated as a single band corresponding to a molecular mass of 23.4 kDa (Fig. 1b), a value close to that reported [11]. The cross-linked preparation obtained showed a slower migration with a rather diffused band corresponding to an average molecular mass of 48 kDa (Fig. 1, inset) suggesting it may be a dimer.

Attempts were made to immobilize native and cross-linked enzyme preparations on Cu-IDA-Sepharose. While the native preparation showed impressive immobilization (Table 1), the cross-linked preparation failed to bind significantly to the adsorbent. This inability of the cross-linked preparation to bind to Cu-IDA-Sepharose may be due to masking or shielding of histidine residues due to cross-linking via glutaraldehyde. This may in turn be due to proximity of lysine residues to histidine in the cross-linked preparation.

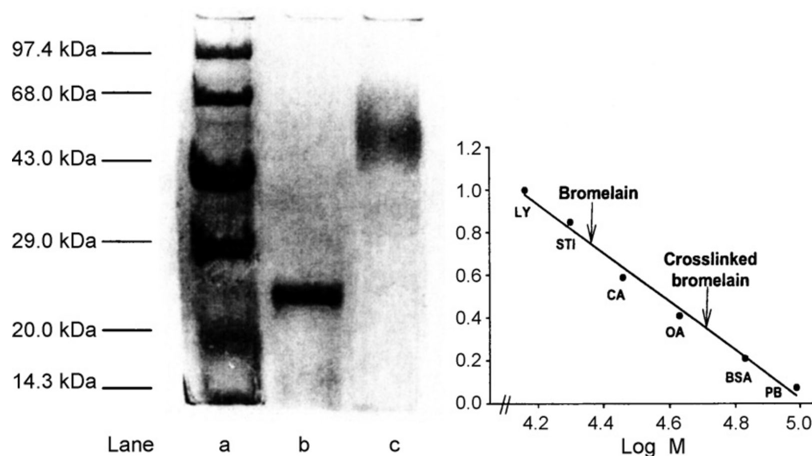

Fig. 1. SDS (reducing) gel electrophoresis of native and soluble cross-linked bromelain preparations and determination of molecular weight. The electrophoretic pattern of native and cross-linked preparations of IgG in 12.5% SDS (reducing) gel are shown. About 0–5 μg of each protein was electrophoresed on slab gels and stained with silver nitrate (cross-linked preparations fail stain with Coomassie brilliant blue). Lane a: molecular weight markers; lane b: native bromelain; lane c: cross-linked bromelain. (Inset) Molecular weight determination of native and cross-linked bromelain by the theoretical treatment of Weber and Osborn.

3.3. Effect of temperature

Temperature activity profiles of native, Cu-IDA-Sepharose-immobilized and soluble/cross-linked bromelain preparations were studied. All the preparations were optimally active at 60 °C. While the immobilized preparation was slightly stable at higher temperatures, the cross-linked preparation exhibited significant stability compared to native enzyme (Fig. 2A). Native, Cu-IDA-Sepharose-immobilized and soluble/cross-linked bromelain preparations were investigated for their ability to resist inactivation induced by incubation at 60 °C. The temperature stability profiles at 60 °C as a function of different time intervals show significantly higher retention of activity by cross-linked and immobilized preparations over the native enzyme during the entire duration of the experiment; the effect was most marked in the case of the cross-linked enzyme. In native preparations, only 20 min were required for 50% inactivation. Cross-linked and immobilized preparations retained more than 50% of their residual activity even after exposure to 60 °C for 100 min. Immobilized and cross-linked preparations retained 50% and 70% activity, respectively, after 100 min (Fig. 2B).

The higher thermal stability in the case of the cross-linked preparation likely results from stabilization of the native enzyme at several places in the soluble enzyme–enzyme complex by virtue of inter- and intra-molecular cross-links, thereby preventing enzyme unfolding when subjected to heat stress. Stabilization of the immobilized preparation can be explained if the histidine is present at or near a temperature-labile region which, when coupled to Cu-IDA-Sepharose, blocks the unfolding of enzyme. Other enzymes immobilized on Cu-IDA-Sepharose have also shown impressive thermal stability [22]. Decreased autolysis may also contribute to the observed stabilization in both cross-linked and immobilized bromelain preparations [23,24].

3.4. Effect of pH

The activity profiles of native, Cu-IDA-Sepharose-immobilized and soluble/cross-linked bromelain preparations were investigated in 0.2 M sodium phosphate or Tris–HCl buffer of various pHs at 37 °C. Immobilized and cross-linked preparations differed in their optimum pH as compared to the native preparation (Fig. 2C). The optimum pH of native, cross-linked and immobilized preparations were 7.5, 8.0 and 6.0, respectively. These differences may be due to differences in the microenvironment of the preparations. Further, the cross-linked preparation was more active in the alkaline pH range, while the native and immobilized preparations could not survive the alkaline pH. Murachi and Yamazaki [25] reported no changes in the conformation of bromelain up to pH 10.0. Beyond that point, conformational changes occurred through at least two distinct stages, with a concomitant loss of activity. The extended stability of the cross-linked preparation in the alkaline range may be due to resistance to unfolding due to inter- and intra-molecular cross-linking.

3.5. Kinetics/agitational effect and reusability

Enzyme kinetics data evaluated by non-linear regression (Sigma Plot; Enzyme kinetic module, data not shown) indicate apparent K_m values ± standard errors of mean (S.E.M.) for native, Cu-IDA-Sepharose-immobilized and soluble/cross-linked bromelain preparations to be 1.08 ± 0.090, 0.42 ± 0.072, and 1.56 ± 0.129 mg/ml, respectively. These results suggest a high affinity for substrate in the case of the metal affinity-bound preparation. While the differences in the K_m values were only moderate, the low K_m value observed for the metal affinity-bound preparation relative to the native or even the cross-linked enzyme using a macromolecular substrate is difficult to explain. Possible explanations include favorable microenvironmental effects of the IDA-Sepharose support and/or favorable conformational changes resulting from the metal affinity binding. Also, better accessibility to the substrate on the IDA-Sepharose support due to enzyme orientation may contribute to this effect. The higher K_m value observed for the cross-linked preparation over the native and immo-

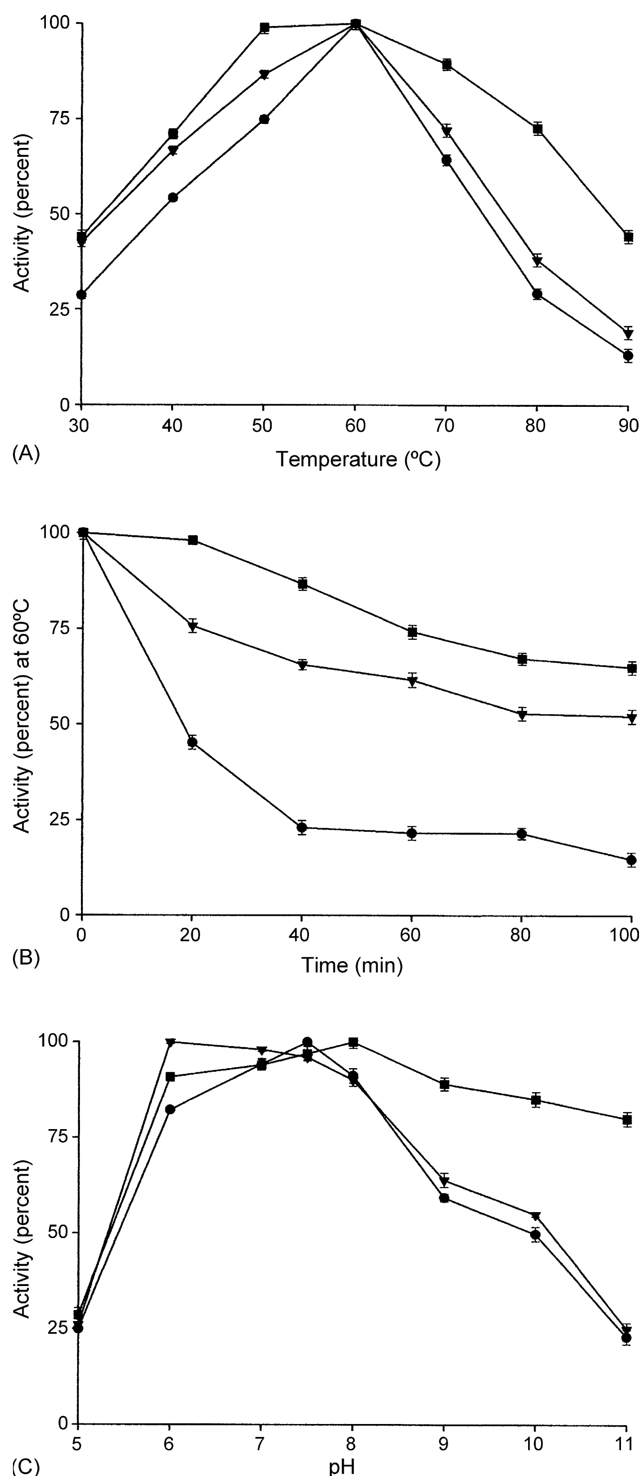

Fig. 2. Effects of temperature (A), pre-incubation at 60 °C on thermal inactivation (B) and pH (C) on activity profiles of native, Cu-IDA-Sepharose-immobilized and soluble/cross-linked bromelain preparations. Appropriate aliquots of native (●), immobilized (▼) and cross-linked (■) bromelain preparations were assayed for enzyme activity at indicated temperatures under standard conditions of pH and substrate concentration. The 100% activity corresponds to 25 enzyme units. Each point represents the mean of three experiments carried out in triplicate. Standard errors of mean did not exceed 1.90.

bilized preparations may be due to steric hindrance in the approach of substrate, caused by unfavorable inter- and intramolecular cross-linking. The V_{max} values of the soluble and the immobilized preparations were comparable. However, the V_{max} of the cross-linked preparation was 20% lower, suggesting inactivation.

Given the lone site of attachment of bromelain to the support in the metal affinity-bound preparation, it was considered essential to investigate the strength of the association of the enzyme to the metal affinity support. The effect of agitation at 2000 rpm for different time intervals on a rotary shaker was investigated for possible desorption of the bound bromelain preparation. Immobilized preparations displayed impressive mechanical stability with no significant loss in the enzyme activity of bromelain when agitated at 2000 rpm for 60 min (data not shown). The affinity-bound preparation was highly reusable, with only 25% loss in activity and/or leaching. It was also stable for several weeks at 4 °C. Elution of bromelain bound to Cu-IDA-Sepharose could be easily performed in the presence of 10 mM EDTA.

IDA-Sepharose loaded with the divalent metal ion Cu^{2+} is known to be a potential solid support for immobilization of enzymes/proteins of biotechnological relevance [5,20,26]. The remarkable stability of bromelain on Cu^{2+}-IDA-Sepharose is noteworthy, considering that binding of this protease apparently involves a single available histidine. Stem bromelain is an interesting protease that contains a single oligosaccharide chain [27,28], a free sulfhydryl group and three intrapeptide disulfide bonds [29–32]. We have also successfully attempted oriented immobilization of bromelain on a Sepharose-Con A support based on oligosaccharide–Con A interactions [31]. Considering the applications of bromelain on macromolecular substrates in both industrial and analytical settings, metal- and lectin-affinity support-based favorable oriented immobilization seems promising. Commercial/industrial use of bromelain is in the baking, beer industry [33] and in meat tenderizing industry [34] where low pH (6.0) and high temperature stability (10–50 °C) attributes are desirable. Given immobilized bromelain stability at low pH (shift in optimum to pH 6.0) and high temperature, it can sure be used with merit, with the added advantage that it can be recovered. Bromelain though physiologically beneficial [8–10] has implications if overdosed through its use in homogenous reaction phases, from where it cannot be recovered. Also, the oriented immobilized preparation of bromelain may provide an analytical tool to decipher mechanistic explanation to its function in physiology.

Acknowledgements

Facilities provided by Aligarh Muslim University are gratefully acknowledged. PG was Senior Research Fellow of the Council of Industrial Research, New Delhi, India. TM was Masters student, working for his Masters thesis with MS. The authors are also thankful to the Department of Science and Technology for providing lab facilities under its FIST programme and to the University Grants Commission for the DRS to the Department of Biochemistry.

References

[1] M. Saleemuddin, Adv. Biochem. Eng. Biotechnol. 64 (1999) 203.
[2] J. Turkova, J. Chromatogr. B: Biomed. Sci. Appl. 722 (1999) 11.
[3] H. Wang, J. Wu, J. Li, Y. Ding, G. Shen, B. Yu, Biosens. Bioelectron. 20 (2005) 2210.
[4] G. Chaga, Biotechnol. Appl. Biochem. 20 (1994) 43.
[5] L.L. Liu, L.X. Zeng, T. Liu, D. Le, Chin. J. Biotechnol. 21 (2005) 789.
[6] B. Solomon, Z. Hollander, R. Koppel, E. Katchalski-Katzir, Meth. Enzymol. 135 (1987) 160.
[7] H. Varshney, M. Saleemuddin, J.I. Rhee, K. Schugerl, Process Biochem. 37 (2001) 275.
[8] J.M. Braun, B. Schneider, H.J. Beuth, In Vivo 19 (2005) 417.
[9] H. Lotz-Winter, Planta Med. 56 (1990) 249.
[10] A.S. Meyer, C. Koser, J. Adler-Nissen, J. Agric. Food Chem. 49 (2001) 3644.
[11] G. Vanhoof, W. Cooreman, in: A. Lauwers, S. Scharpe (Eds.), Drugs and Pharmaceutical Sciences, Pharmaceutical Enzymes, vol. 84, Marcel Dekker Inc., (Bromelain) New York, 1997, p. 131, chapter 7.
[12] T. Murachi, T. Tsudzuki, K. Okumura, Biochemistry 14 (1975) 249.
[13] T. Murachi, M. Yasui, Y. Yasuda, Biochemistry 3 (1964) 48.
[14] M. Kunitz, J. Gen. Physiol. 30 (1947) 311.
[15] A. Arroyo-Reyna, A. Hernandez-Arana, Biochim. Biophys. Acta 1248 (1995) 123.
[16] J. Muller, T. Zwing, Biochim. Biophys. Acta 705 (1982) 117.
[17] R.J. Sundberg, R.B. Martin, Chem. Rev. 74 (1974) 471.
[18] T.T. Yip, Y. Nakagawa, J. Porath, Anal. Biochem. 183 (1989) 159.
[19] M. Farooqui, PhD Thesis, Department of Biochemistry, Faculty of Life Science, Aligarh Muslim University, Aligarh, India, 1999.
[20] J. Porath, Trends Anal. Chem. 7 (1988) 254.
[21] S.S. Hussain, G. Lowe, Biochem. J. 117 (1970) 341.
[22] P. Sosnitza, M. Farooqui, M. Saleemuddin, R. Ulber, T. Scheper, Anal. Chim. Acta 368 (1998) 197.
[23] F.H. Arnold, J.H. Zhang, Trends Biotechnol. 12 (1994) 189.
[24] S. Afaq, J. Iqbal, Elect. J. Biotechnol. 4 (2001) 1.
[25] T. Murachi, M. Yamazaki, Biochemistry 9 (1970) 1935.
[26] F.H. Arnold, Biotechnology (NY) 9 (1991) 151.
[27] T. Murachi, A. Suzuki, N. Takahashi, Biochemistry 6 (1967) 3730.
[28] J. Scocca, Y.C. Lee, J. Biol. Chem. 244 (1969) 4852.
[29] P. Gupta, R.H. Khan, M. Saleemuddin, Int. J. Biol. Macromol. 33 (2003) 167.
[30] P. Gupta, R.H. Khan, M. Saleemuddin, Arch. Biochem. Biophys. 413 (2003) 199.
[31] P. Gupta, M. Saleemuddin, Biotechnol. Lett. 28 (2006) 917.
[32] A. Ritonja, A.D. Rowan, D.J. Buttle, N.D. Raislings, V. Turk, A.J. Barrett, FEBS Lett. 247 (1989) 419.
[33] T.P. Lyons, Biochem. Soc. Trans. 10 (1982) 287.
[34] K.J. Prusa, E. Chambers IV, J.A. Bowers, F. Cunningham, A.D. Dayton, J. Food Sci. 46 (1981) 1684.