

Bioaffinity based oriented immobilization of stem bromelain

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Abstract Bromelain is a basic, 23.8 kDa thiol proteinase obtained from stem of the pineapple plant (*Ananas comosus*) and is unique in containing a single oligosaccharide chain attached to the polypeptide. This property allowed its affinity binding and favorable orientation on a Sepharose support pre-coupled with the lectin, concanavalin A (Con A). For comparison, bromelain was also immobilized by covalently coupling to the CNBr-activated Sepharose. The preparation obtained was more resistant to thermal inactivation as evident from the retention of over 50% activity after incubation at 60°C for 100 min (as compared to 20% retained by the native enzyme and 30% retained by the covalently immobilized enzyme), exhibited a broader pH-activity profile with the enzyme retaining over 60% activity at pH 11 (as compared to over 25% retained by native and the enzyme immobilized covalently). The native, covalently-coupled and affinity-bound

bromelains had apparent K_m values of 1.1, 2 and 0.54 mg/ml, respectively using casein as the substrate. The V_{max} values remained unaffected on immobilization.

Keywords Bromelain · Concanavalin A · Oligosaccharide · Stability

Introduction

Traditional procedures of enzyme immobilization involve attachment of enzymes to supports via amino acid side chain groups such as amino, carboxylic, -SH groups 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, however be improved by uniformly orienting them favorably on solid supports (Saleemuddin 1999; Turkova 1999; Wang et al. 2005). The strategies currently used for this purpose include the use of monoclonal antibody supports, binding or coupling through lone ligands introduced chemically or genetically (Chaga 1994; Solomon et al. 1987; Varshney et al. 2001).

Bromelain is well studied due to its relatively non-specific action on proteins and antiedemic, anti-inflammatory and coagulation inhibitory

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potential and is an enzyme of commercial utility (Braun et al. 2005; Lotz-Winter 1990; Meyer et al. 2001). Stem bromelain, isolated from the stem of pineapple plant, is a thiol proteinase with a molecular mass of 23.8 kDa and is unusual in containing a single oligosaccharide chain linked to the polypeptide chain (Murachi et al. 1967; Scocca and Lee 1969). The lone glycosyl chain does not seem to play any significant role in enzyme action 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 was affinity-bound to a Sepharose matrix precoupled with the lectin concanavalin A (Con A) and its behavior compared with a preparation coupled to cyanogen bromide activated support as well as the soluble enzyme.

Materials and methods

Bromelain (EC 3. 4. 22. 32), concanavalin A (Con A), α -D-methylmannopyranoside and Sepharose 4B were from Sigma. Other chemicals were of analytic grade and purchased from Sisco Research Laboratory, Bombay, India.

Immobilization of con A/stem bromelain on Sepharose 4B

Sepharose was activated as described by Porath et al. (1967). Appropriate amount of Con A/bromelain was added to the CNBr-activated Sepharose. The amount of protein/enzyme units immobilized was calculated by subtracting the amount of protein/enzyme units added to support from that remaining in the supernatant and washings. Stem bromelain coupled to Sepharose represent randomly immobilized preparation.

Activation of Sepharose 4B-bound Con A

Matrix bound Con A was activated by the method of Yariv et al. (1968). Con A-Sepharose was suspended in 0.1 M sodium acetate buffer, pH 5.2 containing 1 mM each of CaCl_2 , MgCl_2 , MnCl_2 and 0.15 M NaCl and stirred for 12 h in the cold.

Immobilization of stem bromelain on Con A-Sepharose 4B

Appropriate units of bromelain in 0.2 M sodium phosphate buffer, pH 7.0 were added to 2 ml of activated Con A-Sepharose and incubated overnight at 4°C. The bromelain-Con A Sepharose complex was then washed to remove unbound/excess bromelain, which was, quantitated for calculating bound enzyme units. Stem bromelain bound to Sepharose-Con A represent oriented immobilized preparation.

Caseinolytic assay of Stem bromelain

Proteolytic activity of bromelain was measured using casein as a substrate (Murachi et al. 1964). The standard incubation mixture contained 0.2 M sodium phosphate buffer of pH 7.5, 5 mM L-cysteine, 0.1 M KCl and appropriate quantities of bromelain in 0.5 ml. Enzyme preparations were pre-incubated for 5 min at 37°C and the reaction initiated by addition of 0.5 ml 1% (w/v) casein. Reaction was stopped after 30 min with 1 ml 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 number of enzyme units of native (free) and various immobilized preparation were taken for comparison of various stability parameters. One unit of bromelain was the amount that resulted in change of 0.001 absorbance (at 670 nm) per min, under the stated conditions. Activity assay were calibrated in adaptation to Kunitz (1947).

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 concentration of stem bromelain was determined using an extinction coefficient $\epsilon_{1\text{cm}, 280\text{nm}}^{1\%} = 20.1$ (Arroyo-Reyna and Hernandez-Arana 1995). The molecular mass of stem

bromelain was taken as 23,800 Da (Vanhoof and Cooreman 1997).

Effectiveness factor

The effectiveness factor (η) value of the immobilized preparation represents the ratio of actual to theoretical activity of the immobilized enzyme (Muller and Zwing 1982). 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. Effectiveness factor of an immobilized enzyme is, among others, a measure of internal diffusion effect and reflects the efficiency of immobilized enzyme activity.

Results and discussion

Immobilization of bromelain on Sepharose 4B/Sepharose 4B-coupled Con A

As shown in Table 1, the higher η value exhibited by the affinity bound bromelain preparation over the enzyme covalently coupled to Sepharose suggests high accessibility of enzyme to the casein substrate in case of the former. The Sepharose coupled Con A and the glycosyl chain may act as long spacers and contribute to the high access of the Con A bound enzyme. Good accessibility of

the glycoenzymes immobilized on Con A supports has been demonstrated in a number of earlier studies (Saleemuddin and Husain 1991). In addition, binding of stem bromelain on Con A via its lone oligosaccharide, as evident from its complete elution with α -methyl-D-glucoside (data not included), may also contribute to the high accessibility of the active site of the enzyme. Con A binding to bromelain-derived oligosaccharides has been previously studied by Wilson and Altmann (1998). Bromelain contains 15 lysine residues (Vanhoof and Cooreman 1997) and theoretically most of these may constitute points of contact with the activated Sepharose support, resulting in a preparation with random orientation of the enzyme. It is also not unlikely that coupling of the enzyme may be through one or multiple side chain amino groups. This may explain the relatively low access of bromelain coupled to the CNBr activated support towards casein.

Enzyme kinetics data evaluated by non-linear regression (Sigma Plot; Enzyme kinetic module) indicate apparent K_m values (standard error of means (SEM) for native, covalently coupled and affinity bound preparation to be 1.1 ± 0.083 , 1.95 ± 0.181 , 0.54 ± 0.063 mg/ml, respectively. The V_{max} values of soluble and the immobilized preparations were comparable. These results suggest high affinity for substrate in case of the affinity bound preparation. While the differences in the K_m values were only moderate, the observed low K_m value of affinity bound preparation over that covalently linked and even the soluble

Table 1 Immobilization of bromelain on -Con A-Sepharose and activated Sepharose

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)	
Affinity binding	11550	6870	4680	3002	0.64
Covalent coupling	11550	5600	5950	2666	0.45

Each value represents the mean of three independent experiments performed in duplicate. Standard error of means was within limits of 6% to the average value. η value of affinity bound to covalently coupled preparation 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

enzyme, on a macromolecular substrate is difficult to explain. Possible explanations include favorable micro environmental effects of the lectin support and/or favorable conformational change resulting from the affinity binding. Also better accessibility to substrate due to long Con A and oligosaccharide spacer between support and enzyme may contribute to the behavior.

Effect of temperature

Native bromelain and the covalently coupled preparation were optimally active at 60°C, but there was a shift in the optimum to 70°C in case of the affinity bound preparation (Fig. 1). The affinity bound preparation also retained greater fraction of maximum activity at high temperatures.

On incubation at 60°C the affinity-bound preparation retained more than 50% of initial caseinolytic activity after incubation for 100 min, while only 30% and 20% activity remained in the covalently coupled and native preparations respectively (Fig. 2). The number of covalent/non covalent linkages between enzyme and the support are known to influence the stability of the immobilized enzyme preparations (Iqbal and

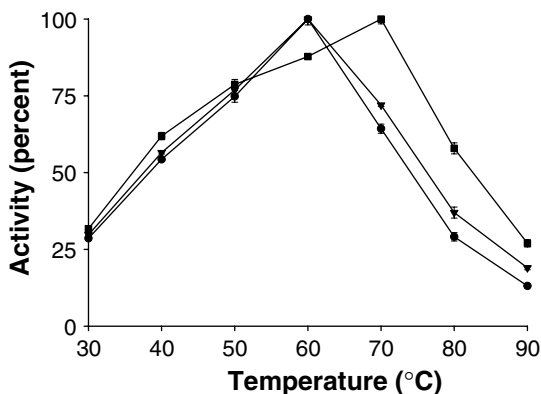


Fig. 1 Effect of temperature on native and immobilized bromelain preparations. Appropriate aliquots of native (●), covalently coupled (▼) and affinity bound (■) bromelain preparations were assayed for enzyme activity at indicated temperatures over 30 min under standard conditions of pH and substrate concentration. 100% activity corresponds to 16 enzyme units. Each point represents the mean of three experiments carried out in triplicate. Standard error of means did not exceed 1.9

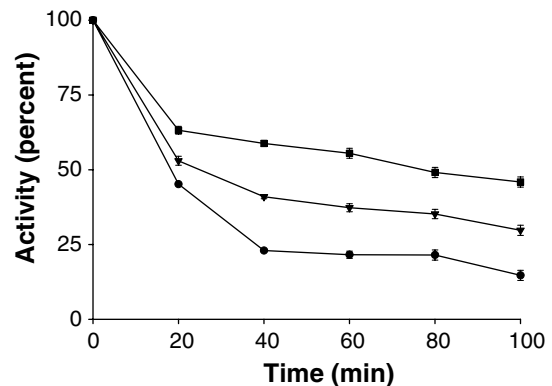


Fig. 2 Effect of pre-incubation at 60°C on thermal inactivation of native and immobilized bromelain preparations. Appropriate aliquots of native (●), covalently coupled (▼) and affinity bound (■) bromelain preparations were incubated at 60°C for indicated time duration, cooled to room temperature and residual activity determined under standard assay condition. Activity of aliquots not subjected to heat inactivation was taken as 100 for calculation of percent activity. 100% activity corresponds to 16 enzyme units. Each point represents the mean of three experiments carried out in triplicate. Standard error of means did not exceed 1.7

Saleemuddin 1983). Bromelain coupled to CNBr activated Sepharose is expected to be connected at several sites on the support due to presence of large number of side chain amino groups (Vanhoof and Cooreman 1997) and unfold less readily at high temperatures. In contrast, the affinity bound preparation is expected to be linked to the support through the lone glycosyl chain.

One possible explanation for the observed stability of the affinity bound preparation may be related to the ability of the affinity bound enzyme to refold after thermal denaturation. Reactivation of the heat denatured enzyme to their native form is facilitated if the polypeptide chain undergoes complete unfolding prior to its exposure to conditions that facilitate refolding (Husain et al. 1992; Klibanov and Mozhev 1978; Monsan and Combes 1988). Clearly the affinity bound preparation with its single connection with the support may have a greater possibility of undergoing complete unfolding and reactivation also due to lack of chemical modification of the constituent aminoacids during the immobilization process. Earlier studies from this and other laboratories have shown that several glycoenzymes attain

greater resistance to various forms of denaturation when immobilized via their glycosyl residues. A study also suggested that the labile region where unfolding of the enzyme may begin, may be located close to the glycosylation site (Ulbrich-Hofman et al. 1993) and blocking of the region leads to remarkable improvement in the stability. In case of the covalently coupled preparation however, attachment through such region may not be feasible forasmuch as side chain amino groups in the region may be masked by the oligosaccharide chains. Decline in activity profile of affinity bound preparation at temperature higher than 70°C may be due to the denaturation of Con A (Sanders et al. 1998), release and inactivation of soluble bromelain.

Effect of pH

Activity profiles of native, covalently coupled and affinity bound bromelain preparations were investigated in 0.2 M sodium phosphate or Tris/HCl buffer of various pH at 37°C. Optimum pH value of bromelain remained unaltered at pH 7.5 on immobilization (Fig. 3). However, unlike the covalently coupled preparation the affinity bound preparation displayed improved stability between

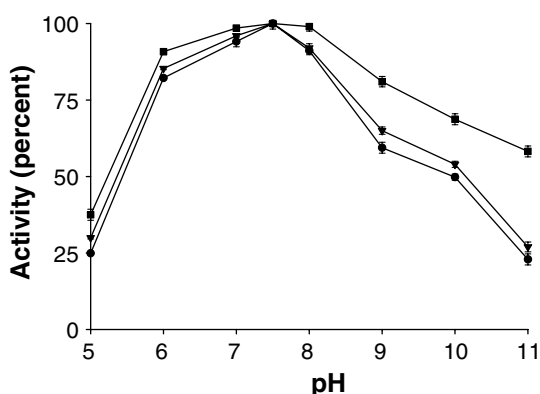


Fig. 3 pH activity profiles of native and immobilized bromelain preparations. Appropriate aliquots of native (●), covalently coupled (▼) and affinity bound (■) bromelain preparations were incubated in 0.2 M sodium phosphate buffer (pH 5–8) or 0.2 M Tris/HCl buffer (pH 9–11) and activity determined under standard assay condition. 100% activity corresponds to 16 enzyme units. Each point represents the mean of three experiments carried out in triplicate. Standard error of means did not exceed 1.8

pH 9.0 and 11.0, which is inconsistent with the model proposed by Murachi and Yamazaki (1970). They have shown that bromelain readily loses catalytic activity beyond pH 10.0. Retention of significant activity especially by the affinity bound preparation may be related to overall improvement in resistance to unfolding and/or facilitation of refolding of the enzyme.

Agitational effect and reusability

In view of the lone site of attachment of bromelain to the support in case of the affinity bound preparation, it was considered essential to investigate the strength of association of the enzyme with the Con A support.

The effect of agitation at 2,000 rpm for different time intervals on a rotary shaker was investigated for possible desorption of bound bromelain preparation. Affinity bound preparation displayed impressive mechanical stability with no significant loss in activity when agitated at 2,000 rpm for 60 min (data not shown). The affinity bound enzyme was also highly reusable with only 25% loss in activity after repeated use for 20 times and was also found to be stable for several weeks at 4°C (data not shown).

There have been few reports suggesting Con A-Sepharose to be an appropriate solid support to yield stable carrier-glycoenzyme species (Arnold et al. 1999; Froehner and Eriksson 1975; Lovenberg et al. 1975). The remarkable stability exhibited by bromelain immobilized on Con A support is interesting considering that binding of the protease apparently involves a single oligosaccharide-Con A interaction.

To the best of our knowledge, this report represents the first study in which an enzyme in its native state has been uniformly and favorably immobilized on a non-antibody support (Con A-Sepharose) without subjecting it to any kind of modification. It is interesting to observe that the uniformly oriented enzyme displays useful stability properties.

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