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Glutaraldehyde-Activated Chitosan Matrix for Immobilization of a Novel Cysteine Protease, Procerain B

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ABSTRACT: Proteases have several applications in the food industry. We report the immobilization of procerain B, a novel cysteine protease, on glutaraldehyde-activated chitosan beads through covalent attachment. Glutaraldehyde not only serves as a cross-linking agent but also links the procerain B on the surface of bead through primary amine group (either lysine side chain or N-terminal) by Schiff base linkage. Immobilized procerain B was characterized for optimum functional range and stability with respect to pH and temperature. The chitosan-immobilized procerain B has broad pH and thermal optima. The effects of substrate concentration and reusability of immobilized beads were also studied. It showed nearly 50% activity until the 10th use.

KEYWORDS: Chitosan, glutaraldehyde, cysteine protease, immobilization, FTIR and SEM

INTRODUCTION

Chitosan is the deacetylated product of chitin, the second most abundant polysaccharide after cellulose in nature.[1] Chitin, the main constituent of the outer skeleton of insects and crustaceans, yields chitosan after alkaline treatment for removal of acetyl groups.[2,3] Waste shells of shrimps, prawns, crabs, lobsters, and crayfish are generally used as raw materials for commercial production of chitin and chitosan. Recently, fungi and insect larvae are being explored as alternate sources of raw materials for chitin production.[4,5] Recombinant approaches have been applied to clone the gene responsible for production of chitosan. Because of its easy availability, biocompatibility, and inert and hydrophilic nature, it has several applications in different fields.[6−10] It can be used as an encapsulating agent for slow and prolonged release of drug[11−14] as a chelating agent for cleaning of industrial waste[15] in surgery and grafting[16−18] and in purification techniques as ion exchange and affinity chromatography.[19−21] Chitosan is an ideal matrix for immobilization of enzymes. It can be used in the form of a gel, membrane, bead, or powder. Hydroxyl and amino groups present in chitosan favor the immobilization process by adsorption and covalent linkage.[22−24]

Immobilized enzymes are more advantageous than their soluble forms, as there are multiple limitations of soluble enzymes, such as structural instability after isolation from the natural environment, narrow functional range, sensitivity toward a trace amount of inhibitors, and enzyme contamination of end product, mainly in food and pharmacy industries where ultrapure products are required. The use of immobilized enzymes can not only circumvent these problems but also has some extra advantages, such as repeated use of the same batch of enzyme and better control of the catalytic period.[25−30] Immobilized proteases can be used in the dairy industry for the production of cheese. Additionally, casein hydrolysate with a high free amino acids content can be produced by immobilized proteases. Immobilization is the attachment of enzymes with an inert insoluble matrix through which the substrate can easily pass and product can diffused out.

Proteases are enzymes that degrade polypeptides into smaller oligopeptides and amino acids. Five different catalytic types of proteases have been recognized, depending on the serine, threonine, cysteine, aspartic, or metallo group involved in enzyme catalysis.[31] Many industries are exploiting the hydrolytic property of proteases, mainly the food, detergent, pharmaceutical, and leather industries. Several proteases have been isolated and reported from different animal, plant, and microbial sources. Proteases from various parts of plants have been characterized, and most of them are found to be cysteine proteases. High-temperature stability and broad substrate specificity make plant cysteine proteases a preferred choice for industrial use.[31]

Proteases are thought to be involved in a range of biological processes, including senescence, perception, signaling, recycling of damaged proteins, and execution leading to plant defense.[32] We have previously reported a novel cysteine protease from the latex of a medicinal plant *Calotropis procera* with a molecular mass of 25.7 kDa and broad pH and temperature optima.[33] Because of its broader functional range, high thermal stability, and compatibility with detergents, it may have several industrial applications in food, detergent, dairy, and leather industries.[34] In the present study, we aim to immobilize the procerain B on chitosan beads through covalent linkages and characterize the immobilized product.

MATERIALS AND METHODS

Materials. The enzyme was purified from the latex of plant *C. procera* by the method of Singh et al.[33] Chitosan (with a 75% degree of deacetylation and molecular mass of 310 kDa), bovine serum albumin (BSA), azocasein, protease inhibitor, and Bradford reagent were purchased from Sigma Chemicals Co. (St. Louis, MO). Tris buffer, dialysis tubing, and glutaraldehyde were obtained from Fluka Biochemika (Germany). Trichloroacetic acid (TCA) was purchased from Hi-Media

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Laboratories (India). All other chemicals used were of the highest purity and commercially available. All reagents were prepared in Milli Q water (Millipore, United States).

Methods. *Protein Concentration.* The protein concentration at different stages of purification was determined by absorbance at 280 nm as well as by the method of Bradford[35] using BSA as a standard.

Protease Activity. The hydrolyzing activity of the protease was determined with denatured natural substrates casein and azocasein using the method of Dubey and Jagannadham.[36] The enzyme solution (5 μg) was incubated in a final volume of 500 μL of 50 mM Tris-HCl buffer, pH 7.5, at 37 °C for 10 min. Casein and azocasein solutions (1%) (w/v) were prepared in the same buffer at the same pH and added to the enzyme solution, making the final reaction volume to 1 mL, and the reaction mixture was incubated for 30 min at 37 °C. The reaction was stopped by adding 0.5 mL of 10% TCA, incubated further for 10 min at room temperature, and centrifuged (10000 rpm for 10 min). The absorbance of the soluble peptides in the supernatant was measured at 280 nm. In the case of azocasein, as a substrate, 0.5 mL of supernatant after TCA precipitation was mixed with an equal volume of 0.5 M NaOH and incubated for 15 min. The development of color was measured spectrophotometrically by taking the absorbance at 440 nm. A control assay, without the enzyme, was done and used as a blank in all spectrophotometric measurements. One unit of enzyme activity was defined as the amount of enzyme, under given assay conditions, that give rise to an increase of 0.001 units of absorbance at 280 nm or an increase of 0.001 units of absorbance at 440 nm per minute of digestion. The number of units of activity per milligram of protein was taken as the specific activity of the enzyme.

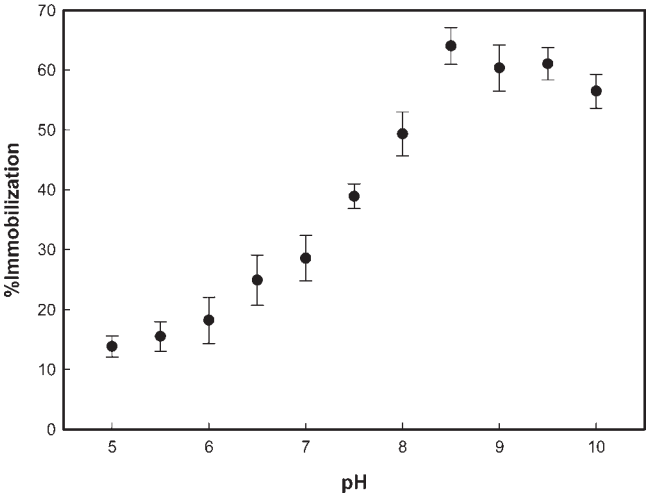

Figure 1. Effect of pH on immobilization of procerain B in the range of pH 5−10. The optimum pH for immobilization of procerain B was nearly 8.5 with 65% immobilization.

Preparation of Chitosan Beads. Because of the high solubility of chitosan at lower pH, the chitosan beads of uniform size were prepared by dissolving powdered Chitosan in milli Q water containing 1.5% (v/v) glacial acetic acid. The different concentrations (1.0−3.0%, w/v) of chitosan were tried for bead preparation, and the most appropriate concentration was used for further experiments. Uniform beads were prepared by casting the stock solution of chitosan with a 12 mL syringe. The syringe was fixed at appropriate height (from larger height the beads became disk shaped), and the stock solution was added dropwise in 1 N KOH solution containing 25% (v/v) ethanol under stirring to form spherical beads, and solution was allowed to stand for 1−2 h for ripening of beads. The beads of diameter nearly 2.0 mm and uniform shape obtained were immediately washed with Milli Q water and stored in water at 4 °C until activation with glutaraldehyde.

Immobilization of Protease. Before immobilization, the chitosan beads were activated with glutaraldehyde. The beads were treated with different concentrations of glutaraldehyde, in the range 0.5−3.0% (v/v) at 37 °C. After incubation, the beads were washed extensively with 50 mM Tris-HCl buffer, pH 8.5, for complete removal of unreacted glutaraldehyde until the absorbance was lower than 0.01 at 280 nm and stored in the same buffer until further use. For immobilization, the activated beads were incubated with varying concentrations of purified protease at 4 °C. The beads were washed with 50 mM Tris-HCl buffer, pH 8.5, to remove unbound protease. The activity and protein content (by Bradford method) of chitosan beads were determined. To determine the optimum time required for their activation, the beads were kept in 3% glutaraldehyde for different time intervals. Furthermore, the activated beads were incubated in desired concentration of protease

Table 1. Optimization of Immobilization Conditions for Procerain B on Glutaraldehyde-Activated Chitosan Beads

	glutaraldehyde concentration (%)	activation time (h)	coupling time (h)	protein concentration in immobilization mixture (mg/mL)	immobilization (%)	activity per bead
	1	2	20	0.2	58.33 ± 0.79	0.069 ± 0.006
variation of glutaraldehyde	2	2	20	0.2	72.58 ± 1.23	0.085 ± 0.013
concentration	3	2	20	0.2	84.39 ± 0.32	0.097 ± 0.015
	4	2	20	0.2	84.51 ± 0.62	0.099 ± 0.008
	3	0.5	20	0.2	66.05 ± 0.44	0.083 ± 0.009
variation of activation time	3	1	20	0.2	70.49 ± 0.93	0.090 ± 0.015
	3	2	20	0.2	84.56 ± 0.69	0.100 ± 0.023
	3	3	20	0.2	86.69 ± 0.41	0.102 ± 0.027
	3	4	20	0.2	85.17 ± 1.07	0.101 ± 0.017
	3	3	8	0.2	43.55 ± 0.35	0.052 ± 0.007
variation of coupling time	3	3	16	0.2	65.60 ± 0.92	0.069 ± 0.011
	3	3	24	0.2	86.98 ± 0.72	0.102 ± 0.031
	3	3	32	0.2	64.46 ± 0.45	0.068 ± 0.019
	3	3	24	0.2	87.50 ± 1.15	0.105 ± 0.015
variation of protein	3	3	24	0.4	74.02 ± 0.73	0.139 ± 0.087
concentration	3	3	24	0.6	48.60 ± 0.65	0.161 ± 0.073
	3	3	24	0.8	24.05 ± 1.98	0.163 ± 0.083

solution for different time intervals to determine the optimum coupling time.

The percent immobilization (percent enzyme activity retention) was calculated as follows:

$$\text{immobilization } (\%) = \frac{\text{total activity of immobilized protease}}{\text{total activity of soluble protease}} \times 100$$

The total activity of immobilized protease was determined by subtracting total activity of unbound protease from total activity of soluble enzyme.

Fourier Transform Infrared (FTIR) Spectra of Chitosan Beads. To confirm the activation of chitosan beads with glutaraldehyde, the FTIR spectra of normal chitosan beads and glutaraldehyde activated beads were taken with UNICAM Mattson 1000 FTIR spectrophotometer and compared. For FTIR spectra samples were crushed with potassium bromide (KBr) to form a very fine powder. This powder was then compressed into thin pellet for analysis.

Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) Analysis of Beads for Immobilization. The detailed surface morphology of the normal chitosan bead, glutaradehyde-treated beads, and immobilized beads were analyzed by SEM. Samples were subjected to gold coating, then imaged, and photographed by using SEM (LEO 1430 VP) operated at an acceleration voltage of 10.00 kV. An EDX spectrum was also collected to analyze percentage elemental composition of bead surface.

pH and Temperature Optima. The activity of the immobilized enzyme was measured as a function of varying pH to determine the pH optima of the immobilized procerain B. Seven beads were used for activity measurement. The buffers used were 0.05 M glycine-HCl (pH 2.0−3.5), 0.05 M Na-acetate (pH 4.0−5.5), 0.05 M Na-phosphate (pH 6.0−7.5), 0.05 M Tris-HCl (pH 8.0−10.0), and 0.05 M sodium carbonate (pH 10.5−12.5). The substrate solution of azocasein or hemoglobin was prepared in the respective buffers. Immobilized procerain B on beads was equilibrated in 0.5 mL of the buffer at a given pH for 15 min and added to the substrate solution of the same pH. The assay procedure was the same as described above. Because of the insolubility of azocasein below pH 4.0, hemoglobin was used as a substrate for activity measurements at lower pH.[37]

The effect of temperature on the activity of immobilized procerain B was also studied using azocasein as the substrate. Seven beads were incubated at the desired temperature in the range of 15−95 °C for 15 min in 50 mM Tris-HCl buffer, pH 7.5, and then used for the activity measurement at the same temperature. Prior to the assays, the substrate solution was also equilibrated at the corresponding temperature in the same buffer. At each temperature, a control assay was carried out without the enzyme used as a blank.

Stability. The ability of immobilized enzyme to retain its activity under various conditions such as extreme pH and temperatures was studied. Seven beads were incubated at different pH values in the range of 1−12 for 12 h at room temperature, and the residual activity was measured as described earlier using azocasein as the substrate. Similarly, beads were incubated at temperatures from 10 to 95 °C for 15 min and assayed for residual activity.

Effect of Substrate Concentration on the Reaction Velocity. The effect of increasing substrate concentration on the reaction velocity of the enzyme hydrolysis was studied using azocasein as substrate at pH 7.5 and 37 °C. Seven beads were used, and the concentration of azocasein was in the range of 1−160 μM. Assays were performed as already described under proteolytic activity measurements. A blank was used at the specific substrate concentrations without the enzyme. A Lineweaver−Burk plot was plotted, and the value of Michaelis−Menten constant (K_m) was calculated.

Reusability of Immobilized Protease. The reusability of the immobilized enzyme with repeated use of beads was tested. The assay was performed as described in the section, activity assays of protease. After each reaction, the chitosan beads with immobilized protease were removed and washed with 0.1 M Tris-HCl buffer, pH 7.5, to remove any residual substrate within the chitosan beads and stored in the same buffer at 4 °C until further use. They were then reintroduced into fresh reaction medium, and the enzyme activity was determined at optimum conditions.

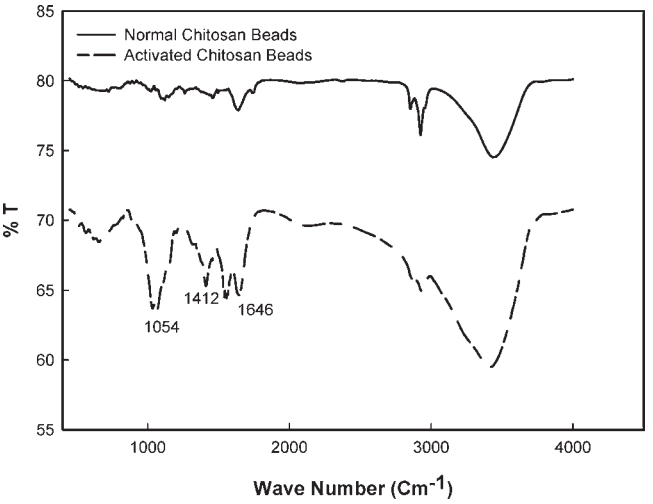

Figure 2. Comparison of FTIR spectra of normal chitosan beads (solid lines) and glutaraldehyde-activated beads (dotted lines) for confirmation of glutaraldehyde activation of beads. The peak at 1412 cm^{-1} represents the C−N bond and results in a cross-linking reaction. Other peaks at 1646 and 1054 cm^{-1} may attributed to C═N and polysaccharide structure.

RESULTS AND DISCUSSION

Enzymes have been used for industrial purposes for a long time, and proteases are one of the most important industrial enzymes. The use of immobilized enzymes rather than free form is more fascinating and offers added advantages. We have already studied the possible applications of newly discovered cysteine protease, procerain B.[34] Immobilization can further increase its applicability in various industries. In the present study, we investigated the optimal immobilization conditions for procerain B on chitosan beads and characterized the immobilized enzyme. The chitosan beads were prepared and then activated by glutaraldehyde. Glutaraldehyde not only activates the beads but also cross-links the chitosan to provide resistance against lower pH. The two terminal aldehyde groups of glutaraldehyde react with amino groups of D-glucosamine units of different chains, resulting in cross-linking of those chains through glutaraldehyde. The irreversible Schiff's base linking of aldehyde with amino group provides operational stability to beads. Spherical chitosan beads of uniform size were prepared, and the immobilization of procerain B on glutaraldehyde activated chitosan beads was successfully optimized (3% glutaraldehyde with 3 h of activation and 24 h of coupling time with a 0.2 mg/mL protein concentration in immobilization mixture), resulting in 87.50 ± 1.15% immobilization. The immobilized product was characterized for optimum functional range and stability. The immobilized procerain B has an optimum temperature of 45−65 °C and two pH optima (one in acidic and other in neutral pH range). The operational stability and reusability of immobilized procerain B were greater than soluble form.

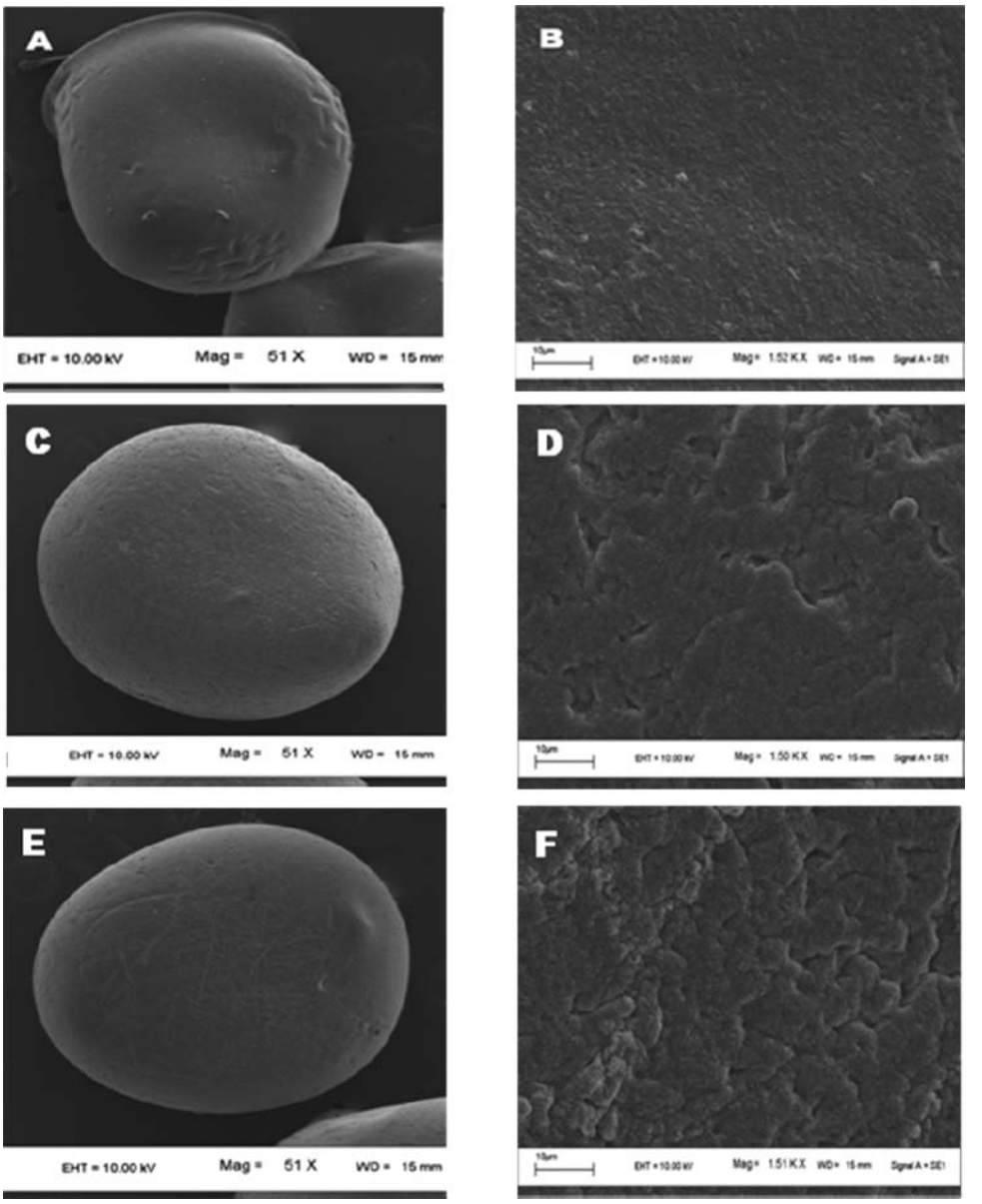

Figure 3. SEM images of beads and detailed surface view of (A and B) normal chitosan beads, (C and D) glutaraldehyde-activated chitosan beads, and (E and F) immobilized chitosan beads.

Effect of pH on Immobilization of Procerain B. pH is a key parameter for immobilization of enzyme through the amino group on a matrix. The ionization state of different functional groups present on the surface of any enzyme mainly depends on the pH of enzyme solution. The effect of pH on the immobilization of procerain B was studied in the range of pH 5−10. The optimum pH for immobilization of procerain B was nearly 8.5 with 65% immobilization (Figure 1). At lower pH, the percentage of immobilization is decreased due to solubility of chitosan at lower pH, and at higher pH, it may be due to an improper ionization stage of surface amino groups.

Optimization of Procerain B Immobilization on Chitosan Beads. The immobilization of procerain B on chitosan beads was optimized in terms of glutaraldehyde concentration for activation, time of activation, coupling time, and concentration of enzyme solution in immobilization mixture. Different concentrations of glutaraldehyde in the range of 1−4% were tried for the activation of chitosan beads, and the percentage immobilization of procerain B was observed; 3% glutaraldehyde was the most effective concentration with 84.39% immobilization (Table 1). Lower concentrations of glutaraldehyde were not sufficient to generate enough aldehyde groups on the surface of chitosan beads to immobilize most of the protease molecules, and at a higher concentration, the beads became fragile.

Table 2. EDX Analysis of Bead Surface[a]

atomic % of elements	A	B	C
C	52.78	52.42	49.28
N	9.95	8.99	15.86
O	37.27	38.59	34.86

[a] A, normal chitosan beads; B, glutaraldehyde-activated chitosan beads; and C, immobilized chitosan beads.

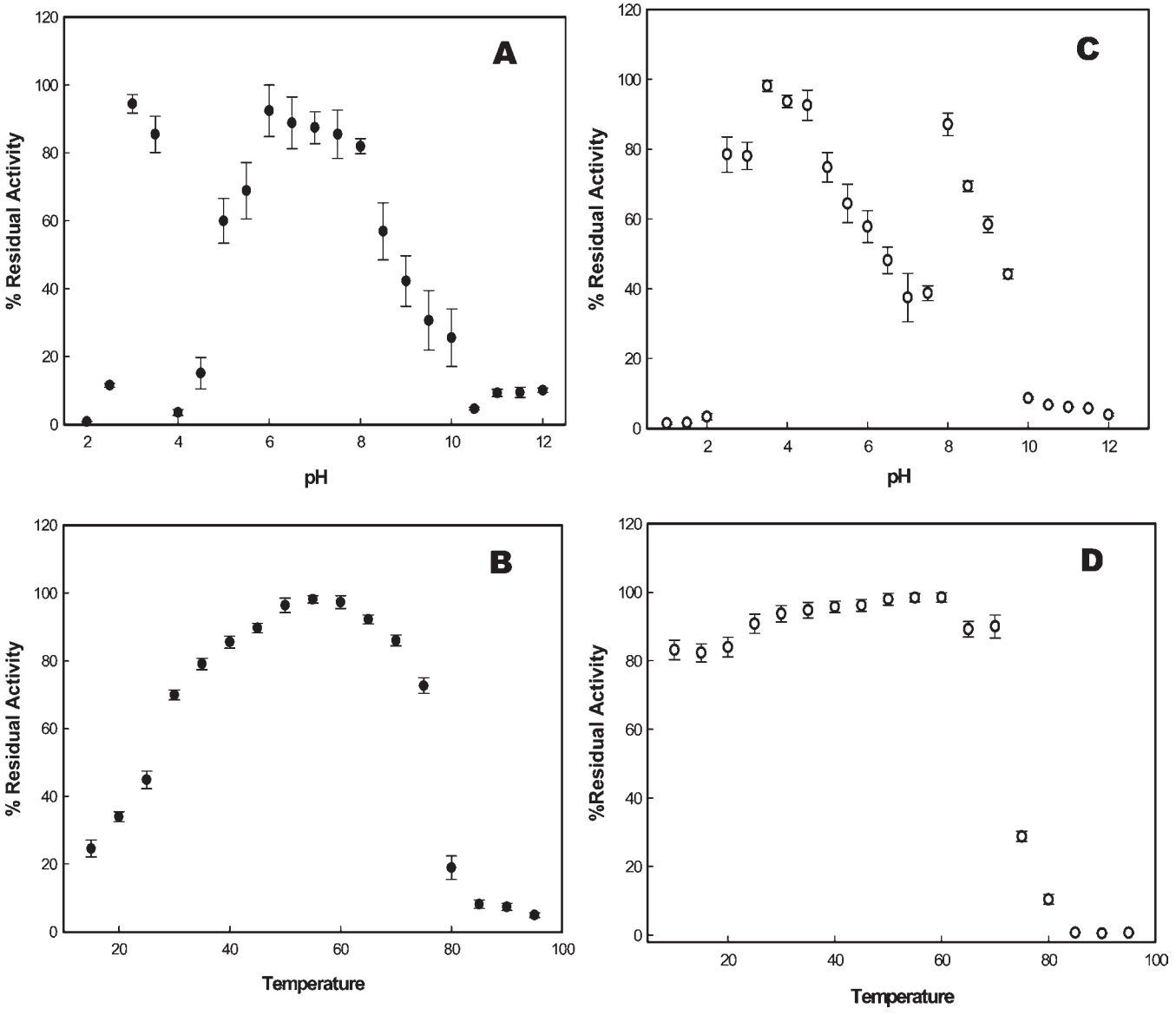

Figure 4. Effects of pH (A and C) and temperature (B and D) on activity (●) and stability (○) of immobilized proceratin B. The precise cause of such reproducible pH optima and stability profile is not understood, but it is likely to be due to interaction of enzyme with chitosan matrix.

Different time intervals in the range of 0.5−4 h were tried for activation with 3% glutaraldehyde, and corresponding immobilization was observed. Maximum immobilization (86.69%) was at 3 h of activation (Table 1). The higher concentration of glutaraldehyde and longer time for activation cause cracking of beads due to the fragile nature. The third parameter was coupling time for the formation of bond between the aldehyde group of glutaraldehyde and the amino group of the side chain of lysine present on the surface or terminal amino group of the enzyme. Different coupling times (8−32 h) were tried for optimum immobilization, and at 24 h, the immobilization was maximum (86.98%). At longer coupling times, the immobilization again was decreased (Table 1) due to leaching. We have optimized the concentration of procerain B in the immobilization mixture according to the application requirements. To economically use the enzyme for immobilization, we have tried different concentrations of enzyme (0.2−0.8 mg/mL) in immobilization mixture. The optimum concentration was 0.2 mg/mL with 87.50% immobilization (Table 1). At this concentration, most of the procerain B molecules get immobilized on activated surface of beads. A higher concentration of procerain B in immobilization mixture leads to an increase in per bead enzyme activity required in some applications. The optimal per bead activity was observed at 0.6 and 0.8 mg/mL concentrations (Table 1). The cross-linking and activation with glutaraldehyde were confirmed by comparison of FTIR spectra of normal and activated beads (Figure 2). The peak at 1412 cm^{-1} represents the C−N bond results with a cross-linking reaction. The aldehyde groups of glutaraldehyde molecules react with the amino group of chitosan through schiff base linkage. Linkage of both terminal aldehyde groups with separate chitosan molecules results in cross-linking, while the linkage of only one aldehyde group results in the activation of surface for immobilization. Other peaks at 1646 and 1054 cm^{-1} may attributed to C═N and polysaccharide structure. SEM images showed the detail view of normal, activated, and immobilized bead surfaces. The surface

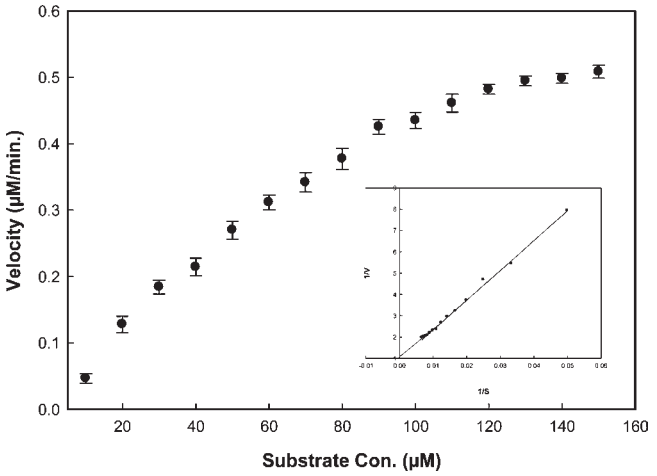

Figure 5. Effect of substrate concentration on reaction velocity of immobilized procerain B. The K_m value for azocaseine was calculated from the Lineweaver−Burk plot shown in the inset.

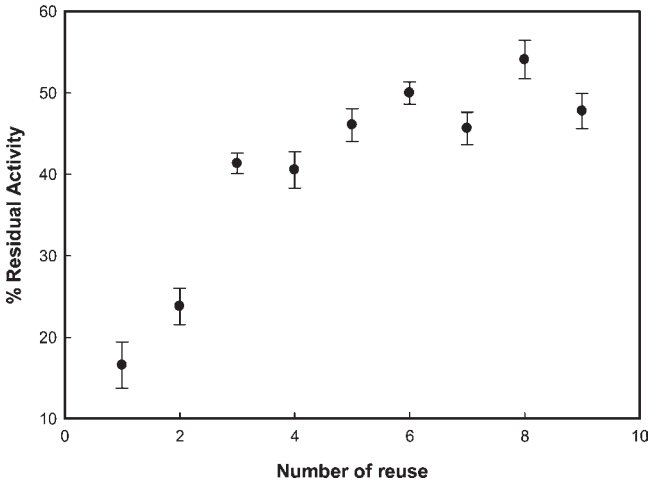

Figure 6. Reusability of immobilized procerain B. After each reaction, the beads with immobilized procerain B were removed and washed with 0.1 M Tris-Cl buffer, pH 7.5, to remove any residual substrate within the chitosan beads and stored in the same buffer at 4 °C until further use.

morphology of activated and immobilized beads was changed. The roughness of bead surface increased with glutaraldehyde treatment and immobilization (Figure 3). This may be due to activation of the surface with glutaraldehyde and attachment of the enzyme on the activated surface. The increase of nitrogen percentage in EDX analysis of bead surface in the case of immobilized beads was due to the presence of proteins (procerain B) on the surfaces of beads (Table 2).

pH and Thermal Optima of Immobilized Procerain B. After immobilization, chitosan-immobilized procerain B showed two very interesting pH optima, one in acidic pH (pH 3.0) and the other around pH 6.0 (Figure 4A), which shows the better applicability of immobilized procerain B in comparison to free form having only one pH optima in the range of pH 6.5−8.5.[28] Two pH optima may be due to the interaction of the enzyme with a chitosan matrix. However, the precise cause was not understood. The temperature optimum of immobilized procerain B was 55 °C (Figure 4B), while the free enzyme shows maximum activity in the range of 40−60 °C.[33] The conformational flexibility of procerain B was affected by immobilization. An increase in the temperature optima is a clear reflection of conformational rigidity, which makes it resistant to denaturation.

Stability. After immobilization, the pH stability pattern of procerain B got a strange and highly reproducible pattern. The immobilized procerain B showed nearly 80% activity in the acidic range, pH 3 to pH 5.0, and in basic range at pH 8.5 (Figure 4C), while the soluble form was more stable around neutral pH where the immobilized procerain B was least stable. This may be due to interaction of the enzyme with a chitosan matrix. However, the precise cause of such stability profile as a function of pH is not understood. Plant cysteine proteases are well-known for their higher thermal stabilities and widely used in the process where a higher temperature is required. Immobilization may further increase their stability and industrial importance. Immobilization on chitosan beads causes restricted deformation in three-dimensional structure of procerain B due to temperature and leads to increased thermal stability. The temperature stability of immobilized procerain B was investigated, and we found that it showed more than 80% activity up to 70 °C (Figure 4D), while soluble procerain B shows a similar activity up to 65 °C only.[33] Thus, immobilization leads to a slight increase in thermal stability, which makes it industrially more valuable.

Effect of Substrate Concentration on Activity of Immobilized Procerain B. The immobilized procerain B followed the Michaelis−Menten equation with azocasein as a substrate (Figure 5). At higher concentrations of substrate, the enzyme activity is saturated. The K_m of immobilized enzyme was 148.72 μM, which is lower than that of free enzyme (210 ± 31 μM).[33]

Reusability of Immobilized Protease. Reusability is one of the most important advantages of immobilization. Immobilized procerain B showed nearly 50% activity until the 10th use (Figure 6). The activity of immobilized procerain B was lower at initial reuses, but it regained activity up to the fifth use.

In brief, spherical chitosan beads of uniform size were prepared, and the immobilization of procerain B on glutaraldehyde-activated chitosan beads was successfully optimized, resulting in 87.50 ± 1.15% immobilization. The immobilized product was characterized for optimum functional range and stability. The immobilized procerain B has an optimum temperature of 55 °C and two pH optima (one in acidic and the other in neutral pH range).

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■ ABBREVIATIONS USED

TCA, trichloroacetic acid; BSA, bovine serum albumin; SEM, scanning electron microscopy; EDX, energy dispersive X-ray; FTIR, Fourier transform infrared

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