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Chemical modification and immobilization of papain

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Abstract: Papain, an endolytic cysteine protease (EC: 3.4.22.2), from *Carica papaya* latex has been chemically modified using succinic anhydride. This reagent reacts with the amino group of the lysine residues in the enzyme, thereby changing its net charge from positive to negative. The resultant enzyme had its optimum pH shifted from pH 6 to 8 and there was no change in the temperature optima of 70 °C. The modified papain had a specific activity of about 62.8 IU mg^{-1} of protein at pH 8.0 at 30 °C, whereas for the native enzyme it was 46.57 IU mg^{-1} under same conditions. Stability of the modified papain was further increased by entrapping in alginate/starch beads. The immobilized papain retained its activity even after six cycles of hydrolysis. The wet beads, when dried at 50 ± 2 °C, increased the storage stability of the immobilized enzyme. The succinylated papain is active in various organic solvents and hence can be successfully used in biotransformations as well as being used as a proteolytic component in detergents.

Keywords: papain; succinic anhydride; immobilization; detergent; lysine; biotransformations

INTRODUCTION

The protease enzyme is incorporated in the detergents that are used to remove the protein-containing stains of blood, egg-yolk, chocolate, etc.[1] During washing the protease enzyme hydrolyzes the protein stains in the fabric into peptides. But when used in detergents, it needs stability at alkaline pH values and also at low temperature (20–30 °C).[2] Subtilisin, a serine protease from genetically modified *Bacillus licheniforms*, currently used in detergents is made to be stable at alkaline pH values.[3]

Papain is an endolytic cysteine protease (EC: 3.4.22.2), and can be used as an alternative to substilisin, provided that it is stable and active at alkaline pH values and at low temperatures. Papain is less expensive than substilisin and normally has high thermal stability. Papain molecules consist of a single peptide chain of 211 amino acid residues[4] out of which 11 are lysine residues. The amino acid residues are folded into two parts, which form a cleft. The active site of papain has an Asn-His$^+$-Cys$^-$ (catalytic triad) residue, which exists as a zwitterion.[5] Papain does not have lysine residues in its active site, and hence it can be chemically modified easily using succinic anhydride (Scheme 1). Succinic anhydride reacts specifically with the ε-amino group of lysine residues and changes its charges from positive to negative, leading to a shift in pH optima from 6 to 8.[6] Protein with negatively charged groups causes a localized lowering of pH in the surrounding liquid, thus it acquires resistance towards deactivation in highly alkaline detergent liquid.

Immobilization of papain by entrapment in alginate beads after chemical modification will increase its stability and reusability in alkaline conditions.[7,8] Sodium alginate is cheap, commercially available in abundant quantities, and hydrophilic in nature. Polyvalent cations such as Ca^{2+}, Al^{3+}, Ba^{2+}, etc bring about cross-linking of different polymer molecules to form a stable gel (see eqn (1)). The ionically-linked gel structure is thermostable over the range of 0–100 °C.

$$2\text{Na (alginate)} + Ca^{2+} \longrightarrow \text{Ca (alginate)}_2 + 2Na^+ \quad (1)$$

MATERIAL AND METHODS

Papain (crude), 1 Anson unit g^{-1}, and casein (95%; w/w protein) were purchased from SISCO Laboratory, Mumbai, India. Bovine Serum Albumin (BSA) was from Sigma, St Louis, USA, succinic anhydride was from Merck, India, and sodium alginate from CDH, India. All the other reagents used are of analytical quality.

Purification of papain

Crude papain, which contains other proteases, was further purified by ammonium sulfate precipitation at 4 °C; the 40–50% cut-off fraction was taken for the experiments.

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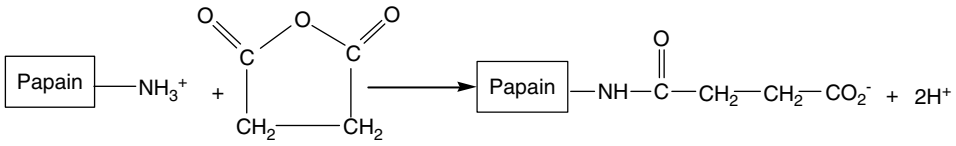

Scheme 1. Succinic anhydride modification of papain.

Activation of papain

Papain in 50 mmol dm^{-3} phosphate buffer (pH 6.0) was mixed with 50 mmol dm^{-3} L-Cysteine–HCl and 3 mmol dm^{-3} ethylene diamine tetra acetic acid (EDTA), and incubated for 30 min at 30 °C and centrifuged. The supernatant was taken for further experiments.[9]

Protease assay

Casein (2%) was used as the substrate for activity measurement. The reaction media contained 0.5 cm^3 of substrate and 0.5 cm^3 of enzyme (0.5 g in the case of immobilized enzyme). The reaction was carried out at 30 °C for 10 min and stopped by the addition of 1 cm^3 10% trichloro acetic acid (TCA). The mixture was centrifuged and to 0.5 cm^3 of supernatant was added 5 cm^3 of 0.5 mol dm^{-3} Na_2CO_3 and kept for 10 min and 0.5 cm^3 of Folin Ciocalteau's reagent added.[10] The tubes were incubated for 30 min in the dark for color development. The color was read against the reagent blank at 660 nm in a spectrophotometer (Shimadzu UV 2100, Japan). The protease activity was expressed as mmol of tyrosine released per minute per cm^3.

Protein estimation

Protein was estimated by Lowry's Method using Bovine Serum Albumin (BSA) as standard.[11]

Succinylation of papain

Succinic anhydride at different concentrations from 0.4 μmol to 1 μmol was added slowly with constant stirring to papain suspension in 50 mmol dm^{-3} phosphate buffer, pH 8.0. This immediately brought about a drop in pH and hence 1 mol dm^{-3} NaOH was added to the solution to maintain the pH at 8.0 during the reaction. The reaction was allowed to proceed for 1 h at 25 °C, after that it was stopped by lowering the pH to 7.5 using 1 mol dm^{-3} HCl. The specific activity of the succinylated papain was assayed at pH 8.0 at 40 °C.

Free amino group measurement

Free amino groups were determined by the ninhydrin method.[12]

pH and temperature optima of native and succinylated papain

Native and succinylated papain were incubated for 30 min at various pH values (from 5 to 10) and the temperature varied from 20 °C to 90 °C, and the activity of the enzymes was assayed.

Immobilization of papain

Activated papain (0.5 cm^3) was mixed with 4.5 cm^3 of 3% sodium alginate solution. The beads were formed by dropping this polymer solution into 0.2 mol dm^{-3} $CaCl_2$ solution at room temperature. The beads were left in 0.05 mol dm^{-3} calcium chloride solution for 6 h to cure. The calcium alginate beads were about 2–3 mm in diameter. Both native and modified papain was immobilized in alginate/starch beads and stored in the 0.05 mol dm^{-3} $CaCl_2$ solution at 40 °C. Starch beads were prepared using 6%(w/v) gelatinized corn starch.

Protease assay for immobilized papain

Protease assay was carried out by the above mentioned method for both native and modified immobilized papain (0.5 g). Assay was done at distinct time intervals to check the stability of the immobilized enzyme.

Reuse stability and storage stability of immobilized enzyme beads

The immobilized enzyme beads were used in repeated cycles for the hydrolysis of casein (2%) for 30 min. Each time the beads were washed in water and reused. The proteolytic activity was determined as before.

To study the storage stability, the wet beads were air dried at 50 ± 2 °C for 6 h and stored for 1 week. The residual proteolytic activity was then determined.

RESULTS AND DISCUSSIONS

The crude papain was purified by ammonium sulfate precipitation to remove other enzyme proteins consisting of chymopapain, carican and endopeptidase. Initially papain was first fractionated at 40–50% saturated ammonium sulfate cut-off, further fractionated with 48% saturated NH_4SO_4 precipitation. The specific activity was found to be 46.57 IU mg^{-1} at pH 8, at 30 °C, for the above fraction. Succinic anhydride was used for the chemical modification of papain since the reagent reacts specifically with the ε-amino groups of protein and the succinyl residue is stable to most normal treatments. The parameters for the reaction of enzyme with succinic anhydride were optimized to get the maximum enzyme activity. It was found that papain treated with 0.5 μmol of succinic anhydride had the highest specific activity of 64 IU mg^{-1} and hence was used for further studies (Fig 1). The modified enzyme was found to have an optimum activity of 71.78 IU mg^{-1} protein at an optimum pH of 8.0, at 70 °C. The maximum activity of native papain was 62.79 IU mg^{-1} at the optimum pH of 6.0, at

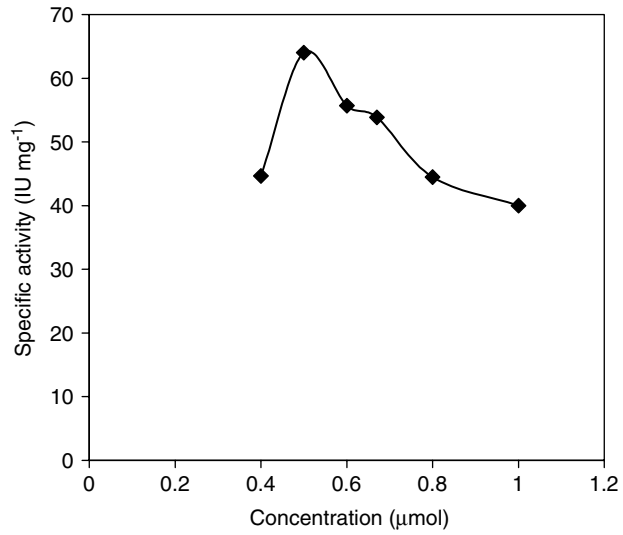

Figure 1. Effect of concentration of succinic anhydride on the activity of the modified papain at pH 8.0, 25 °C.

70 °C. Nine lysine residues out of 11 were succinylated after treating with succinic anhydride by the present method. Succinylation of papain reverses the positive charge on the ε-amino groups of the side chain of the lysine residue with the negative charge of the succinyl group, thus shifting the pH optima from 6.0 to 8.0 but the temperature optima remained the same. Enveloping the enzyme with negatively charged groups might cause a localized lowering of pH with respect to the surroundings. This will shift the pH optimum of the enzyme.[13] The modified papain did not lose its activity at high pH and temperature conditions.

The primary aim of modifying the papain was to increase the enzyme's ability to withstand alkaline pH conditions at different temperature settings in domestic washing machines as well as for biotransformation applications. The efficiency of modified papain over a pH range of 5 to 10 at 20 °C after incubation of 30 min is given in Fig 2. Native papain loses its activity gradually above pH 6.0 and in alkaline conditions, and shows only 50% of its original activity at pH

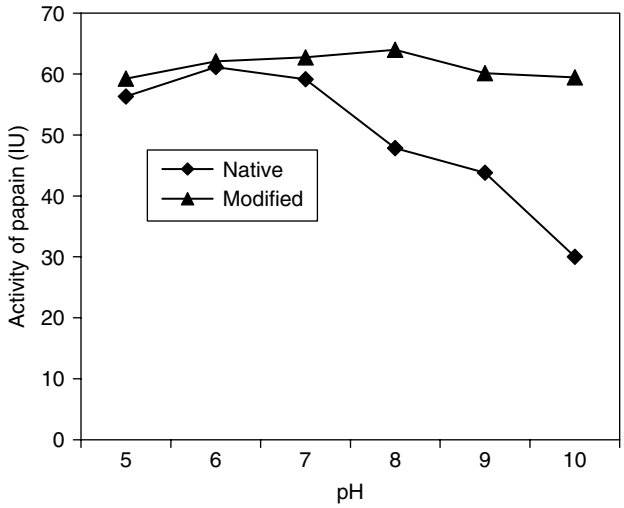

Figure 3. Activity of modified papain at 40 °C, at different pH values.

10.0 The modified papain is stable till pH 8.0, where the activity is maximum, thereafter there is only a marginal decrease in activity till pH 10.0. The normal cloth-washing temperature is 20 °C so it was chosen for investigating stability at various pH values.

The activity profile of modified papain at 40 °C at different pH values after incubation of 30 min is given in Fig 3. Both the modified and native papain show almost the same activity till neutral pH, thereafter the modified papain shows excellent pH stability. The native papain loses 50% of its activity at the alkaline pH of 10.0.

The activity profile of modified papain at 60 °C (warm settings) is almost the same as at 40 °C (Fig 4). Modified papain shows excellent stability in alkaline conditions whereas activity of the native papain gradually decreases at pH values above neutral.

The activity profile of modified papain at 90 °C (hot setting) is different from that at lower temperatures (Fig 5). The modified papain is stable up to pH 8.0, thereafter the activity decreases to 40 IU. Here also the modified papain is better than native papain, which loses its activity rapidly at pH values above neutral.

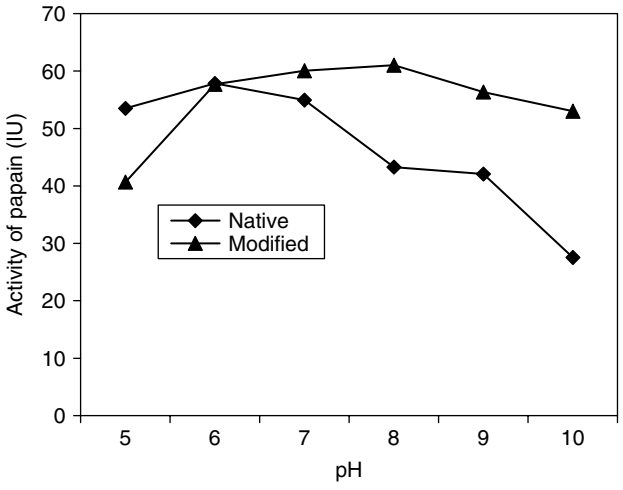

Figure 2. Activity of modified papain at 20 °C, at different pH values.

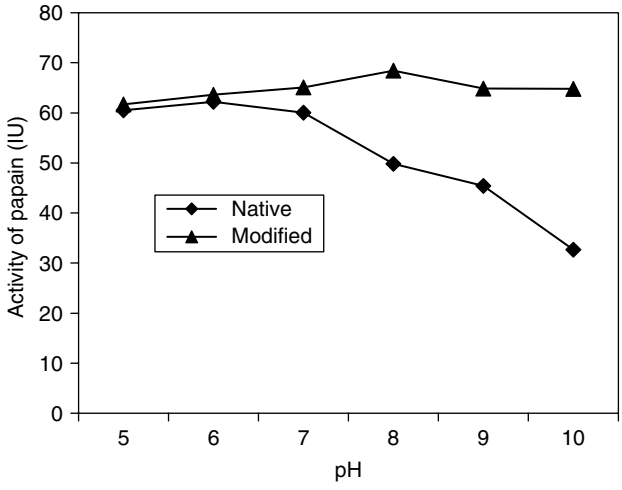

Figure 4. Activity of modified papain at 60 °C, at different pH values.

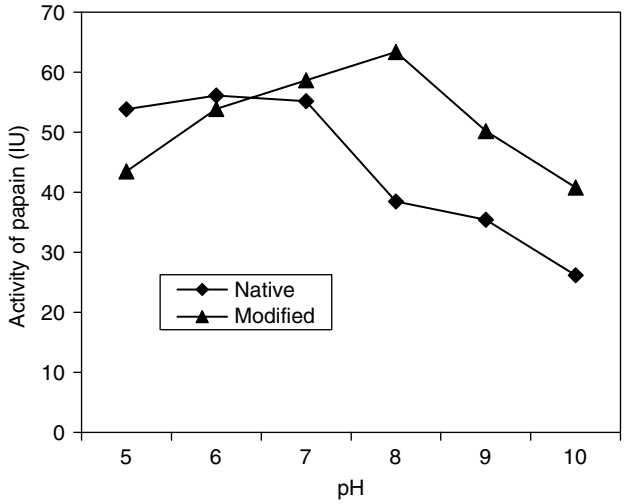

Figure 5. Activity of modified papain at 90 °C, at different pH values.

The active site of papain contains Cys-His-Asn as a catalytic triad involved in the hydrolysis of proteins. The modified papain retains its catalytic activity even after succinylation of the lysine residue.

Papain was then entrapped in calcium alginate or gelatinized corn starch in order to improve its stability during use at alkaline pH values and under storage. Immobilization of the enzyme was carried out with both native and modified papain. Reuse stability of the immobilized calcium alginate enzyme beads was checked and the values are given in Fig 6. It was noticed that the enzyme is quite stable and has retained the activity even after six cycles of reaction. From the second cycle onwards the activity became stabilized. The drop in activity happened after the first cycle for both native and modified immobilized enzyme. This is because the unbound enzyme present in the beads was leached out. The enzyme entrapped on Ca-alginate beads had an average size of 2–3 mm but starch enzyme particles

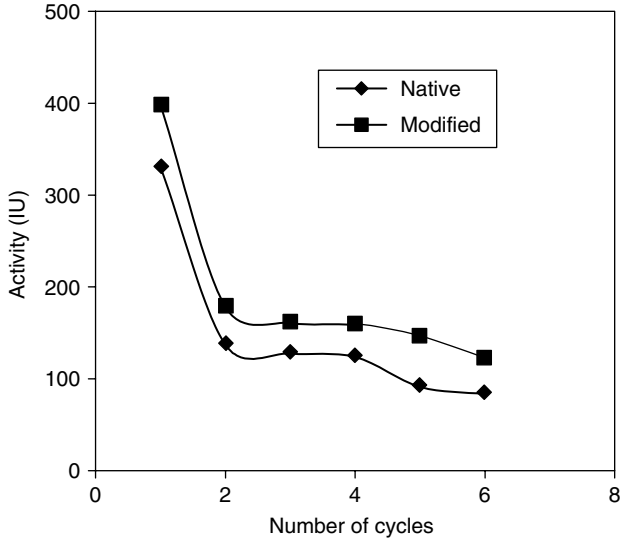

Figure 6. Reuse stability of modified and immobilized papain beads (wet).

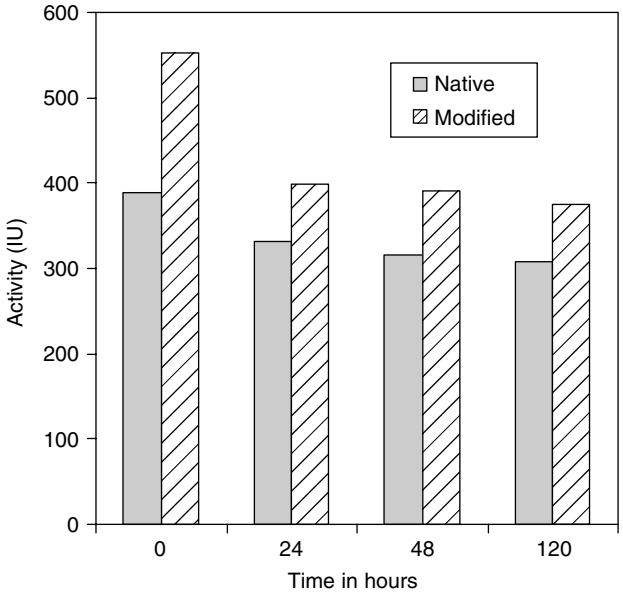

Figure 7. Comparison of the activity of the immobilized native and modified papain dry beads stored at room temperature.

were around 1 mm. Both alginate and starch beads were dried at 50 ± 1 °C for 6 h under vacuum to remove part of moisture and to harden the beads to increase the strength and storage stability. After the drying process, the beads' size was reduced to less than 0.5 mm.

The storage stability of both types of dry beads was checked at distinct time intervals up to 120 h. It was found that the activity retention was 67% for modified papain and 79% for native papain and hence the enzyme did not lose much activity in 6 days (Fig 7). If the beads are dried further the enzyme became entrapped strongly in the matrix and the pore size of the matrix reduced drastically, causing loss of activity. The dried beads can be safely incorporated into the detergent powders without much activity being lost during storage.

CONCLUSIONS

From our studies, it is evident that both alginate and corn starch is a cheap nontoxic matrix for immobilization of the modified papain and the alginate beads were found to be more stable for continuous use. Succinylation of papain increases its stability and activity at alkaline pH values. The modified papain can also be used in peptide synthesis and biotransformations.

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