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# Chemical modification of papain for use in alkaline medium

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**Abstract**

Chemical modification is a useful method to recognize and modify functional determinants of enzymes. Papain, an endolytic cysteine protease (EC3.4.22.2) from *Carica papaya* latex has been chemically modified using different dicarboxylic anhydrides of citraconic, phthalic, maleic and succinic acids. These anhydrides reacted with five to six amino groups of the lysine residues in the enzyme, thereby changing the net charge of the enzyme from positive to negative. The resultant enzyme had its optimum pH shifted from 7 to 9 and change in temperature optima from 60 to 80 °C. The modified papain also had a higher thermostability. Stability of the modified papain was further increased by immobilization of the enzyme either by adsorption onto inert matrix or by entrapment in polysaccharide polymeric gels. Entrapment in starch gel showed better retention of enzyme activity. Incorporation of modified and immobilized enzymes to branded domestic detergent powders was found to have very good activity retention. The papain entrapped in starch gel showed better stability and activity retention than in other carbohydrate polymers when added to domestic detergent powders.


*Keywords:* Papain; Chemical modification; Dicarboxylic anhydrides; Immobilization; Washing detergents

## 1. Introduction

Proteases are one of the most important industrial enzymes, accounting for nearly 60% of total worldwide enzyme sales. Proteases are mainly incorporated in the cloth washing detergents to remove the protein strains of blood, egg yolk, chocolate, etc. [1]. The use of protease enzyme in washing clothes is that it hydrolyses the protein strains in the fabric into peptides. But when used in detergents, it requires stability at alkaline pH values and also at low temperatures [2]. The serine protease, subtilisin from genetically modified *Bacillus licheniformis* is now widely used in detergents due to its stability in alkaline conditions [3].

Papain is a food grade, highly active endolytic cysteine protease (EC3.4.22.2) from *Carica papaya* and is one of the widely used industrial enzymes. It can be used as an alternative to subtilisin, provided that it is stable in harsh conditions and is active at low and high temperatures. Papain is less expensive than microbial enzymes, and has got a wide range of specificity and good thermal stability amongst other proteases. Therefore, it has got high potential to use in detergents. Papain molecules have a molecular weight of 23,000 Da and an isoelectric point of 9.5. Papain molecules consist of a single peptide chain of 211 amino acid residues folded into two parts that form a cleft and having 11 lysine residues [4]. Papain preferably cleaves peptide bonds involving basic amino acids and it also has an esterase activity. It is used for cell isolation, in breweries, food and pharmaceutical as digestive enzyme, leather, cosmetic and textile industries. The catalytic site of the enzyme contains a catalytic triad Asn-His+-Cys−, which exists as zwitterions. Lysine residues are not a part of active site in papain; hence it can be chemically modified by different dicarboxylic anhydrides of citraconic, phthalic, maleic and succinic acids. These anhydrides react specifically with the $\varepsilon$-amino group of lysine residues and change its charges from positive to negative, leading to a shift in pH optima of the enzyme from 7 to 9 [2]. Immobilization of papain by covalent binding to organosilica [5], chelating sepharose [6], vinyl alcohol/vinyl butryl copolymer membranes [7], epoxy polymer [8], and cold-plasma functionalized polyethylene and glass surfaces [9], nitrilon fibre carrier [10], fibrous polymer of polyvinyl alcohol [11] has been carried out in the past mainly for biomaterial use. Currently there is a strong interest in the use of renewable and non-toxic supports for enzyme immobilization to make the process ecofriendly in many modern industrial applications. Our earlier studies on modified papain entrapped in cheap and

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non-toxic matrices like corn starch gel have shown promising results [12]. We report the modification of papain to increase the pH optima and subsequent immobilization in carbohydrate gels and the resultant effect in the reaction kinetics.

## 2. Experimental

### 2.1. Chemicals

Crude papain (7.7 IU/g) and cysteine-hydrochloride were purchased from Sisco Research Laboratory (Mumbai, India). Bovine serum albumin and citraconic anhydride was from Sigma (St. Louis, MO, USA). All anhydrides of phthalic, maleic and succinic acids were obtained from Merck (India). Casein was purchased (95%, w/w, protein) from S.D. Fine Chemicals (Mumbai, India). Celite was procured from New Modern Chemical Corporation, India. (High flow super cell—a naturally occurring diatomaceous earth, calcined at 500 °C to remove protein and other impurities, composition $SiO_2$ 83.7% and $Al_2O_3$ 5.3% and having particles of 5–20 μm in diameter, 85% of it is made up of tiny interconnected pores with an average porosity of 9 mm.) Branded domestic detergents are from Henkel Spic Ltd., India. All the other reagents used are of analytical quality.

### 2.2. Purification of papain

Crude papain, contaminated with other proteases was purified by ammonium sulphate fractional precipitation at 4 °C; the 45% ammonium sulphate cut-off fraction was taken for the experiments after dialysis.

### 2.3. Activation of papain

Reducing the disulphide bonds of the cysteine molecules of the protein by suitable reagents activates papain [13]. For activation, papain was taken in Tris buffer (pH 9.0, 0.1 M) and was mixed with 0.01 M EDTA, 0.06 M mercaptoethanol and 0.05 M cysteine-hydrochloride giving a final pH of 6.2 and was incubated for 30 min at 30 °C.

### 2.4. Protease assay

Proteolytic activity of native and modified papain was determined at 60 °C in 50 mM glycine–NaOH buffer, pH 10.5, using casein as substrate [14]. One unit of protease was equivalent to the amount of enzyme required to release 1 μmol of tyrosine/ml/min. Protein concentration was estimated as described by Lowry's et al. [15] using bovine serum albumin as standard.

### 2.5. Preparation of succinic, maleic, phthalic and citraconic papain

Chemical modification of papain was carried out by standard procedure [16]. Papain was suspended in 0.1 M Tris buffer at pH 9.0. Optimized concentration of different anhydrides was added gradually with constant stirring to the papain suspension. The pH was maintained with 1N NaOH throughout the reaction. The reaction was allowed to proceed for 1 h at 25 °C and was then stopped by lowering the pH to 7.5 using 1N HCl. Modification by citraconic anhydride was carried out according to the procedure described by Dixon and Perham [17].

### 2.6. Free amino group measurement

Free amino groups were determined by the Ninhydrin method [18].

### 2.7. Method of immobilization

The enzyme was immobilized by adsorption onto celite, kaolinite, and by entrapment to Hibiscus leaf mucilage and corn starch. The enzyme was immobilized by adsorption onto celite, kaolinite with a ratio of 2:1 (w/v). After 10 min of equilibration at 28 ± 2 °C, it was spread as a thin layer in a Petri plate and dried in a vacuum desiccator at 28 ± 2 °C for 12 h without any agitation. The mucilage from common garden hibiscus plant was collected by macerating the leaf in water and taking out the mucilage by filtration under vacuum. The mucilage is reported to be composed of mainly glucomannan residues. The enzyme was immobilized in starch with a ratio of 2:1 (w/v). It was dried overnight in vacuum desiccator at 28 ± 2 °C for 12 h.

### 2.8. Determination of optimum temperature, pH and kinetic parameters

Optimum temperature, pH, $K_m$ and $V_{max}$ were determined by changing each of the parameters by keeping all the other conditions constant and the protease activity was assayed. The activity of native and modified enzyme was studied in a temperature range of 20–90 °C at pH 10.5. The activity profile was studied in different pH range of 6.0–10.5 at 60 °C using 50 mM buffer (pH 6–8, phosphate buffer; pH 9–10.5, NaOH/glycine buffer). The kinetic studies were done by changing substrate concentration from 0.25 to 2.5%. The thermostability profile was studied by incubating the enzyme at various temperatures (60 and 70 °C) for different durations and then assaying the residual enzyme activity.

### 2.9. Estimation of protease activity of modified and immobilized enzyme in detergents

To study the effect of the cloth washing detergent on the activity of modified and immobilized papain, 100 mg of protease free detergent was introduced into the reaction mixture and was incubated for a definite period of time and then the residual enzyme activity was found out. Measurements were made also standard conditions to check the effectiveness of the detergent in water.

## 3. Results and discussion

Chemical modification is an easy and low cost method to stabilize the enzyme. The thermal stability of enzymes has

been reported to be remarkably improved by the modification with methoxy polyethylene glycol and succinimide [19,20]. The other compounds used for the modification of enzymes are glucosamine and acetic anhydride [21].

### 3.1. Modification of lysine residues

Here a simple and selective procedure is adopted for modification of ε-amino groups of the lysine residues of papain by using different dicarboxylic anhydrides. The crude papain was purified by ammonium sulphate fractional precipitation which removed other enzyme proteins such as chymopapain, caricain and endopeptidase. Primarily papain is fractionated at 20–45% saturated ammonium sulphate precipitation, which amounts only to 6–8% of the crude papain. The specific activity was found be 1.650 IU/mg at pH 10.5 at 60 °C. The dicarboxylic acid anhydrides are used for the chemical modification of proteins; which specifically reacts with the ε-amino groups of protein and the modified enzyme is stable to most normal treatments. (Excess of the chemical treatment inactivates the enzyme.) There is a marked change in the thermal stability and activities of the modified enzymes. It was found that papain modified with 0.4 μmol of anhydrides retained the maximum enzyme activity. The modified papain did not lose its activity significantly at conditions of high pH and temperature unlike the native enzyme. Five to six lysine residues of the enzyme out of eleven were modified after treating with acid anhydrides by the present method.

### 3.2. Effect of pH

The main aim of papain modification was to enhance the enzyme's ability to withstand alkaline conditions. The effectiveness of modified papain over a pH range of 6–10.5 at 60 °C after incubation of 10 min (Fig. 1). The unmodified papain loses its activity gradually above pH 7.0. The optimum pH of native papain was at 7.0 and in the case of modified papain; the pH was shifted to the alkaline side giving an optimum pH of 9.0. This was possible due to the reaction of acid anhydride with the lysine groups of the papain, resulting in a net anionic charge, thereby shifting the pH optima to the alkaline side. It was observed that out of 11 lysine residues, 5–6 lysine groups present in the molecule were modified (Table 1). Enzyme with negatively charged groups might cause a limited lowering of pH with respect to the surroundings [2]. This will in turn shift the pH optimum of the enzyme. The modified papain did not lose its activity significantly at this high pH and temperature conditions. The modified papain was found to be stable at 60 °C till pH 9.0, where the activity was the highest; thereafter the activity showed a marginal decrease.

Table 1
Estimation of lysine residues

| Enzyme | Reacted lysine residues |
|---|---|
| SA | 6.2 ± 2 |
| MA | 5.7 ± 7 |
| PA | 6.1 ± 8 |
| CA | 5.9 ± 5 |

### 3.3. Effect of temperature

The protease activity of native and modified papain increased gradually with temperature and the maximum activity was obtained at 60 °C. The exception was in the case of succinyl and citraconic papain, where the maximum activity was at 70 and 80 °C (Fig. 2). High optimal temperature is an important characteristic useful for alkaline proteases as detergent additives as observed in the case of subtilisin from *Bacillus* sp.

### 3.4. Thermal stability

The stability of the enzyme in various temperatures was studied and was found that the modified papain had better thermal stability at 60 and 70 °C (Figs. 3 and 4). The half life of the modified papain was more than 18 h at 60 °C as against the native papain which was only 6 h. At 70 °C the modified papain had a

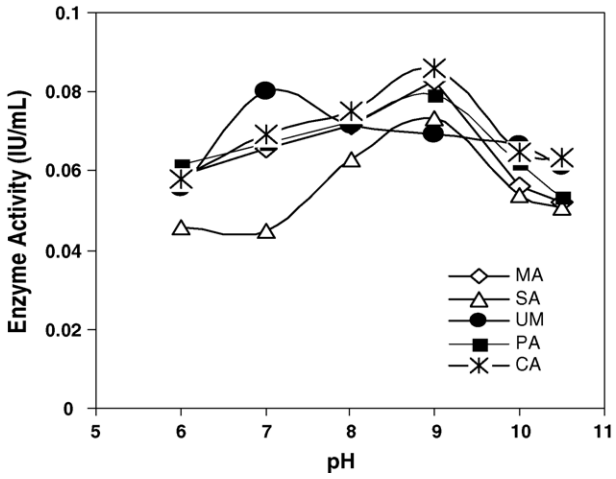


Fig. 1. pH profile of modified papain.

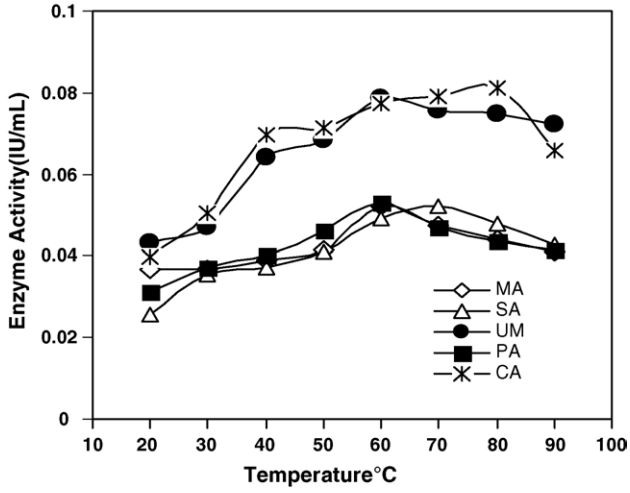


Fig. 2. Temperature profile of modified papain.

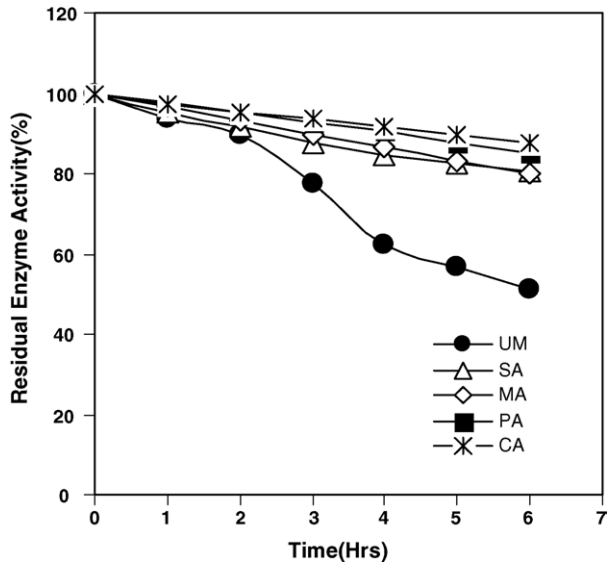


Fig. 3. Temperature stability of modified papain at 60 °C.

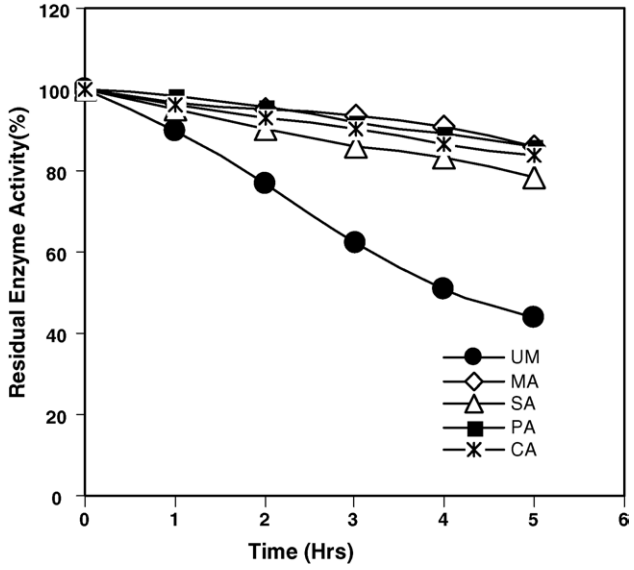


Fig. 4. Temperature stability of modified papain at 70 °C.

half life of nearly 14 h while native papain had only 4.5 h. There is a significant improvement in the thermal stability may be due to the enhancement of the order and compactness of the structure, thus favoring intramolecular stabilizing forces and consequently increasing the stability of the enzyme as was observed in the case of glucose oxidase [22]. Thermostability depends on the microenvironment of the enzyme and its subunit reorganization. The stabilization of papain was brought about by the modified lysine residues with bulky groups which provide new opportunity for the occurrence of the hydrogen bonding, hence prevents the unfolding and denaturation of the protein. Also charge repulsions contributes to the conformation and stability of proteins, this repulsion between charged groups present in the protein is the main driving force for protein to be stabilized in open conformation [23].

### 3.5. Kinetic studies

When the substrate concentration was plotted against the ratio of the substrate concentration to the rate of the reaction (Hanes–Woolf plot), the primary plot was obtained and Michaelis–Menten constant ($K_m$) and velocity maximum ($V_{max}$) were obtained automatically from the secondary plot values using Sigma plot (7.01 Version) 2001 software. Hanes–Woolf equation can be represented as,

$$\frac{[S]}{v} = \frac{1}{V_{max}}[S] + \frac{K_m}{V_{max}}.$$

where [S] is the substrate concentration, $V_{max}$ the maximal velocity and $K_m$ is the binding of substrate at (1/2)$V_{max}$ (Michaelis constant). Hanes–Woolf plot avoids both the misleading impression of the experimental error and the uneven distribution of the points by Lineweaver–Burk plot and the angular distortion of the errors of the Eadie–Hofstee plot [24]. The modified papain (citrconic papain) showed a high $K_m$ value compared to native and other modified papain which indicates a lower affinity towards the substrate (Table 2). There is not much difference in the $V_{max}$ values of the other modified enzymes which is around 3.0–3.5. The $k_{cat}$ value is varied between $0.69 \times 10^{-5}$ and $0.81 \times 10^{-5}$. The highest $k_{cat}$ is with citraconic papain followed by maleic papain and then native papain. The catalytic efficiency $k_{cat}/K_m$ was highest in the native papain ($9.26 \times 10^4$) followed by phthalic papain ($8.64 \times 10^4$) and maleic papain (Figs. 5 and 6). Citraconic papain had the lowest catalytic efficiency $4.8 \times 10^4$. Among the modified papain, phthalic papain was found to be the best in catalytic efficiency. Among the dibasic acids used in the study, phthalic is the bulkiest group attached to the lysine residues which in turn improves the catalytic activity by slightly altering the enzyme conformation.

### 3.6. Immobilization of enzymes

Immobilization of enzyme through physical method is still the most commonly used technique to improve the reuse stabil-

Table 2
Kinetic constants of native and modified papain

| Enzyme | $K_m$ (mol/ml) | $V_{max}$ (mol/s/g) | $k_{cat}$ (mol/s/mol enzyme) | $k_{cat}/K_m$ |
|---|---|---|---|---|
| UMP | $8.2 \times 10^{-6}$ | $3.2 \times 10^{-5}$ | 0.76 | $9.2 \times 10^4$ |
| PA | $8.2 \times 10^{-6}$ | $3.0 \times 10^{-5}$ | 0.71 | $8.6 \times 10^4$ |
| CA | $1.6 \times 10^{-5}$ | $3.4 \times 10^{-5}$ | 0.81 | $4.8 \times 10^4$ |
| MA | $1.1 \times 10^{-5}$ | $3.3 \times 10^{-5}$ | 0.79 | $7.0 \times 10^4$ |
| SA | $1.3 \times 10^{-5}$ | $2.9 \times 10^{-5}$ | 0.69 | $5.14 \times 10^4$ |

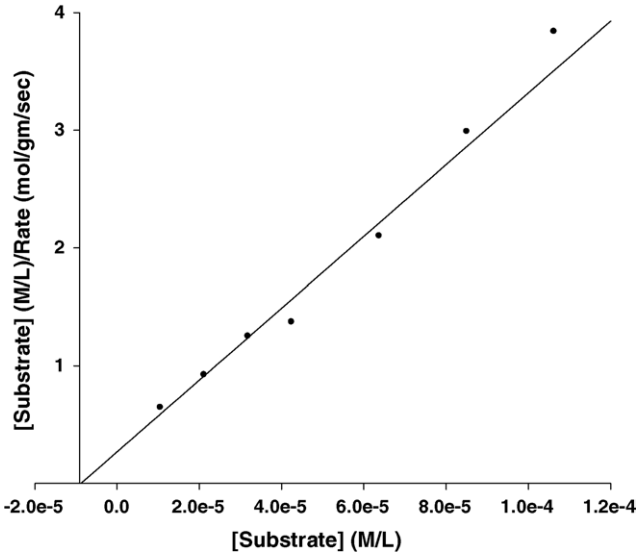


Fig. 5. Hanes–Woolf plot of native papain.

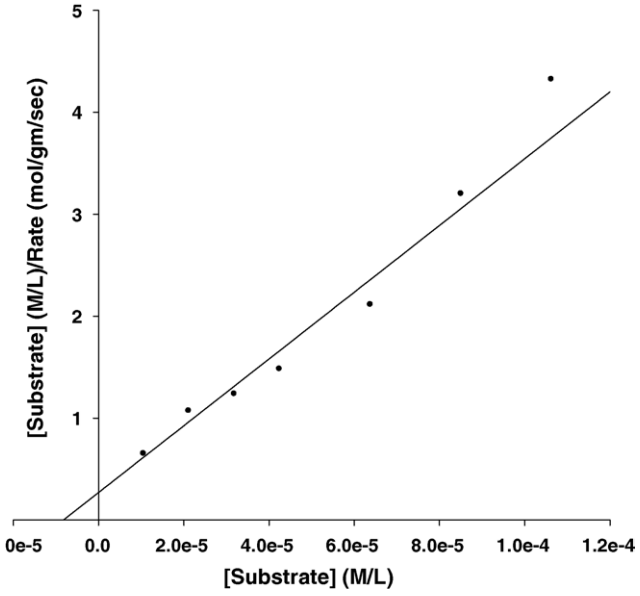


Fig. 6. Hanes–Woolf plot of phthalic papain.

ity of the enzyme because it is the easiest to perform and the least expensive. Both native and modified papain was immobilized by adsorption onto celite, kaolinite clay, and entrapped in polysaccharide gels such as Hibiscus leaf mucilage (glucomannan) and corn starch (glucose polymer) (Table 3). Adsorption on celite was found to be good having 61% retention of activity. In this method, the forces between a support and the enzyme include

Table 3
Immobilization of papain in different matrices

| Immobilization matrices | Activity retention (%) |
|---|---|
| Hiflow supercel | 61.6 ± 2 |
| Kaolinite clay | 31.4 ± 7 |
| Leaf mucilage | 20.0 ± 4 |
| Starch gel (corn) | 98.1 ± 4 |

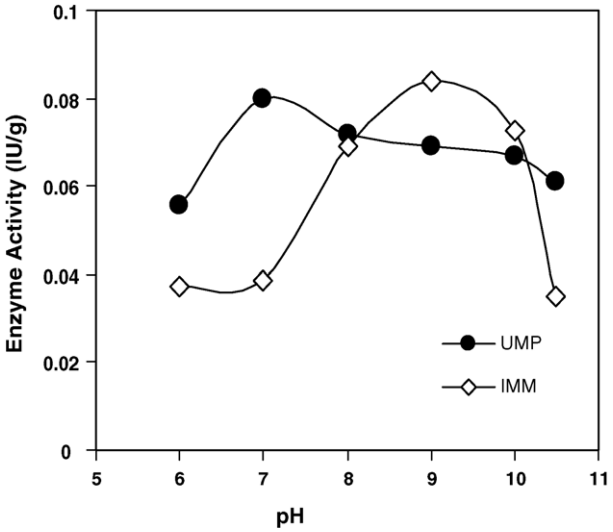


Fig. 7. Optimum pH of immobilized papain in starch gel.

hydrogen bonding, Van der Waals forces and hydrophobic interactions. Entrapment in corn starch gel was the best among the immobilizations with 98.14% activity retention. Starch is a very good immobilization medium, renewable resource, hydrophilic in nature, non-toxic, non-reactive and neutral keeping in view of the use of the immobilized papain for detergent formulations. Among these different immobilization matrices, starch gel showed better activity retention. Hence, it is used for further experiments.

### 3.7. Optimum pH and temperature of immobilized enzyme

The optimum pH and temperature of the immobilized papain in corn starch gel was studied and the data shows that the pH optima was changed to the alkaline side and there is a marked deviation in activity profile from the native enzyme with the temperature (Figs. 7 and 8). The immobilized enzyme also showed better thermal stability at higher temperatures of 60–70 °C,

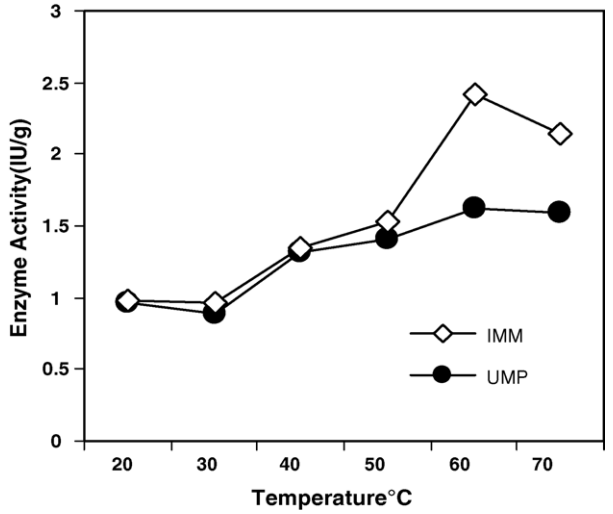


Fig. 8. Optimum temperature of immobilized papain in starch gel.

Table 4
Immobilization of modified papain in starch gel

| Immobilization of modified enzyme | Activity retention (%) |
|---|---|
| Maleic papain | 77.9 ± 7 |
| Succinyl papain | 78.3 ± 6 |
| Phthalic papain | 78.7 ± 6 |

mainly due to the microenvironment surrounding the enzyme inside the gel which prevents unfolding of peptide chains.

### *3.8. Activity retention of modified enzyme in starch*

The modified papains were also immobilized by entrapment method. Phthalic, maleic and succinyl papain was chosen for the studies. The modified papain was entrapped in corn starch gel and the best activity retention was for both maleic and succinyl papain which was found to be about 78% of the initial activity (Table 4).

### *3.9. Effect of detergents on modified and immobilized papain*

An ideal detergent enzyme should be stable and active in the detergent solution for a longer period of time and should have adequate temperature stability to be effective in a wide range of washing temperatures. Detergent enzyme should be stable at high pH and temperature, withstand oxidizing and chelating agents, functional at low enzyme level and have a wide specificity. Proteases offer a multifunctional role in the overall cleaning process as they improve the washing capacity of detergents. Protease enzymes enhance the cleaning of protein –based soils, such as grass and blood, catalyzing the breakdown of the constituent proteins in these soils through hydrolysis of the amide bonds between individual amino acids. In addition they decrease the redeposition of proteins, especially hydrophobic proteins such as those found in blood, resulting in improved overall whiteness. The activity profile of the modified enzymes when incorporated directly to the detergents was studied. Succinyl and phthalic papain showed good activity retention and retained 50% of the original activity (Table 5). The activity of the modified and immobilized enzyme in detergents was studied. The activity of native and immobilized enzyme was increased in detergents, compared to its native form. Studies on the effect of detergents on protease activity of both modified and modified and then immobilized papain showed an increase in activity (Fig. 9). The change in activity may be due to the favorable alkaline pH of the modified papain.

Table 5
Activity retention modified enzyme in detergents

| Modified enzymes | Activity retention after 1 h (%) |
|---|---|
| Maleic papain | 32.9 ± 3 |
| Phthalic papain | 52.4 ± 0 |
| Succinyl papain | 48.6 ± 6 |

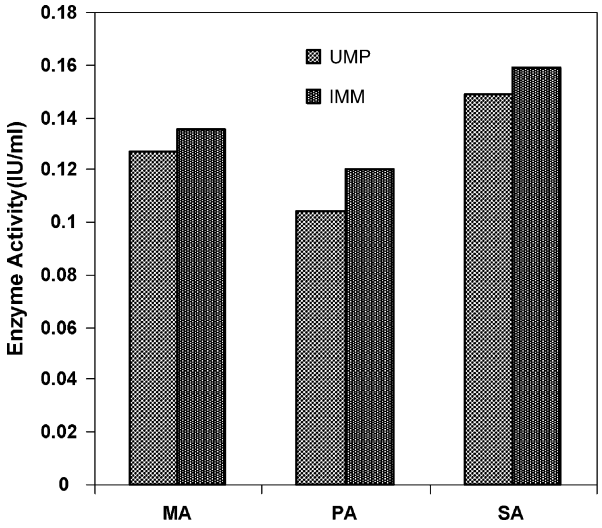


Fig. 9. Modified and immobilized enzyme in detergents.

## 4. Conclusion

Chemical modification of amino groups of the enzyme with acid anhydrides of dicarboxylic acids are a simple method and may provide a very valuable strategy for giving proteins some new and useful characteristics related to the stability and catalytic effect. In addition, the chemical modification may be used to change the physical property, substrate specificity, or even the type of reaction catalyzed by a particular enzyme. Our studies have shown that the amino groups of papain can be modified by anhydrides of maleic, phthalic, citraconic and succinic acids giving useful properties to the enzyme. The optimum pH of the modified papain was shifted to pH 9.0 with all the dibasic acid anhydrides. Phthalic papain was found to be the best modified enzyme. The immobilization of the chemically modified enzymes in a suitable matrix, by adsorption or gel entrapment is a useful strategy for reuse of the enzyme as well as improving the thermal stability of the biocatalyst. Starch was the best matrix for immobilization by entrapment. The modified enzyme showed enhanced catalytic properties and was more resistant to thermal and autolytic inactivation. We have succeeded in enhancing the resistance to heat as a denaturing condition utilizing a simple modification without noticeable effects on activity. Modified enzymes were found to be more robust than native enzyme when incorporated in domestic cloth washing detergent formulations.

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