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Immobilization of Modified Papain with Anhydride Groups on Activated Cotton Fabric

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Abstract Papain (EC 3.4.22.2) has been chemically modified using two novel reagents including different anhydrides of 1,2,4-benzenetricarboxylic and pyromellitic acids. Then, the modified papain was immobilized on the activated cotton fabric by a two-step method. The number of free amino groups in the modified protein was investigated through the 2,4,6-trinitrobenzenesulfonic acid method. Energy dispersive spectrometer was used to characterize papain immobilization. Some parameters of both modified and native papain immobilized on cotton fabric, such as optimum temperature, optimum pH, and the stabilities for reservation in various detergents were studied and compared. The resultant papain had its optimum pH shifted from 6.0 to 9.0. Compared with immobilized native papain, the thermal stability and the resistance to alkali and washing detergent of immobilized modified enzyme were improved considerably. When the concentration of detergent was 20 mg/ml, the activity of the immobilized pyromellitic papain retained about 40% of its original activity, whereas the native papain was almost inhibited. This work demonstrated that the cotton fabric immobilized modified papain has potential applications in the functional textiles field.

Keywords Papain · 1,2,4-Benzenetricarboxylic anhydride · Pyromellitic anhydride · Modification · Immobilization · Cotton fabric · Detergent

Introduction

Papain (EC 3.4.22.2) is a powerful proteolytic enzyme, belonging to the cysteine protease family. It can be isolated from the latex of the papaya fruit. The enzyme plays a key role in biotechnology and has a range of important applications in cell isolation, leather, cosmetic, textiles, detergents, food, and pharmaceutical industries [1]. However, its uses are quite limited due to poor recovery, yield, limited reusability, rapid inactivation in the soluble

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state, and high enzyme cost [2]. A number of strategies have been proposed to overcome such a limitation including the use of immobilization and chemical modification [3, 4].

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 [5, 6], and its active site consists of Cys-25, His-159, and Asp-158 [7]. Lysine residues are not a part of the active site in papain, so the modification of the papain surface with different anhydrides appears to be a good strategy to improve biocatalyst performance by chemical binding. In the alkaline condition, the electronegativity of –COOCO is its tendency to attract bonding electrons toward itself to form an amide [8]. Various modifiers such as succinic, phthalic, and maleic anhydrides have been used to modify papain since the anhydrides are used for modification of papain [9]. In this work, two novel modifiers, 1,2,4-benzenetricarboxylic and pyromellitic anhydrides, containing larger molecular weight and more carboxylic groups, were developed to modify papain.

Immobilization of papain after chemical modification will increase its stability and reusability in alkaline conditions [10]. Up to the present moment, many natural polymers are often preferred to use as the matrix of enzyme because of their non-toxic, low cost, free availability, and biodegradability characteristics [11]. Among the natural polymers, cotton fabric, the ubiquitous homopolysaccharide made up of cellulose, is particularly abundant, inexpensive, and available. Several authors investigated the crosslinking potential of cellulose with glutaraldehyde. Xu et al. [12] reported the use of glutaraldehyde and glyoxal to impart higher levels of wet strength to paper, comparing their different reactivities with cellulose. Choi et al. [13] tested the use of these two aldehydes for replacement of formaldehyde in durable press finishing of cotton, reporting higher efficiency of glutaradehyde in the crosslinking with cellulose. In their work, the reaction of cellulose with glutaraldehyde took place directly due to the cellulose in cotton fibers, which is relatively inert because the inter- and intra-molecular hydrogen bonds formed between the hydroxyl groups lower the reactivity of the hydroxyl group with organic or inorganic reagents [14], while the grafting allows easy and controllable introduction of new groups with a high surface density and long stability of the grafted layers. Therefore, we have chosen the chemical grafting approach for the surface modification of the cotton fabric. According to the best knowledge of the authors, there is no report in the literature on the immobilization of modified papain on the activated cotton fabrics.

In the present work, the modified papain was firstly immobilized on the activated cotton fabric by the two-step method. Then, the modified and native papain both immobilized on the fabric were characterized by energy dispersive spectrometry (EDS). The efficiency of enzyme activity was evaluated by investigating the thermostability, pH stability, retaining activity in detergent, and enzymatic kinetics. Papain has many practical applications in cosmetics, textiles, and detergents [1], which could provide a direction to develop new functional textiles.

Materials and Methods

Materials

Papain (EC 3.4.22.2, 6 U/mg), albumin, 2,4,6-trinitrobenzenesulfonic acid (TNBS), 1,2,4-benzenetricarboxylic anhydride, and pyromellitic anhydride were purchased from Sigma-Aldrich Trading Co., Ltd (Shanghai, China). Acetone and pyridine were procured from Shanghai Chemical Reagent Co., Ltd (China), characterized for analytical grade. L-Cysteine

(biological reagent) and all the other reagents of analytical grade were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Cotton fabric of twill weave (20 tex × 16 tex, 50 ends/cm × 24 picks/cm, tex = $G/L \times 1000$, where G (grams) and L (meter) are the weight and length of the fiber, respectively) was desized and bleached before use. Branded domestic detergent was purchased from NICE Group (China). Distilled water was used throughout this research.

Surface Modification of Cotton Fabric

A laboratory procedure was used to incorporate reactive functional groups onto cotton fabrics. The treatment was carried out in two steps:

1. Desized cotton fabric samples were added to 1 M NaOH solution, incubated in a 30 °C bath equipped with shaker at a rotation of 180±5 rpm for 60 min. After alkaline treatment, the samples were washed with distilled water, followed by washing with acetone for 3 cycles. Then, the treated fabrics were immersed in acetone containing *p*-toluenesulfonyl chloride with a liquid-to-solid ratio of 20:1 (*v*/*w*) at 45 °C for 3 h. Souring treatments were carried out similarly with the process as mentioned above and air-dried.
2. The cotton fabrics treated with the first step were dipped in an aqueous solution containing 0.1 M ethyl diamine at room temperature for 1 h under magnetic stirring and 0.02 M 25% glutaraldehyde was added, then rinsed thoroughly with distilled water to remove the residual reagents, and dried at room temperature.

Modification by 1,2,4-Benzenetricarboxylic Anhydride and Pyromellitic Anhydride

Papain (6 mg/ml) was suspended in 50 ml 0.05 M Tris–HCl buffer pH 8.0. Fifty milligrams of different anhydrides was added slowly with constant stirring to the papain suspension. This immediately brought a drop in pH, and hence, sodium hydroxide was gradually added to the solution to maintain the pH at 8.0 using a microburette during the reaction. The reaction was allowed to proceed for 1 h at 25 °C. The samples were dialyzed exhaustively against distilled water at 4 °C.

Determination of Free Amino Group Content

Determination of free amino groups in native and modified papain was carried out according to the TNBS method [15]. The free amino groups remained in the modified papain, upon reaction with TNBS, from a highly chromogenic derivative, which can be measured at 335 nm. Data were finally expressed as relative concentrations (percent), which were calculated from the following equation: α (%)=1−β (%), where α is the fraction of amines consumed by the anhydrides, corresponding to β, which is the protein–NH_2 attached with TNBS.

Immobilization of Papain

The prepared cotton fabric (1 g), incubated in 50 ml Tris–HCl buffer pH 8.0, was added in native and modified papain solution (6 mg/ml), respectively. The flask was shaken at 170 rpm at 4 °C. Following immobilization for 10 h, all the fabric samples were washed with distilled water several times until no significant amount of papain activity was detectable in the washing solution and then dried in the vacuum oven.

Enzymatic Assays

A given amount of immobilized native papain or modified papain and 1.6 ml 0.01 Tris–HCl buffer pH 8.0 were put into a tube, mixed with 0.4 ml papain activator consisting of 0.02 M ethylene diaminetetraacetic acid and 0.05 M L-cysteine pH 8.0 and incubated for 10 min at 40 °C to reduce the disulfide bonds of the cysteine molecules of the protein. Then, 1 ml of 1% casein in pH 8.0 Tris–HCl buffer was added. After the enzymatic reaction was carried out at 45 °C for 15 min, 3 ml of 5% trichloroacetic acid solution was added into the tube and mixed, and the mixture was statically equilibrated at room temperature for 10 min. The following procedure was the same as the determination of the soluble papain activity described by Kasy and Wildi [16]. The absorbance of the filtrate obtained was determined at 280 nm using a quartz cuvette with 1 cm path length. One unit of enzyme activity was defined as the tyrosine content formed per minute at 45 °C and pH 8.0. The relative activity (percent) was the ratio between the activity of every sample and the maximum activity of samples.

Protein Determination

Protein concentration was determined by the Bradford method [17], using the Coomassie protein assay reagent with bovine serum albumin as the standard. After column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm.

Characterization of Papain Immobilization and Spectral Measurements

Scanning electron microscope (SEM, JSM-5600LV) equipped with an EDS (Oxford) was used to characterize the particle size, the morphology, and the chemical composition of the cotton fabric before and after papain immobilization. Secondary electron images were acquired using an accelerating voltage of 10 kV. A Fourier transform infrared (FT-IR) spectrometer (Nicolet, NEXUS-670, USA) with 2 cm^{-1} resolution was used for sample analysis. The scans were repeated 200 times in spectral range from 4,000 to 400 cm^{-1}. KBr was used as the mulling agent. The conformational change of papain was further confirmed with UV–vis spectroscopy [18]. The UV–vis spectrum was recorded on a UV-2102PC spectrophotometer (UNICO, Shanghai, China) in the range 240–340 nm equipped with 1.0-cm quartz cells.

Influence of Temperature on Enzyme Activity and Stability

The effect of temperature on the activity of immobilized native and modified papain was determined by assaying the enzyme activity in a standard procedure mentioned above. The temperature was varied from 30 to 90 °C at pH 8.0. Thermal stability of the immobilized enzyme was determined by measuring residual activity after incubating the enzyme for 1–4 h in a circulating water bath at 80 °C pH 8.0. Heated samples were cooled immediately in ice water, and the residual enzymatic activity was determined as described above.

Influence of pH on Enzyme Activity

The effect of pH on the activity of immobilized native and modified papain was determined by assaying the enzyme activity at different pH values ranging from 5.0 to 11.0 at 45 °C, using 0.05 M concentrations of the following buffer systems: Tris–HCl (pH 5.0–10.0) and Tris–NaOH (pH 11.0). The residual enzymatic activity was determined as described above.

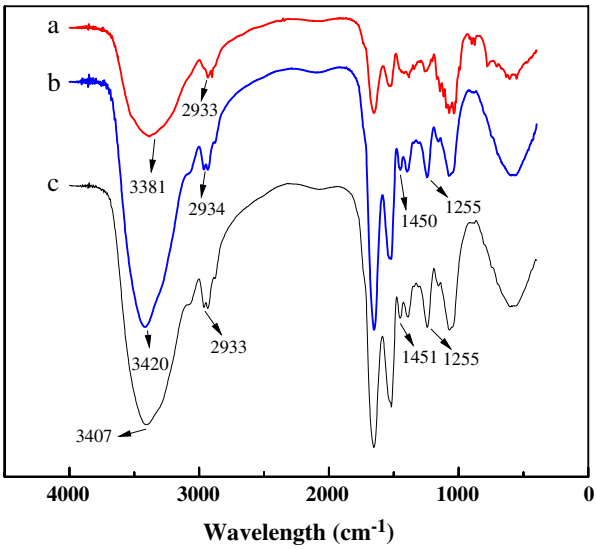


Fig. 1 FT-IR spectra of *a* UP, *b* BP, and *c* PP

Proteolytic Activity in Detergent

For each sample, a measured amount of detergent powder in distilled water was used to give a final concentration of 5–25 mg/ml. A suitable dilution of the native and modified enzyme was made in the detergent solution, respectively, and then assayed for the proteolytic activity at 45 °C. Casein was used as the substrate.

Determination of Kinetic Constant

The kinetic constants of immobilized native and modified enzyme were determined as the concentrations of casein ranged from 0.1% to 0.5% at pH 8.0. The K_m and V_{max} of the enzyme were calculated from the intercept and slope according to the Hanes–Woolf plot. The incubation temperature was 45 °C.

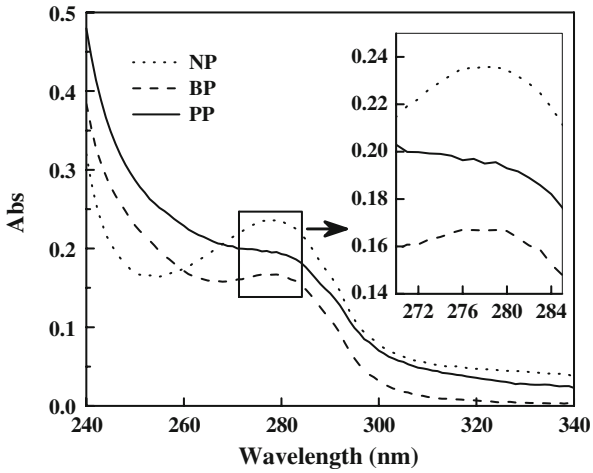


Fig. 2 UV–vis spectra of native and modified papain in distilled water

Table 1 Degree of modification.

Enzyme	α (%)	Relative activity (%)
UP	0	100
BP	45.24±0.88	96.73±0.12
PP	40.35±0.57	95.88±0.96

Data presented are average value ± SD of n=3 experiments

Results and Discussion

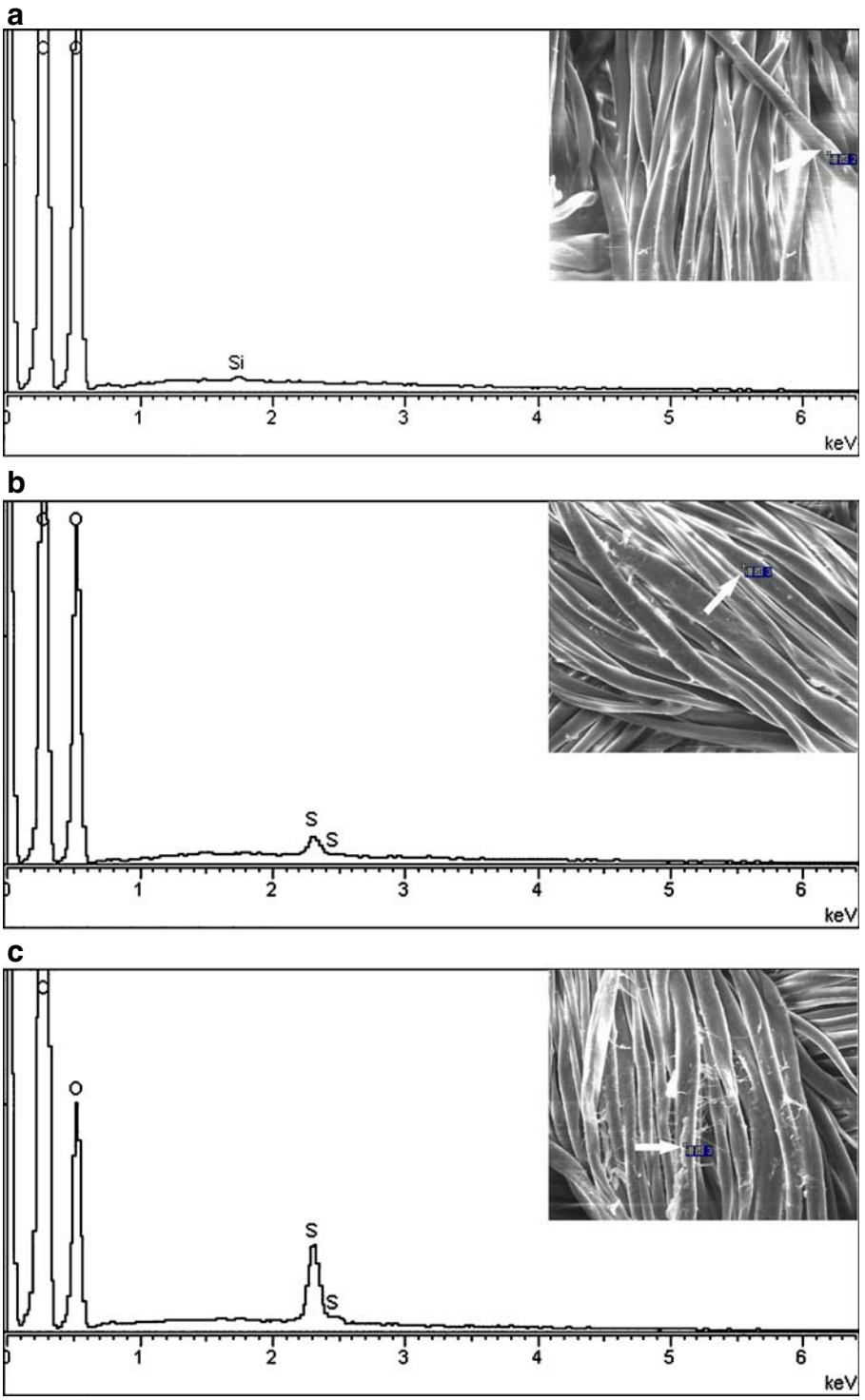
Modification of Papain

FT-IR, UV Spectral Studies FT-IR spectroscopy has been proven to be a powerful tool for providing conformational and structure dynamics information of proteins. Figure 1 illustrates the formation of new absorption peaks in the FT-IR spectra and changes in functional groups of papain after being chemically modified. Higher absorption in the region from 3,381 to 3,420 cm^{-1} corresponding to the –OH stretching of the –OH groups present in the unmodified papain (UP), 1,2,4-benzenetricarboxylic papain (BP), and pyromellitic papain (PP). The absorption at 2,933 cm^{-1} was due to the stretching vibration of CH_2. After chemically modified, a relatively higher absorption at the characteristic peak (1,652 cm^{-1}) and (1,519 cm^{-1}) of amide I and amide II was observed. They both have a relationship with the secondary structure of the protein [19]. In addition, the benzene ring absorption band overlapped with amide I at 1,600 cm^{-1}, 1,255 cm^{-1} due to C–O stretching of the ester group, and 1,450 cm^{-1} due to C–N stretching. These findings confirmed that modification had taken place.

UV–vis absorption measurement is a very simple method and applicable to explore the structural change and to know the complex formation [20]. The UV–vis absorption spectra of native and modified papain from 240 to 340 nm in distilled water was shown in Fig. 2. UP has strong absorbance with a peak at 280 nm and the spectrum of BP showed a blue shift of 4 nm in the emission maximum from 280 to 276 nm. Moreover, PP did not show any evident aggregation but present a broad band around 270–280 nm. In addition, the intensity of absorption of BP and PP was lower compared to UP. These changes are, most often, due to the interaction between the enzyme and anhydrides, and they may also be due to the unfolding of papain in the presence of chemical modification. Unfolding behavior of protein led to a decreases in the intensity of absorption [21].

Determination of Modification Degree by TNBS Derivatization Concerning the crosslinking effect and its efficiency, papain, modified with 1,2,4-benzenetricarboxylic anhydride or pyromellitic anhydride, was investigated and the results were shown in Table 1. We can induce the percentage of the modified lysine residue. An accurate determination will depend on all uncrosslinked groups participating in the TNBS reaction [22]. From our results (Table 1), it was observed that 1,2,4-benzenetricarboxylic papain with minimum numbers of exposed ε-amino groups, about 44.71% lysine residues of the enzyme were modified by the present method, and the modified papain did not lose its activity significantly. This behavior may suggest that among different anhydrides those with small molecular size are preferentially modified during

Fig. 3 Chemical composition analyses by EDS to the referred region in the corresponding SEM (magnification ×1,000). **a** Before treatment, **b** treatment by the two-step method, and **c** after papain immobilization by glutaraldehyde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the crosslinking reaction and the amino acid at the active site causes no detectable change in the tertiary structure of the enzyme. Also, the two modified papain showed similar profiles for the numbers of ε-amino groups at different chemically modified reagents.

Immobilization of Modified Papain on Cotton Fabric

Surface Characterization of Bioscoured Cotton EDS was used to qualitatively analyze the chemical composition change of the cotton fabric before and after papain immobilization, as shown in Fig. 3. The results show that the silicon element is not seen after chemical treatment (Fig. 3b), whereas S atoms were increased a little because the *p*-toluenesulfonyl chloride that combined with the fabric did not react with ethyl diamine completely. The untreated fabric lacks sulfur element (Fig. 3a). The sulfur element ratio changed significantly appearing only on the papain-immobilized fiber (Fig. 3c). The text result shows that papain was covalently bound onto the cotton fabrics successfully.

In this study, the reaction of cellulose with papain occurs through the aid of a coupling agent, glutaraldehyde. Glutaraldehyde can react with several functional groups of proteins. Furthermore, these groups are generally not involved in the catalytic site, which allows moderate crosslinking to preserve enzyme conformation and thus biological activity.

Effect of Temperature on the Activity and Stability of Immobilized Enzyme As to be expected, the enzyme activity of immobilized native and chemically modified papain increased gradually with temperature, and the maximum activity was obtained at 80 °C (Fig. 4). This confirms that the enzyme favors higher temperature as can be observed from the activity data at 60, 70, and 80 °C. The activity of immobilized unmodified papain (IUP) and immobilized benzenetricarboxylic papain (IBP) were increased stably over a period of 60 to 80 °C without significant change. However, the activity of immobilized pyromellitic papain (IPP) has a vital peak at 80 °C, which may be attributed largely to the particular enzyme structure [23]. Figure 5 is a comparison of the thermal stabilities of immobilized native and chemically modified papain at 80 °C in buffer pH 8.0. Both preparations exhibited a similar trend; after 4 h of incubation, the IPP retained 15% activity while total activity losses were found for the IUP and IBP. Thermal inactivation is believed to be mainly caused by denaturation of tertiary structure through protein unfolding or disruption of the active site of the enzyme [24]. The greater stability of the IPP is partly ascribed to diminished autolysis due to their fixation on the support. Moreover, pyromellitic anhydride possesses two ester function groups per molecule compared with the single group of 1,2,4-benzenetricarboxylic anhydride, which could provide more than one reactive site. Pyromellitic anhydride reacted with the amino groups in the molecule, to generate amide–acid crosslink, and non-covalent hydrogen bonds determine enzyme folding and stability, as well as extraionic interactions with the side chains of the amino acid residues, which had also been found to play a role in enzyme stability [23].

Effect of pH on the Activity of Immobilized Enzyme pH value is a very important parameter to investigate in this study. The tertiary structure of the enzyme depends on pH; the enzyme can be denatured at extremes of pH [25]. Hence, the main objective of papain modification was to improve the stability of the enzyme in extreme environmental conditions in terms of pH in detergent. In Fig. 6, the native or modified papain was active over a broad pH range, displaying over 80% of its activity in the pH range 7.0–10.0. The extended stability of the crosslinked preparation in the pH range may be due to resistance to unfolding due to inter-

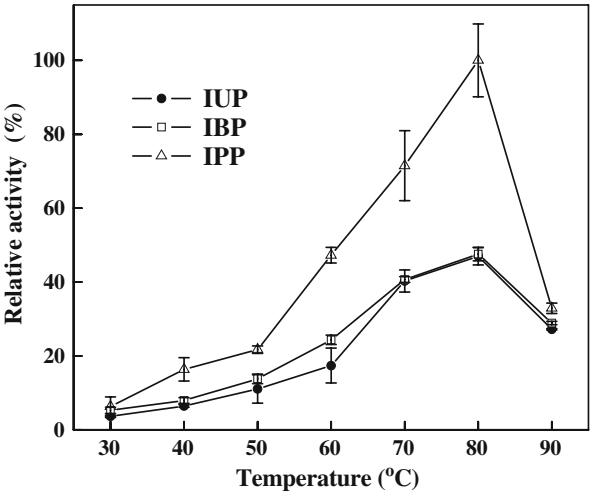

Fig. 4 Effect of temperature on immobilized papain activities. Enzyme activity was determined at different temperatures ranging from 30 to 90 °C at pH 8.0. Relative activities were expressed considering the maximum activity of samples as 100%. Indicated values are means of three experiments, and *error bars* represent the standard errors of means

and intra-molecular crosslinking [26]. The maximum native papain activity was observed at pH 6.0. However, a further decrease from pH 6.0 to 11.0 decreased the native papain activity rapidly to the relative activity of 70%, which indicated that the enzyme was insensitive to acidic conditions. These findings were in agreement with a pH optimum of 6.8 for native papain as reported [27]. Moreover, decline in alkaline pH results in native papain exchange. In modified papain, the enzyme is very stable at this pH and corresponds to the maximum pH at 9.0. It is suggested that the acid anhydride reacts with the lysine groups of the papain leading to a net anionic charge from positive to negative. Enveloping the enzyme with negatively charge groups might cause a localized lowering of pH with respect to the surroundings. Furthermore, the 1,2,4-benzenetricarboxylic anhydride has a free carboxyl, and the acid limb attaching to the enzyme can be effectively used to adjust the alkaline condition.

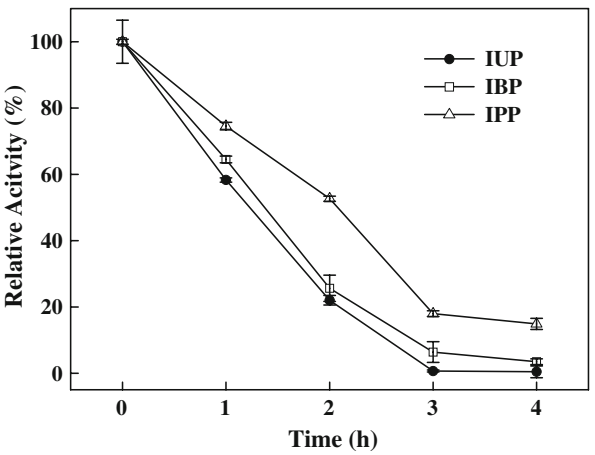

Fig. 5 Thermal stabilities of immobilized papain incubated in pH 8.0 buffer at 80 °C for 1 to 4 h. Relative activities were expressed considering the maximum activity of samples as 100%. Indicated values are means of three experiments, and *error bars* represent the standard errors of means

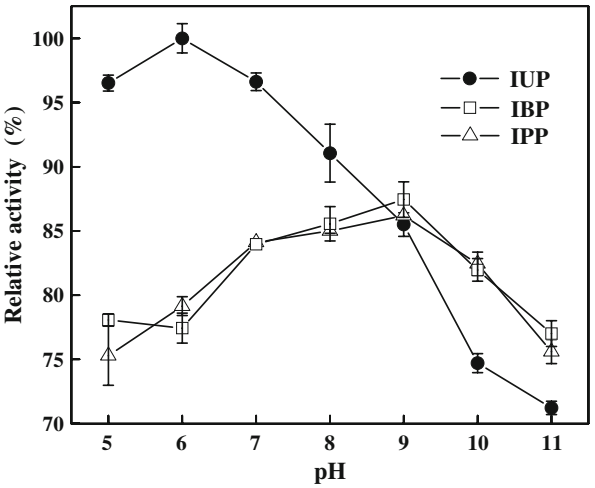

Fig. 6 Effect of pH on immobilized papain activities. Enzyme activity was determined at different pH ranging from pH 5 to 11 at 45 °C. Relative activities were expressed considering the maximum activity of samples as 100%. Indicated values are means of three experiments, and *error bars* represent the standard errors of means

Stability Towards Detergent The use of chemically modified alkaline proteolytic enzymes [28] and papain in detergents has recently been patented [29]. Detergents are very complex formulations containing anionic surfactants, bleaching agents, and water-softening builders. Generally, proteases are susceptible to denaturation by the anionic surfactants [30]. To elucidate the effect of the detergent on the activity of the immobilized enzymes, the native and modified papain immobilized on cotton fabrics were preincubated in the presence of laundry detergent for 30 min at 45 °C.

The data, presented here in Fig. 7, show that the IBP and IPP were more stable in the present test under various detergent concentrations. The IPP still retained about 40% of their activities in the thickest detergent. For IUP, the strong surfactant at 15 and 20 mg/ml caused a significant inhibition of 71% and 86%, respectively. At lower concentrations (0–10 mg/ml) of the IPP applied, there was a very slight decrease in the activity.

The anionic surfactants usually affected negatively on the enzyme activity [31] and the modification of papain caused an increase in the enzyme stability against the anionic

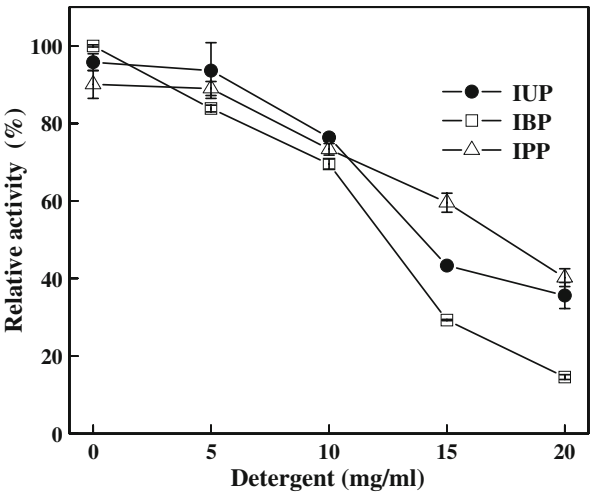

Fig. 7 Effect of detergent on immobilized papain activities. Enzyme activity was determined at different concentrations of detergent ranging from pH 5 to 11. The pH value and temperature were controlled at 8.0 and 45 °C, respectively. Relative activities were expressed considering the maximum activity of samples as 100%. Indicated values are means of three experiments, and *error bars* represent the standard errors of means

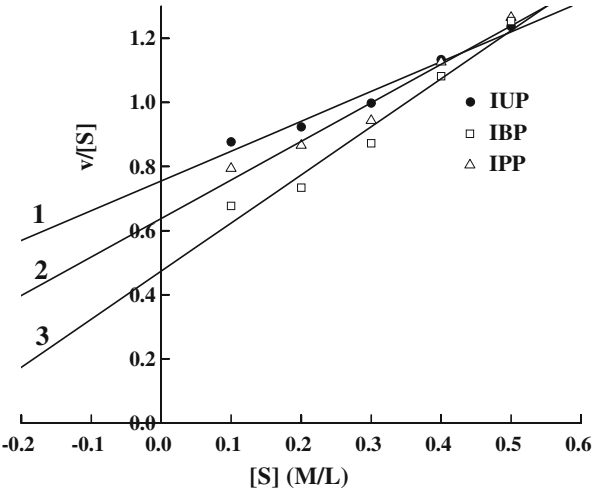

Fig. 8 Hanes–Woolf plots of immobilized native and modified papain. *Curves 1–3* were *1* IUP, *2* IPP, and *3* IBP, respectively

surfactants. The change in activity may be due to the alkaline pH which was caused by the detergent favoring the modified papain. The activity of chemically modified papain on fabrics after washing fastness with the detergent was preserved; thus, as functional cotton textile, it was very efficient to overcome the poor alkaline laundering durability. Moreover, the addition of enzyme led to a distinctly better cleansing power of detergents through hydrolytic degradation of difficult protein-containing solids on fibers.

Kinetic Parameters The experiments were carried out with low initial substrate reservoir concentration so that the Michaelis–Menten kinetics can be approximated by first-order kinetics [32]. Accordingly, the kinetic parameters of the enzyme were investigated including the maximum reaction rate (V_{max}) of the enzymatic reaction and the Michaelis–Menten constant (K_m). These parameters were obtained from Hanes–Woolf plot which can be written as:

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

The 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 [33]. When we compare the K_m values of IUP with that of IPP (Fig. 8), some differences in K_m values indicated that the enzyme modified by pyromellitic anhydride has greater affinity towards the substrate (Table 2). Compared with IUP, there is not much difference between IBP and IPP in the V_{max} value which is around

Table 2 Kinetic constants of native and modified papain.

Enzyme	k_{cat} (mol·s^{-1}·mol enzyme^{-1})	V_{max} (mol·s^{-1}·g^{-1})	K_m (mol·l^{-1})	k_{cat}/K_m
IUP	1.79	1.076×10^{-2}	0.8121×10^{-2}	2.204×10^{2}
IBP	1.604	0.9624×10^{-2}	0.7005×10^{-2}	2.290×10^{2}
IPP	1.386	0.8317×10^{-2}	0.5307×10^{-2}	2.612×10^{2}

0.8–1.0 mol s^{-1} g^{-1}. In addition, the highest k_{cat} is with IUP followed by IBP and then IPP. Between the native and modified papain in the study, IPP was found to be the best in catalytic efficiency. The difference may be ascribed to the structural differences of the enzyme, as pyromellitic anhydride is the bulkiest group attached to the lysine residues which in turn improves the catalytic activity by altering the enzyme conformation.

Conclusion

In this study, two novel modifiers were used to modify papain. The degree of modification was established and maximum modified degree was approximately 45% at papain modified by 1,2,4-benzenetricarboxylic anhydride. The results showed that 1,2,4-benzenetricarboxylic anhydride and pyromellitic anhydride were effective in promoting the activity of papain under extreme environmental conditions. The native or modified papain was then immobilized on the activated fabric as a new carrier. According to the analyses of EDS, it is demonstrated that the two-step method has been successful in papain immobilization on cotton fabric, and the model was based on the relationship between the enzyme and the binding quantity of the activated carrier. Each immobilized modified papain exhibits remarkable stability for medium alkalinity and high temperature, as well as good reusability for detergent.

Evidence indicates that native papain is a proteolytic enzyme and also plays a key role in the interaction between native enzyme and protein, such as casein. But there is little information available in the literature about the effect of modified papain on protein. This research will be done subsequently to investigate the effect of modified papain on different proteins, not only casein but a whole class of casein analogues.

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