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Stabilization of Papain by Modification with Chitosan

Ali KILINÇ, Seçil ÖNAL and Azmi TELEFONCU
Department of Biochemistry, Faculty of Science, Ege University,
35100 Bornova, İzmir-TURKEY
e-mail:akilinc@sci.ege.edu.tr

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Papain (EC 3.4.22.2) was immobilized on chitosan by adsorption and subsequent cross-linking with glutaraldehyde. The immobilized papain displayed a lower specific activity than did the native enzyme. The thermal stability of the immobilized papain, relative to that of the free enzyme, was markedly increased. The storage stability of the conjugated enzyme was enhanced such that more than 85% of the initial activity remained after a month storage at 45°C. The optimum pH of immobilized papain was shifted to the acidic region and enzyme stability was increased at pH levels below 3.5.

Key Words: Enzyme stabilization, immobilized papain, chitosan

Introduction

The chemical modification of enzymes with macromolecules constitutes a useful strategy for improving the stability of these biocatalysts. In this context, the use of several natural or synthetic polymers such as dextran, polyethylene glycol and carboxymethylcellulose has been reported[1−4].

Amino acid residues of enzymes contain reactive groups such as amino (Lys, N-terminus), thiol (Cys), carboxyl (Asp, Glu, C-terminus), aromatic hydroxyl (Tyr) and aliphatic hydroxyl (Ser and Thr). Chemical, ionic or chelation reactions with such groups enable us to attach the amino acids and hence proteins to soluble or insoluble inert supports. Immobilization is one of the best ways of stabilizing enzymes. Immobilization imparts stability to proteins by restricting the movement of the protein molecule by attachment to an inert body via chemical bonds. The various domains are therefore held in the correct orientation to retain activity at least over an extended period of time when compared with enzymes in free solution.

The addition of polyhydroxyl compounds to enzyme solutions has been shown to increase the stability of enzymes[5−8]. This is thought to be due to the interaction of the polyhydroxyl compound with water in the system. This effectively reduces the protein water interactions as the polyhydroxyl compounds become preferentially hydrated and thus the hydrophobic interactions of the protein structure are effectively strengthened. This leads to an increased resistance to thermal denaturation of the protein structure and an increase in the stability of the enzyme. Polysaccharide amines were effective in reducing hydrophobic interactions when they were used as spacers, and they prolonged enzyme life in use when compared to alkane spacer molecules[9,10]. Chitosan is in polycationic form at acidic and neutral pH regions. The association of the polyelectrolyte with the enzyme probably results in the formation of a cage around the enzyme molecule.

Some proteases, including papain, can hydrolyze the peptide bonds of collagen and keratin in the stratum corneum of the skin. The controlled skin damage can trigger skin repair and bring to the surface a layer of smoother, softer skin[11]. Papain is one of the potential active ingredients in cosmetic products. The aim of this work is the stabilization of papain by coupling with chitosan, which is a cationic, bioactive, biodegradable, biocompatible polysaccharide suitable for many applications in cosmetics.

Experimental

Materials

Papain (EC 3.4.22.2) and N-benzoyl-L-arginine ethyl ester (BAEE) were obtained from Sigma Chem. Co. (St. Louis, USA). Glutaraldehyde (25%) was purchased from BDH (Dorset, UK). Chitosan was obtained from Fluka Chemie AG (Buch, Switzerland). All other chemicals were of analytical grade.

Preparation of Chitosan-Papain Conjugate

Chitosan was dissolved in 2% (v/v) acetic acid, precipitated by adding 1 M NaOH up to pH 10.0 and then extensively washed with 30 mM citrate/phosphate buffer pH 6.0 (Buffer-I). Then 100 mg chitosan was suspended in 10 ml of citrate/phosphate buffer and 50 mg papain was added. The suspension was kept at 4°C overnight under stirring and subsequently centrifuged at 10,000xg for 15 min. The resulting pellet was resuspended in Buffer-I containing 2% glutaraldehyde. The suspension was stirred at room temperature for 30 min under vacuum and then centrifugated again at 10,000xg for 15 min. The chitosan-papain conjugate was washed with small aliquots of Buffer-I containing 2M KCl and Buffer-I alone until no protein was detected in the washings.

Determination of Immobilized Protein

The protein content of the chitosan-papain conjugate was calculated by substracting the amount of protein determined in the centrifugate and washings following immobilization from the amount of protein used for immobilization. The protein content in the solutions was determined by the Bradford method[12].

Enzyme Assay

Enzyme activity in native and modified papain was determined titrimetrically, using N-benzoyl-L-arginine ethyl ester (BAEE) as substrate. The reaction mixtures contained 125 mmole of substrate, 25 mmole of cysteine, 10 mmole of EDTA, and the appropriate amount of enzyme in a total volume of 5 ml. The enzymatically liberated N-benzoyl-L-arginine was titrated with 0.01 N NaOH using a pH-stat[13]. One unit enzyme was taken to hydrolyze 1.0 mmole of BAEE per min at pH 6.2 and 25°C.

Stability Tests

In order to evaluate the effect of chemical modification on papain thermostability, two different types of experiment were performed. Firstly, native and modified papain were incubated at different temperatures in 50 mM phosphate buffer, pH 7.0. Aliquots were removed after 10 min incubation, chilled quickly and assayed

for enzymatic activity. Secondly, native and modified papain were incubated at 75°C in 50 mM phosphate buffer (pH 7.0). Aliquots were removed at scheduled times, chilled quickly and assayed for enzymatic activity.

For the estimation of pH stability, native and modified papain were incubated at 25°C in 50 mM citrate/phosphate buffer, pH 2.2-6.2 and 50 mM phosphate buffer, pH 6.2-8.6. Aliquots were removed after 30 min incubation and assayed for enzymatic acitivity.

The storage stability of the enzyme preparations was also studied. Native and modified papain preparations were stored at 25°C and 45°C, and enzymatic activity was measured at scheduled times. It is generally accepted that a month's stability of an enzyme at 45°C is roughly equal to that of one year at room temperature[8].

Results and Discussion

Chitosan was selected as a carrier for papain immobilization on account of the following properties:
(i) polycationic, (ii) hydrophilic, (iii) biocompatible and biodegradable, (iv) bioactive. Papain was immobilized by cross-linking the enzyme and chitosan by means of glutaraldehyde. The optimal cross-linking incubation time and glutaraldehyde concentration were found to be about 30 min and 2%, respectively. Above this incubation time the immobilization yield appeared to increase slightly but the specific activity of the immobilized enzyme decreased. The chitosan-papain conjugate contained about 18 mg protein/100mg chitosan (Table).

Table Some properties of native and chitosan-linked papain (cross-linking process time: 30 min; glutaraldehyde concentration in the coupling medium: 2%).

Property	native papain	chitosan-papain conjugate
Protein content (%)	81.7	15.3 (18 mg/100 mg chitosan)
Specific activity (*mmole/min.mg protein*)	12.4	10.2
pH optimum	6.5	6.0
Temperature optimum (°C)	65	75
Storage stability *(% activity retained after one month at 45° C)*	16	85

The recovery yield of papain activity was about 82% right after immobilization. The specific activity reduction in immobilized enzymes with respect to their native forms has been widely reported[14,15]. Structural changes by covalent attachment and diffusional limitations could be the reasons for these activity losses[16].

The stability of papain was dramatically improved by conjugation with chitosan-compared with that of native enzyme. In order to evaluate the effect of the modification on enzyme thermostability, two different types of experiments were performed. Figure 1 shows the thermal stability profile of chitosan-bound papain after 10 min of incubation at different temperatures. The thermal stability of immobilized papain was markedly increased relative to that of the native enzyme. After conjugation, T50 (the temperature at which 50% of initial activity was retained) was increased from 75°C to 83°C. As shown in Figure 2, the heat stability of chitosan-linked papain at 75°C was improved dramatically.

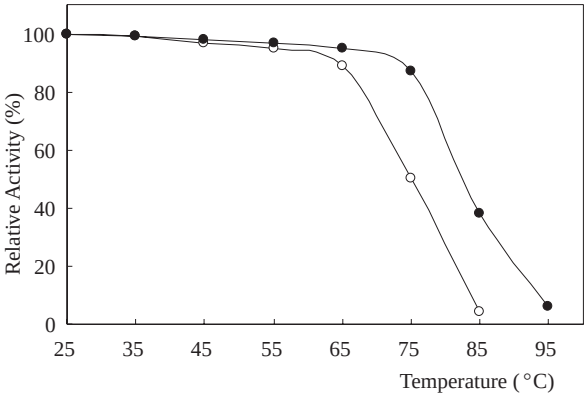

Figure 1. Thermal stability profile of native (o) and immobilized papain (•). Native and immobilized papain preparations were incubated at preset temperatures in 50 mM phosphate buffer (pH 7.0) for 10 min and then assayed under standard test conditions.

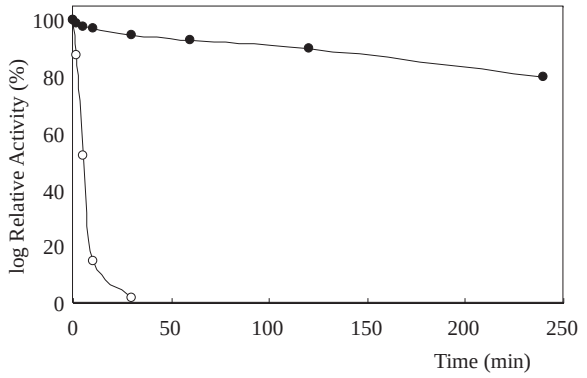

Figure 2. Heat stability of native (o) and immobilized papain (•) at 75°C. The enzyme preparations were incubated at 75°C in 50 mM phosphate buffer (pH 7.0) for scheduled times and then assayed at standard test conditions.

The storage stability of papain was also dramatically improved by conjugation with chitosan compared with that of native enzyme. About 85% of initial activity remained after a month of storage at 45°C (Fig. 3).

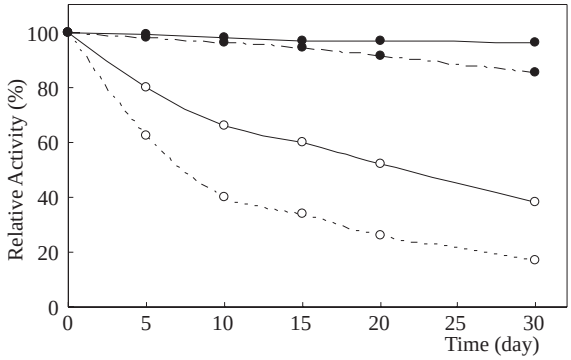

Figure 3. Stability of native (o) and chitosan modified papain (•) when stored at 25°C (—) and 45°C (- - -).

As shown in Figures 1-3, the thermal stability and storage stability of chitosan-conjugated papain were improved. The conformational stabilization of papain molecules due to intramolecular cross-linking is the most important factor underlying this effect[17]. Protein aggregation plays an important role in thermal denaturation of enzymes and the electrostatic repulsion between the enzyme molecules linked to chitosan (a cationic polymer) could be another factor for the improved thermostability of the modified enzyme.

The activities of native and immobilized papains were determined at different pH values. As can be seen from Figure 4, the pH optimum of the chitosan-papain conjugate was shifted to the acidic region relative to native papain. The immobilization of enzymes to charged supports often leads to displacements in the pH activity profile, ascribable to unequal partitioning of H^+ and OH^- between the microenvironment of the immobilized enzyme and the bulk phase due the electrostatic interactions with the matrix[16,18].

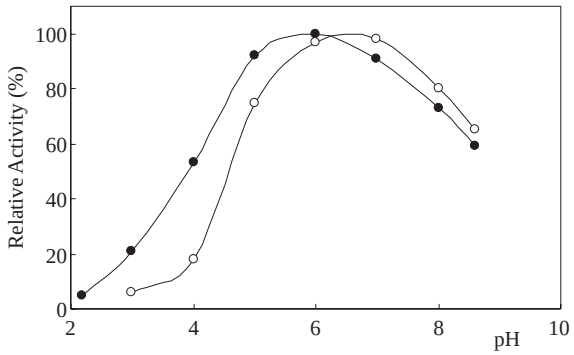

Figure 4. pH-activity curves for native (o) and immobilized papain (•).

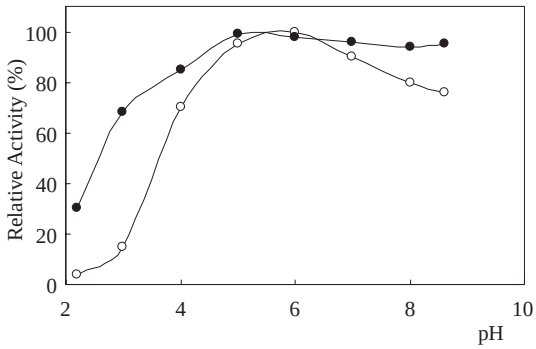

Figure 5. Effect of pH on the stability of native (o) and immobilized papain (•). The test solution (1 ml) in the appropriate buffer and containing papain or immobilized papain was incubated at 25°C for 30 min. The esterase activity was determined under standard test conditions.

The pH stability of free and immobilized papain was determined in the pH range 2.2-8.6. Figure 5 shows that pH stability was increased for chitosan-papain conjugate in the range between 2.2 and 6.2. Since chitosan is positively charged at acidic or neutral pH values, this polysaccharide is able to form intramolecular salt bridges with anionic groups of the protein. This electrostatic interaction could improve the stability of papain, because intramolecular salt bridges are, in fact, one of the principal forces contributing to the maintenance of the active conformation of enzymes[19].

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