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Preparation and Properties of Papain Immobilized onto Metal Ions Cross-linked Chitosan Beads

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ABSTRACT

Chitosan beads were prepared by using a cross-linking agent Cu(II) and Zn(II) metal ions and the metal ion- chitosan beads were employed in papain immobilization processes. Studies on free and immobilized papain systems for determination of optimum pH, optimum temperature, thermal stability and reusability were carried out. The results showed that free papain have been optimum pH 6,5 and optimum temperature 55 ^{o}C while the immobile papain had optimum pH 8 and optimum temperature 85 ^{o}C. The thermal stability of the immobilized papain, relative to that of the free papain, was markedly increased. The residual activity of papain immobilized on chitosan bead- metal ion was about 25% after 12 cycles of batch operation.

Keywords: papain, immobilization, chitosan beads, metal ions

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INTRODUCTION

Enzymes have a wide variety of biotechnological, biomedical, and pharmaceutical applications. They are used as biosensors in bioengineering, clinically as therapeutic agents, in modern diagnostic tool, and as catalyst for chemical and biochemical reactions. A large research of work has been devoted to the polymeric carriers, especially to immobilization of the proteins onto carriers [1-4]. Since the recovery yield and reusability of free enzymes as industrial catalysts are quite limited, attention has been paid to enzyme immobilization which may offer advantages over free enzymes; for example, possibility of continuous process, controlled product formation, ease of enzyme removal from the reaction mixture, and adaptability to various engineering designs.

Chitosan, a poly-N-acetyglucosamine, is a transformed oligosaccharide obtained by deacetylation of chitin, and it is the second most abundant biopolymer after cellulose [2,4]. Chitosan exhibits a unique set of characteristics such as biocompatibility, biodegradability, nontoxicity, hydrophilicity, remarkable affinity to proteins, and high mechanical strength. These characteristics make chitosan as desirable biomaterial for enzyme immobilization [2,4,5,10]. It can provide an optimal microenvironment for the immobilized to maintain relatively high biological activity and stability. Increasingly over the last decade chitosan–based biomaterials were employed as enzyme immobilization in the form beads and membranes [6,7].

In this study, chitosan beads were crosslinked with Zn(II) and Cu(II) for matrix papain immobilization. Various attempts have been made to stabilize papain for a more efficient use. Papain and other proteolytic enzymes have been immobilized by radiation polymerization of various monomers [8,16]. Covalent coupling of papain has also been shown in different studies performed by several workers [9,11,12]. However, the biomatrices with entrapped enzymes tend to leak proteins with time. This resulted in the activity losses as well as contamination of the product with the enzymes, which is not acceptable for pharmaceutical applications. The covalent coupling of enzyme can produce a considerable loss of activity due to the influence of the coupling conditions and to conformational changes in enzyme structure. However, irreversible binding of enzyme to the carrier during covalent coupling does not allow the recovery of the carrier from the carrier-enzyme complex [11,13,14]. A method is, therefore, needed in which the carrier should be easily regenerated and reused without reducing the immobilization yield. Attempts have been made in this direction and a metal chelate regenerable carrier has been used to immobilize the papain. This immobilization is based on the ability of protein side chains of cysteine, histidine and tryptophan to substitute weakly bonded ligands in the metal complexes. This method has a big potential and may be more versatile since it allows a selection among many chelating metal ions.

MATERIALS AND METHODS

Papain (EC. 3.4.22.2) and Casein were obtain from Sigma Chem. Co. (st. Louis, USA). Chitosan was obtained from shell of shrimp with Meyer Methods (1989). All other chemicals were of analytical grade.

Preparation of Swollen Chitosan Beads

To prepare highly swollen beads, an amount of chitosan flakes (1g) was completely dissolved in 0.1l of 1-mol/l acetic acid. The resulting solution was sprayed into 125 of ml deionized water containing 15 g NaOH and 25 ml of 95% ethanol through a nozzle (1.2 mm diameter). The chitosan beads were swelled and washed with deionized water until the solution became neutral. The diameter of wet beads approximately 2.3 mm. The BET surface area of swollen beads was not measured because the drying was difficult. The morphology of chitosan beads was analysis with SEM.

Determination of Immobilization Papain

The protein content of the chitosan-papain conjugate was calculated by subtracting the amount of protein determined in the centrifuged and washings following immobilization from the amount of papain used for immobilization. The papain in the solutions was determined by the Bradford method [13].

Immobilization Papain in Different pH

Papain was dissolved in 15 mM PBS with pH ranging from 4.0 to 8.0, respectively. Each kind of chitosan beads-Zn(II) was incubated individually with above papain solution and shaken in a vibration for 12 h. After equilibration, the pH of each solution was detected with a pH meter and adjusted to certain value with PBS. The adsorption capacity of papain in different condition was calculated by following equation.

Activity Assay of Free and Immobilized Papain

The activity of soluble papain was determined by the method of Kunitz as described by others using casein as substrate at 37ºC and pH 8.2. The enzyme activity of immobilized papain was determined in a similar manner except that the reaction mixture was continuously stirred during the reaction. One unit of enzyme activity is the amount of enzyme, which produces TCA soluble peptides or amino acids giving a blue color equivalent to that of 0.5 mg tyrosine per minute at 37ºC.

Effect of pH and Temperature on the Papain Activity

The pH stabilities of the free and immobilized papain were by immersing the sample in PBS 15 mM in the pH range 4-10. Their thermal stabilities were assay by a standard activity assay in the themperature 40 to 90 ^{o}C.

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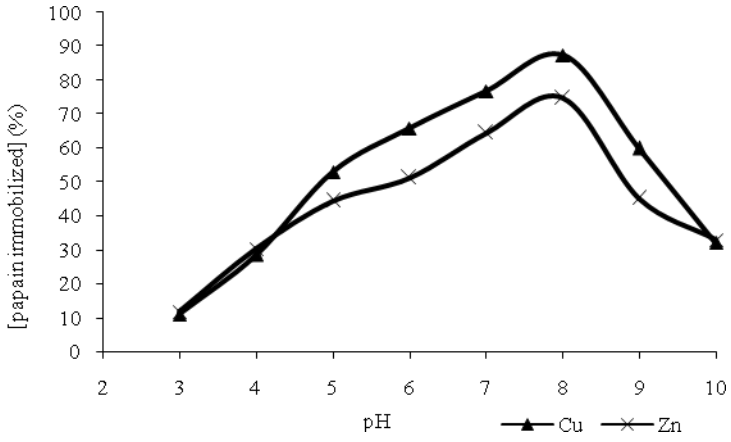
Reusability of Immobilized Papain

For the reusability, after each reaction run, the immobilized papain was removed and wash with water to remove any residual substrate on the matrix. It was then reintroduced into fresh reaction medium and enzyme activity was assayed at optimum condition.

RESULTS AND DISCUSSION

Immobilization Efficiency in Different pH

The effect of pH on the adsorption of papain onto chitosan beads-Zn and chitosan beads-Cu chelated could be found that the maximal immobilized capacity onto matrix in pH 8.0. The decrease in the papain adsorption capacity in more acidic and more alkaline regions could be attributed to electrostatic repulsion effects between the opposite changed groups. Proteins have no net charge at their isoelectric points, and therefore the maximum adsorption from aqueous solutions is usually observed at their isoelectric points. The isoelectric pH papain is 8.75. Papain immobilized on chitosan flake found that maximal immobilized capacity onto chitosan flake in pH 7.5 [13].



Picture 1. Effect of pH value on papain immobilization

However, in the present study, the maximum immobilization was not at this pH, but had slighty shifted toward more neutral pH values. This could be due to preferential interactions between molecules and metal ion incorporated in polymeric matrix at neutral pH. So, in the following experiment, the adsorption of papain on matrix was conducted in pH 8.0. Chitosan do not carry a charge at neutral pH. If the pH value decrease in solution, chitosan beads can be charged positively at lower pH because hydrogen ions can bind to free amino groups. However, limited number of available amino groups on cross-linked chitosan beads reduces number of bound hydrogen ions.

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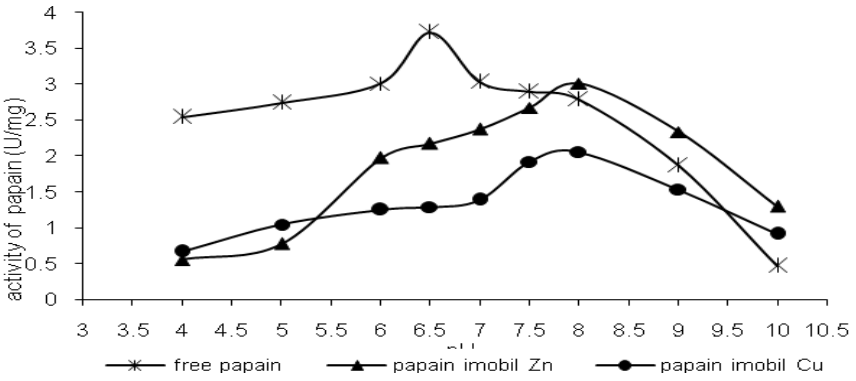
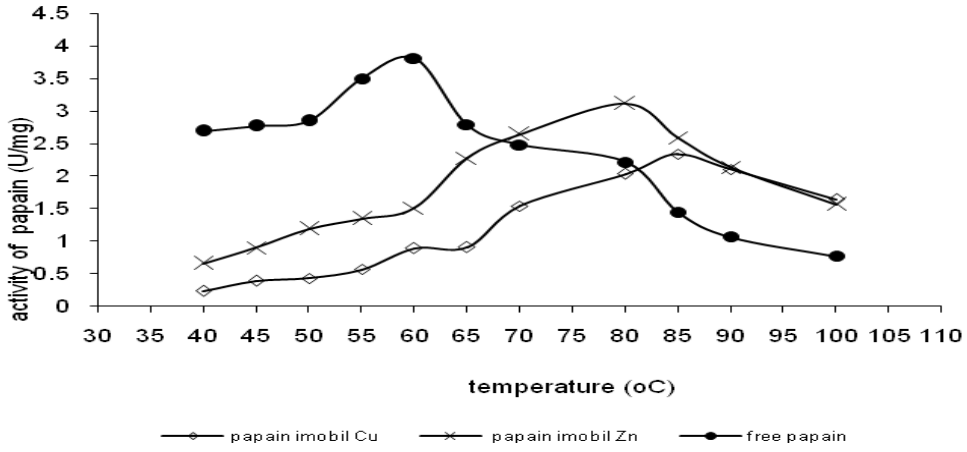
Effect of pH and Temperature on the Papain Activity

The pH dependence of the immobilized papain activity was compared with that of the free enzyme for casein in the pH range of 4.0-9.0 at 65 ^{o}C. It can be seen from Fig. 1 that optimum pH for the immobilized papain shifted slighty from 6.5 to 8.0 when compared with free one. The relative activity of the papain immobilized was improved in a broad pH range compared with the free one. The immobilization of enzymes to charged supports often leads to displacements in the immobilized enzyme and the bulk phase due the electrostatic interactions with the matrix.



Picture 2. Effect of pH on papain activity

The temperature dependence of the activities of the free and immobilized papain was studied in 15 mM PBS at temperature range 40-100 ^{o}C and temperature profiles of free and immobilized papain shown in Fig.2. The optimum temperature range for free and immobilized papain was found to be about 50-60 and 75-85 ^{o}C respectively. The conformational flexibility of the papain was affected by immobilization. The immobilization of papain on chitosan beads with Zn(II) bifunctional agent caused an increase in papain rigidity which is commonly reflected by increase in stability towards denaturation by raising the temperature[8,13].



Picture 3. Effect of temperature on papain activity

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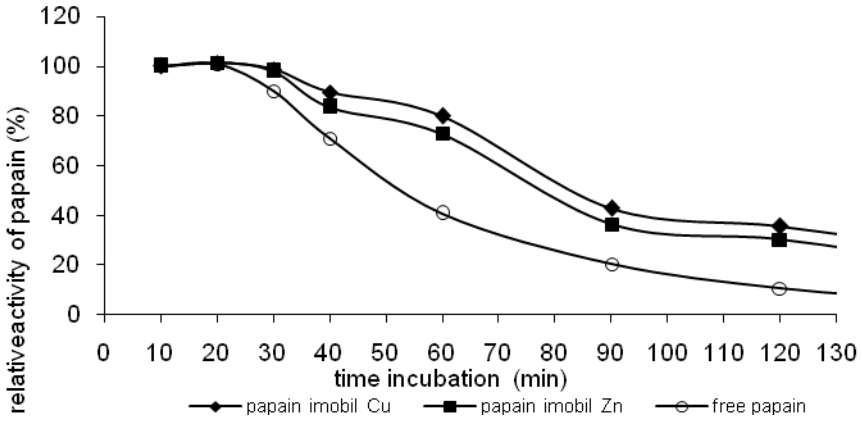
The thermal stability of immobilized papain was markedly increased relative to that of the native enzyme. The thermal stability pf chitosan beads-Zn(II) papain at 80^{o}C was improved dramatically.



Picture 4. Thermal stability of papain

Reusability of Immobilized Papain

To investigate the reusability, the enzyme-immobilized chitosan beads-Zn(II) was washed with deionized water after one catalysis run and reintrodused into a casein solution for another hydrolysis. Fig 4. shows the effect of repeated use on the activity of the immobilized papain. It can be seen that the activity of the immobilized papain decay with recycled. The residual activity of papain immobilized on chitosan beads-Zn(II) was about 25% after 12 cycles of bath operation. The activity loss could be related to the inactivation of the enzyme caused by the denaturation of the protein and the leakage of protein and metal ions from the support's surface.

CONCLUSIONS

The main advantage of immobilization papain on chitosan beads-Zn and chitosan beads-Cu consists its simplicity, university, stability and cheapness. In this study, a novel metal immobilized adsorbent was prepared. Papain could be directly immobilized on the prepared chitosan beads-Zn and chitosan beads-Cu. Papain immobilized has high stability and activity retaining.

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