Research Article

Navvabeh Salarizadeh, Sadegh Hasannia*, Reza Hassan Sajedi, Navid Lamei, Afshin Mohsenifar and Kambiz Akbari Noghabi

Comprehensive study of Serratia peptidase immobilization from Serratia sp. ZF03 onto chitosan nanogels

Serratia sp. ZF03 kaynaklı Serratia Peptidaz’ın Kitosan Nanojellerin Üzerine İmmobilziasyonunun Kapsamlı İncelenmesi

Objective: In the present work, we have extended the study and immobilized the metalloprotease enzyme in glutaraldehyde cross-linked chitosan nanogels to scrutinize the enzyme’s features including stability over its soluble free form.

Method: The immobilized metalloprotease was characterized using scanning electron microscopy (SEM), followed by Fourier transform infrared (FTIR) spectroscopy. The enzyme is optimally active at 50°C and pH range of 8.0–10.

Results: Thermal stability of the enzyme enhanced when immobilized on the nanogel. After 5 min of incubation at 50°C, immobilized enzymes retained 60% of their original activity, while negligible activity (23%) was observed in the case of the free enzyme.

Conclusion: The results obtained here provide a powerful demonstration of the benefits of taking the glutaraldehyde cross-linked chitosan matrices to enhance metalloprotease stability. The high stability of the immobilized enzyme serves to improve its performance for possible application on the industrial scale.

Keywords: Serratia peptidase; Chitosan nanogel; Enzyme immobilization.

Özet

Amaç: Bu çalışmada metalloproteaz enziminin glutaraldehit ile kitosan nanojellerine çapraz bağlanarak, çözünür formdaki stabilitesi de dahil olmak üzere enzim özelliklerine en ince ayrıntısına kadar değerlendirilecek kapsamlı bir çalışma sunulmuştur.

Metot: İmmobilize metalloproteazlar taramalı elektron mikroskopisi (TEM) ve sonrasında Fourier transform infrared spektroskopisi (FTIR) kullanarak karakterize edilmiştir. Enzim 50°C ve 8,0-10,0 arasındaki pH optimum çalısması sonucunda sivi, immobilize enzimler, serbest enzimlerin aktivitesine oranla (%23), %60 oranında aktivitesini korumuşlardır.

Bulgular: Enzimin termal stabilitesi nanojellerin üzerinde immobilizasyonu sonucunda artırılmıştır. 50°C’de 5 dakika inkübasyondan sonra, immobilize enzimler, enzimlerin aktivitesine oranla (%23), %60 oranında aktivitesini korumuşlardır.

Sonuç: Sonuçlara göre, metalloproteaz stabilitesini artırarak glutaraldehit ile çapraz bağlı kitosan matrisindeki enzim özelliklerinin derece yararlı sonuçlar vermiştir. Yüksek stabilite immobilize enzimler için endüstriyel kullanımındaki performanslarını artırıcı etki göstermiştir.
**Anahtar kelimeler**: Serratia peptidaz; Kitosan nanojel; Enzim immobilizasyonu.

**Introduction**

Immobilization of biocatalysts in biological applications has attracted worldwide attention. Immobilization of enzymes as biocatalysts on suitable supports has advantages that it improves stability, storage, economically cost-effectiveness in the large scale of biochemical processes, and provides protection from environmental changes and enzymatic degradation. Immobilized enzymes have usually better pH, stability and thermostability than their free enzymes. Increase or decrease of enzymatic activity depends on the immobilization technique and the nature of the nanomaterial applied as a carrier. A wide variety of materials has been used for enzyme immobilization. These materials according to chemical structures can be classified as natural and synthetic matrices [1] because of the unique properties of chitosan, this natural and biocompatible polymer, is extremely useful for drug delivery systems. Chitosan nanogel has reactive amino groups and hydroxyl groups available and considerable affinity to proteins [2]. Amino acid residues of enzymes have reactive groups, ionic and hydrophobic groups, so that covalent linkage, ionic binding, and physical adsorption with carriers is performed through these amino acid residues [3]. Currently, immobilized enzymes are used for enzyme therapy and clinical diagnosis in medical applications [2, 4]. In this research, synthesis, characterization and immobilization of metalloprotease onto chitosan nanogels cross-linked with glutaraldehyde (GA) were investigated. The metalloprotease isolated from *Serratia* sp. ZF03 has been called serrapeptidase (serratia peptidase). The enzyme has been characterized and purified from different strains of *Serratia marcescens* found in soil, water and the gut of larvae of insects [5–7]. It is mainly used as an anti-inflammatory agent. This enzyme has wide-spread applications in arthritis, chronic bronchitis, sinusitis, atherosclerosis, wound debridement and fibrocystic breast disease [8–10]. Entrapment of this metalloprotease has been investigated by PLGA, Eudragit S100 microspheres and liposome nanoparticles, but immobilization of enzyme on insoluble matrix has not been reported [11–13]. Because of the therapeutic and commercial significance of metalloprotease, this study attempted to investigate immobilization of enzyme on glutaraldehyde cross-linked chitosan nanogels.

**Materials and methods**

**Materials**

Chitosan with medium molecular weight, NaBH₄, DEC, trichloroacetic acid (TCA) and glutaraldehyde, were purchased from Sigma-Aldrich (USA), ammonium sulfate, casein, tryptone, yeast extract, skim milk, glycine, acrylamide, bis-acrylamide, glucose, Tris-HCl, NaCl and parahydroxybenzoic acid were obtained from Merck (Germany).

**Production, expression and purification of enzyme**

In order for optimal production of *Serratia* peptidase protease from *Serratia* sp. ZF03, LB Broth medium containing skim milk 1% and glucose 0.5% was used. Cultivation was carried out at 30°C for 12 h in a rotating shaker, with shaking at 250 rpm. Subsequently, 0.1 mL of the preculture was inoculated into a 1000 mL baffled Erlenmeyer flask containing 200 mL of culture medium, which consisted of the following: tryptone 8 g, yeast extract 4 g, glycine 0.02 g, NaCl 1 g, skim milk 1% (w/v) and 0.5% (w/v) glucose [14]. For the initial purification of the protease, the bacterial culture was centrifuged at 10,000 g for 20 min at 4°C. The culture supernatant precipitated with 67% ammonium sulfate in a cold room. The resulting pellet was then dissolved in 20 mM Tris-HCl buffer (pH 8) and dialyzed using a 25 kDa molecular weight cut off membrane against the same buffer for 48 h with three buffer changes. The dialysate was then loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for subsequent estimation of the molecular weight of the enzyme. SDS-PAGE was carried out according to the method of Laemmli with some modifications [15]. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 for 30 min. Then gels were destained (at room temperature and with shaking) using a 10% (v/v) methanol, 10% (v/v) acetic acid solution.

**Synthesis of chitosan nanogels**

For preparation of chitosan nanoparticles, first, chitosan powder (0.5% w/v) was dissolved in deionized water with pH 4.5 (pH was adjusted with acetic acid). The chitosan solution was sonicated for 30 min with 600 W. Parahydroxybenzoic acid was dissolved in 10 mL ethanol and EDC as a linker and activator of carboxyl groups was
added to it. Then, this solution was gently added to the chitosan solution with continuous stirring and was incubated under these conditions for at least 2 h. After incubation, for precipitation of chitosan, pH 7 was achieved with NaOH, and then centrifuged for 5 min at 600 rpm at room temperature. The precipitated sample was washed with ethanol three times in order to remove the excess material and the precipitation was again dissolved in deionized water. Crosslinking was performed by adding the chitosan nanoparticles into glutaraldehyde solution with continuous stirring for 3–12 h. To stabilize the Schiff base bonds, NaBH4 as reducer was used and the mixture was stirred for 1 h [16]. After that, the excess of glutaraldehyde was extracted by dialysis for 18 h with three buffer changes (Figure 1).

Characterization of nanogels and immobilization of protease

In order to observe the morphology, one drop of each suspension samples was deposited on microscope cover slides and dried at room temperature to obtain a uniform layer of nanoparticles. Samples were coated by a thin layer of metal (gold, Au). The morphology of Au-coated nanoparticles was determined with scanning electron microscopy (SEM) and the size of the nanoparticles was estimated. Chemical characterization of glutaraldehyde-activated chitosan and immobilized nanoparticles was performed using a Fourier transformed spectroscopy Bruker Tensor 27 (Bruker Optik, Germany).

Protease activity assay

The proteolytic activity assay of free and immobilized protease was measured according to the Kunitz method with a little modification using casein substrate [17]. Casein was used at a final concentration of 1.0% (w/v) in a 0.5 mL assay reaction mixture containing 20 mM Tris-HCl buffer 10 mM CaCl2, pH 8.0 and an appropriate amount of enzyme. The reaction mixtures were incubated for 10 min at 37°C and quenched with 0.5 mL 10% (w/v) trichloroacetic acid (TCA) on ice. After 20 min, the quenched reaction was centrifuged at 18,000 g for 10 min and the absorbance of the supernatant was measured at 280 nm, then the specific activity of the free and immobilized protease was measured.

Effect of temperature and pH on the activity of free and immobilized protease

The determination of pH on the activity of free and immobilized enzyme was performed over a range of pH (4–12) at 37°C for 10 min under previously mentioned conditions. A substrate consisting of 1% (w/v) casein in Briton-Robison universal buffer (0.04 M H3PO4, 0.04 M H3BO3, 0.04 M CH3COOH) was used [18]. To determine the optimum temperature, the enzyme was incubated at temperatures between 30°C and 65°C for 10 min with the same substrate in standard assay conditions.

Effect of temperature on the stability of free and immobilized protease

The thermostability of free and immobilized protease was determined by incubating the protease at 50°C for 60 min.
Results and discussion

Purification of protease and SDS-PAGE analysis

Serratia peptidase produced by Serratia sp. ZF03 was precipitated by ammonium sulfate at the 67% saturation level (the most appropriate level of saturation). The precipitated sample was then dissolved in a minimum of Tris-HCl buffer (pH 8) and dialyzed. The purified enzyme was shown to have a molecular weight of approximately 50 kDa by SDS-PAGE (Figure 2).

Synthesis and characterization of chitosan nanogels

Nanoparticle systems are new instruments in the field of biologically active molecules and drug delivery. Among the available nanodevices, polymeric chitosan nanogels are considered as effective carriers for drug delivery, as chitosan is a harmless natural polymer that has properties such as solubility in water, biodegradability and biocompatibility [19, 20]. Chitosan nanogels were prepared by covalent cross-linking using glutaraldehyde and EDC. SEM images of the various forms of nanogel were the proof of synthesis of nanogels (Figure 3). Mean particle size of nanoparticles and protease-immobilized was estimated between 150–180 nm. The samples for SEM observation were coated with Au metal. The result of the FTIR spectrum of chitosan, free and immobilized enzyme showed that protease immobilized onto chitosan nanogels. The absorption peaks in the IR spectrum was attributed to the vibrational frequencies of atoms in molecules. Reduction of the peak in NH₂-chitosan and enzyme in positions of 3425 and 3229 confirmed the binding of NH₂ with carboxyl and aldehyde groups. Also peak of carboxyl groups of enzyme in position of 1630 and aldehyde groups in 1722 was reduced (Figure 4). Because of availability of enzyme to perform reactions and achieve target organ without a long drug delivery, since the targeted drug delivery is from the lungs to the atheroma, particle size and type of immobilization which has been established on the basis of the pulmonary drug delivery.

Effect of temperature and pH on activity and stability of free and immobilized protease

Chitosan nanoparticles which are suitable supports for immobilization of enzyme, were obtained by different processes. The activity and stability of various enzymes attached to these nanoparticles has been investigated [19, 21, 22]. The results of pH and optimum temperature of free enzyme in comparison to immobilized enzyme showed the immobilization process had little effect on pH and temperature profiles, but, the activity of immobilized-protease versus the free enzyme was decreased. The optimum temperature of protease was observed over the range 50–55°C (Figure 5). In the temperature between 40°C and 45°C, immobilized enzyme in two states had 85% and 95% (exposure to bath sonicator) of its maximum activity. In similar conditions and lower temperatures, the activity of free enzyme was actually more than the immobilized enzyme, but, at high temperatures immobilized enzyme showed a slight decrease in activity in comparison to the free enzyme. Below optimal temperature, immobilized enzyme was more stable than that of the free enzyme. The optimum activity of protease was in the pH range 8–10 and after pH 10, activity declined rapidly. Maximum initial activity for free and immobilized enzyme has been retained 20% and 25%, respectively. It appears that immobilized enzyme was more resistant against any changes in pH and was more stable than free enzyme in high pH (11–12) and especially low pH (4–6) conditions (Figure 6). Similar results were observed from immobilization of β-galactosidase on chitosan microspheres and...
Figure 3: SEM images of chitosan nanogels and immobilized enzyme.

Figure 4: FTIR spectra of (A) immobilized enzyme, (B) chitosan, (C) free enzyme.

Figure 5: Temperature profile of free enzyme and immobilized enzyme.

The activity of immobilized and free protease were assayed in different temperatures (30–65°C) in Tris-HCl buffer pH for 10 min at 37°C. The optimum temperature of protease was 50–55°C.

Figure 6: pH profile of free enzyme and immobilized enzyme.
The activity of enzyme was determined in Britton-Robison universal buffer with different pH ranges (4.0–12.0). Protease optimally active in pH range 8–10.

considering that, there were no significant changes in optimum pH and temperature [21]. Other enzymes such as amylase, glucose oxidase, glucosidase, bromelain and ficin immobilized onto chitin and chitosan-based supports [3]. Regarding that covalent crosslinking immobilization decreased protease activity, it is possible more protein bonded onto nanoparticles were obtained by the bath sonicator. Protease activity on nanogels re-exposed by the bath sonicator was decreased further. Reduction of immobilized enzyme activity can be attributed to changes in enzyme properties such as conformational changes induced by immobilization and changes in pH and transfer restrictions [21]. Although covalent crosslinking immobilization led to reduced protease activity but increased thermostability of protease. The result of thermal stability at 50°C showed immobilized enzyme was more stable than free enzyme and its activity reduced with less steep (Figure 7). After incubation in 5 min, free enzyme retained approximately 23% of its activity, whereas immobilized enzyme retained 60% of its initial activity.

nanoparticles [22], chymotrypsin and lipase onto chitosan nanoparticles [21, 23]. In immobilization of chymotrypsin onto chitosan paramagnetic nanogels, enzymatic activity in immobilized enzyme was more than its free counterpart,
Conclusion

In the present study, chitosan nanogels were prepared by covalent crosslinking using glutaraldehyde. Serrapeptidase from *Serratia* sp. ZF03 immobilized onto crosslinked nanogels. The results of FTIR and SEM showed that immobilization of protease has been performed successfully with a bath sonicator. Smaller nanogels help to bind a higher proportion of enzyme. The optimum pH and temperature of the immobilized enzyme did not change remarkably in comparison with the free enzyme. Immobilized enzyme had better stability than free enzyme against environmental conditions. The results show that using the immobilization process increases the protease resistance against environmental fluctuations and improves its efficiency in the pharmaceutical industry and medicine. This method can be effective in pulmonary drug delivery. Due to the important role of this metalloprotease, further enzymatic studies, formulation and immobilization of enzyme on chitosan and other nanoparticles can be carried out in order to understand the mechanism of its activity and industrial applications.

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