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Immobilization of Subtilisin Carlsberg on Modified Silica Gel by Cross-linking and Covalent Binding Methods

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ABSTRACT

Proteases are important enzymes that their role in various industries is undeniable. However, keeping enzymes stable during its activity in harsh conditions is so important. In this study, protease enzyme was immobilized on the porous silica particles and its stability in different temperatures and pHs was evaluated. First silica particles were aminated by 3-aminopropyltriethoxysilane then the protease enzyme was immobilized on the modified silica by glutaraldehyde cross-linking method and the immobilized enzyme's activity was maintained for more than 40 days. Measuring the free subtilisin carlsberg enzyme activity and immobilized enzyme was performed according to the Lowry method. In another part, the effects of different pHs and temperatures on free and immobilized protease were evaluated. The immobilized protease activity was measured in temperature range between 25-75 °C and pH range 6.5-12. The absorption was read in 660 nm. It is shown that the optimum temperature for immobilizaed enzyme is 50 °C. The results showed that immobilized protease is more stable than free protease.

Keywords: Alkaline serine protease, Subtilisin, Silica gel, Immobilization, Cross-linking

INTRODUCTION

Microbial proteases play an important role in industry and commerce. Proteases have many different applications in different industries such as peptide synthesis, protein processing, food industry, detergents, leather industry, pharmaceutical and dairy industry [1].

Among the various proteases, bacterial proteases, in contrast to most animal and fungal proteases are widely used for a variety of applications. The advantages of enzyme immobilization are repeatability and frequent use, better and easier isolation of enzymes from raw materials, increasing contact area with the ingredients, avoiding of enzyme accumulation, increasing the stability of three-dimensional structure, preventing deactivation of enzyme activity in environmental conditions such as temperature and high pH or in presence of oxidants [2,3,4].

Serin proteases, such as Subtilisin Carlsberg, are very important enzyme because of their use in the hydrolysis of proteins and enzymatic analysis of protein sequences. The name of this group of proteases is due to presence of serin residue in their active sites. These proteases are one of the alkaline serin protease groupe and have optimum pH of 9.5 and molecular weight range around 35-90 kDa and isoelectric point near 9. These proteases are produced by various Bacillus species by fermentation method [1]. Their active sites consist of aspartic acid, histidine and serine (Fig. 1). High stability in various conditions (high temperature and alkaline pH) is cause of their superiority over other protease [1,5].

Proteases have been immobilized by various methods on different supports. Using porous supports and covalent binding and cross-linking of enzyme to activated supports is useful and effective [6].

Enzyme immobilization on solid supports that are either organic or inorganic is considerably studied because it is very effective way to increase enzyme stability [7,8,9]. There are many reports about immobilization of various enzymes on inorganic supports, such as immobilization on silica, clay and collagen [10,11,12]. Among inorganic supports, silica particles have been used widely. The sizes

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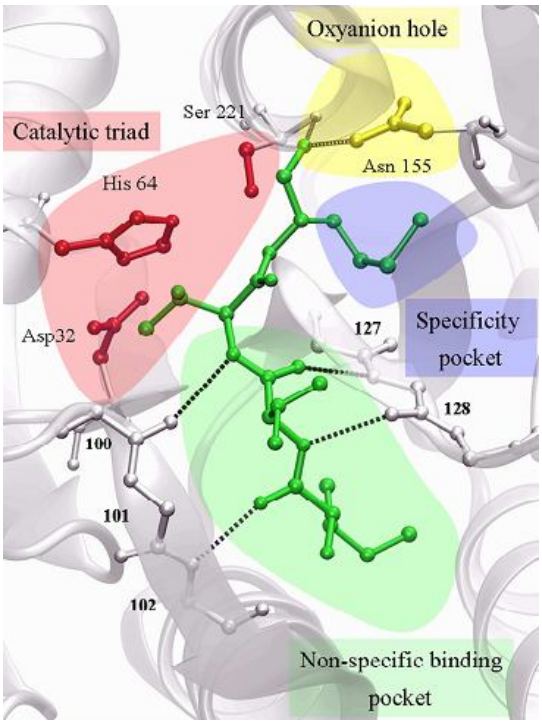


Fig. 1. Catalytic site of subtilisins.

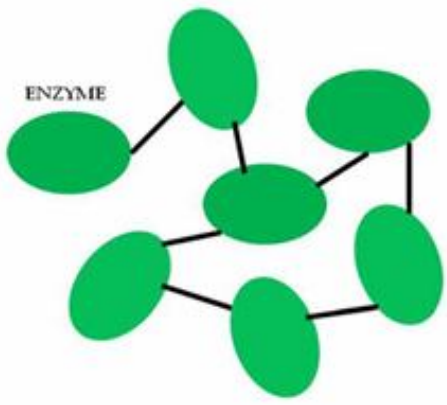


Fig. 2. Crosslinking method for enzyme immobilization.

of these particles are in the range of nanometer, similar to size of enzyme molecules. Other characteristics of these particles are high surface areas, regular structures, high stability to chemical and mechanical forces and resistance to enzyme attack [6]. Immobilization methods are classified in three major classes:

1: covalent binding, 2: entrapment and 3: cross-linking [13].

In cross-linking methods bifunctional (or multi-functional) agents such as glutaraldehyde are used for cross-

linking of enzyme molecules (Fig. 2). Activation of supports by glutaraldehyde is one of the most popular and effective methods for enzyme immobilization. This method is simple and efficient and allows improving enzyme stability. Moreover, glutaraldehyde has also been used for intermolecular and intramolecular cross linking [2,3,4]. Creating a strong connection between the enzyme and support is possible by modification of the support. One of these methods is amination of support and creation covalent bonds between the aminated support and the enzyme [14,15].

In this paper, three methods mentioned above have been used to immobilize protease on silica gel. 1: Covalent binding with silane 2: cross linking with glutaraldehyde and 3: entrapment of enzyme in the porosities of silica.

A wide variety of biomolecules, ranging over proteins, enzymes, antibodies and even whole cells, have been embedded within sol-gel glasses. They retain their bioactivity and remain accessible to external reagents by diffusion through the porous silica. Sol-gel glasses can be cast into desired shapes and are optically transparent, so it is possible to couple optics and bioactivity to make photonic devices and biosensors [16].

The overall objective of this study is to immobilize protease enzyme on silica particles, and increasing the stability of the immobilized enzyme.

In this study, porous silica was selected as support for immobilization of protease enzyme. Due to the very high surface to volume ratio of nanoparticles these supports are highly efficient for enzyme loading. In this study, the silica particle with pore size of 60 Aº is selected by considering enzyme diameter size (4.2 nanometers).

MATERIALS AND METHODS

Materials

Subtilisin Carlsberg (protease frome Bacillus licheniformis), Casein as substrate, silica gel (230-400 mesh), 3-aminopropyitriethOxysilane (APTES), Toloen, Folin Reagent, Trichloroacetic acid (TCA), phosphat baffer and sodium carbonate were purchased from Sigma Aldrich.

Methods

Amination of silica gel. APTES (1 ml) was added to 10 ml of toluene in a Round Bubble Butts (Fig. 3). The mixture was stirred with 150 rpm and then 1 g of silica gel was slowly added. The reaction mixture was refluxed for 3 h. The silica gel was filtered by filter paper, and then was washed with toluene, methanol and acetone. The aminated silica was dried at 80 °C for 18h in oven and kept in desiccator [17]. Aminated silica was ready for further processing.

Immobilization. For immobilization, each time 50 mg of aminated silica was added to 5 ml of 0.1 M phosphate buffer pH 8.5 and then 0.1 ml glutaraldehyde solution 25% (v/v) was added. Glutaraldehyde was used as the cross linker agent for making cross-link between amine groups of subtilisin to amine groups of the modified silica. The mixture was kept at 25 °C for 15 min. This time is activation time of support. After adding glutaraldehyde, color change was observed (Fig. 4).

Then activated support, was filtrated and washed 3 times with distilled water until no free glutaraldehyde remains. On the other word, free Glutaraldehyde molecules that were not linked to the modified support were washed.

Enzyme solution was prepared from 1.3 mg enzyme in 1 ml of 0.1 M phosphate buffer pH 8.5 and was added to previous mixture (50 mg of APTES-silica) and was kept at 25 °C for 24 h without stirring and after this time aminated and activated silica was filtered and washed with Tris-HCl buffer.

Protease activity assay. Measuring the free subtilisin carlsberg enzyme activity and immobilized enzyme was performed according to the Lowry method [18,19]. Enzyme activity at various time intervals, temperatures and pHs was measured by spectrophotometer.

The proteolytic assay was performed by adding 50 mg of immobilized enzyme to the vial then 1ml sodium phosphate buffer 0.1 M pH 8.5 and 5 ml casein solution was added to the vial. The mixture was incubated at 50 °C for 15 min and stirred continuously at 150 rpm and centrifugation was performed at 6000 rpm for 2 min, then 0.5 ml of supernatant was removed and added to 0.5 ml of trichloroacetic acid. And this mixture was sanded for 30 min at 25 °C. Second, centrifugation was performed for 3 min at 5000 rpm. 0.5 ml of supernatant was added to mixture of 5 ml of Na_2CO_3 and 0.5 ml of Folin reagent. Mixture was incubated for 30 min in 37 °C and the

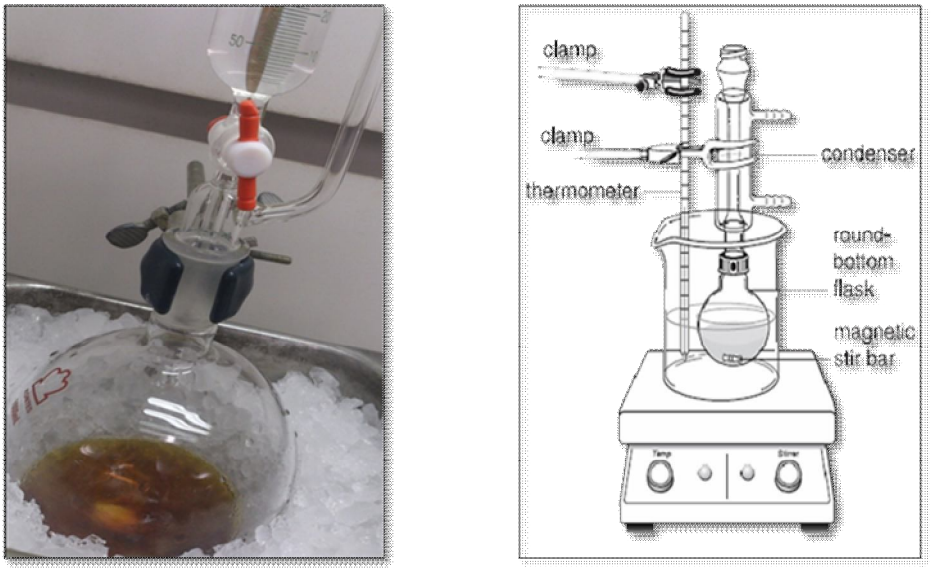


Fig. 3. Reflux process in amination of silica.

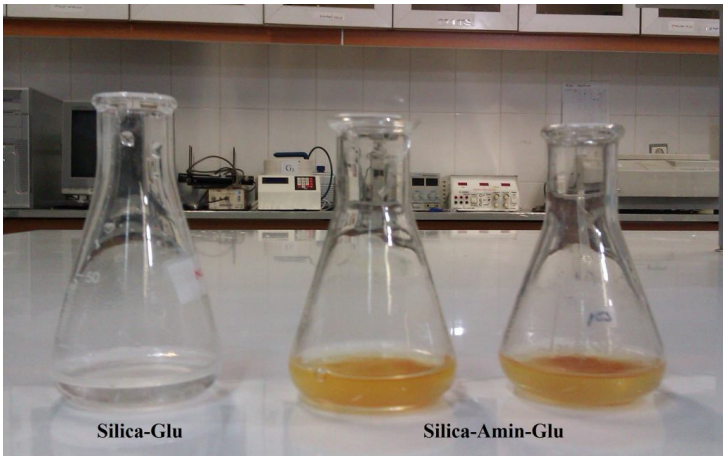


Fig. 4. Color Change of the mixture after adding glutaraldehyde.

absorbance was measured at 660 nm.

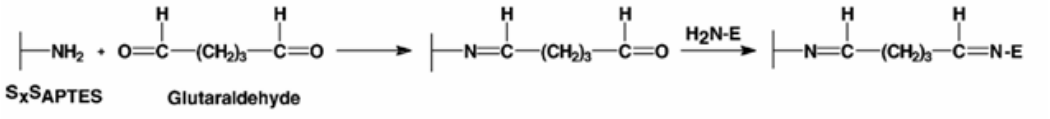
Due to enzyme activity, the tyrosine is liberated along with other amino acids and peptide fragments. Folin & Ciocalteus phenol or Folin's reagent primarily reacts with free tyrosine and produces a blue colored chromophore, which its λ_{max} is 660 nm.

Effect of pH and temperature on enzyme activity. The immobilized protease activity was measured in temperature range between 25-75 °C and pH range 6.5-12. The absorption was read in 660 nm.

Stability evaluation. For stability evaluation, at specified intervals (10 days), the immobilized enzyme activity was measured.

RESULTS AND DISCUSSION

Two stages of immobilization of subtilisin carlsberg on silica supports are shown in (Scheme 1). In the first stage,



Scheme 1. Immobilization on S_xS_{APTES} supports using glutaraldehyde as the activating agent

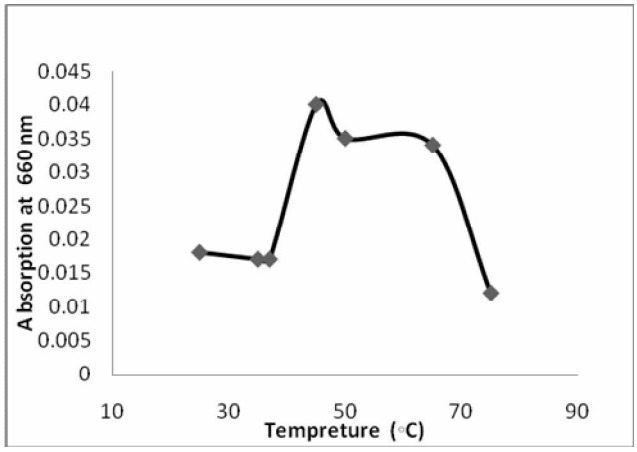


Fig. 5. Effect of tempreture on enzyme activity, reaction tempreture 25, 37, 40, 45, 50, 55, 60, 65, 70 and 75 °C, in phosphat buffer 0.1 M (pH 8.5).

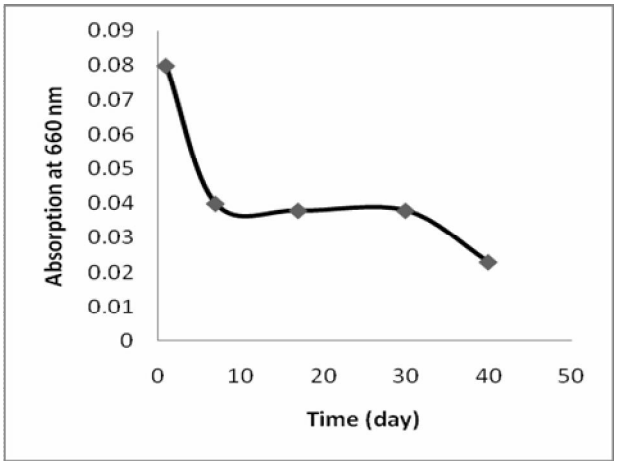


Fig. 7. Immobilized protease activity for 40 days at pH 8.5, tempreture 50 ºC (The activity is measured every 10 days).

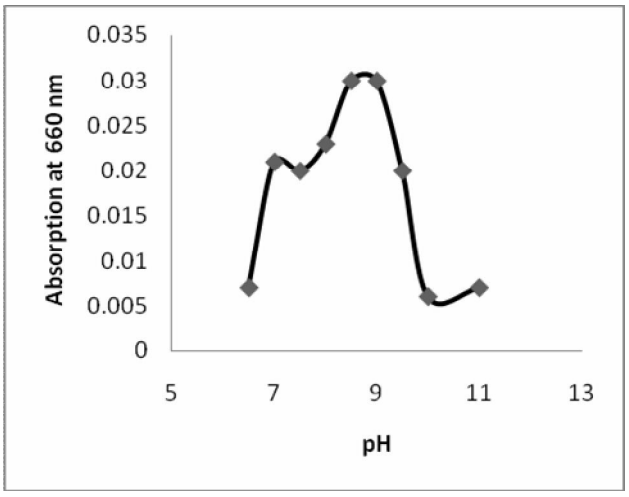


Fig. 6. Effect of pH on enzyme activity (reaction tempreture
is 50 ºC) in phosphat buffer 0.1 M.

silica gel was silanized with APTES and amino groups were added to the support. In second stage, treatment of silica was performed by glutaraldehyde, and the enzyme (with ε-amino group of lysine residues) was immobilized to these supports.

Figure 5 shows immobilized protease activity in different temperatures. As the results show, the activity of immobilized protease is increase from 30 to 50 °C. Also it is shown that immobilized protease is active in the range of 25-75 °C.

Figure 6 shows the immobilized protease activity in different pHs at 50 °C (optimum tempreture). As it is shown the optimum pH is pH 9. Figure 7 shows the immobilized protease activity after 40 days. These results show that the formed imine bond between the glutaraldehyde and amin groups of the enzyme are stable enough to say that this reaction has a good operational stability in aqueous solutions after 40 days. Several similar works have been

done in the past years. For example Guang Yang, *et al.*, in 2010, immobilized lipase enzyme on silica gel by glutaraldehyde cross-linker [20] and Ashok Ganesan, *et al.*, in 2006 immobilized Subtilisin Carlsberg enzyme on silica particles by entrapment method [21], while in this study protease immobilization was investigated and several methods are used to obtain complete and acceptable results that the most important of these results was time stability more than 40 days.

CONCLUSIONS

The results show that immobilized protease is stable in different pHs and temperatures and this method of immobilization could be a good candidate for immobilization of protease in different fields of industry.

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