Immobilization of α-amylase onto magnetic nanoparticles by shaking method

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Abstract

α-amylase was immobilized covalently on Fe_3O_4 magnetic nanoparticles via carbodiimide activation. The efficiency of immobilization and residual activity of the immobilized α-amylase was dependent upon the immobilization temperature and mass ratio of nanoparticles: carbodiimide: α-amylase. Confirmation of the binding was demonstrated by Fourier transform infrared (FTIR) spectroscopy. At room temperature, immobilized amylase displayed more binding efficiency and less residual activity compared with of 4°C, hence 4°C was chosen as optimum immobilization temperature. The 4:1:1 ratio displayed maximum residual activity therefore, it was chosen as a best ratio for immobilization. The optimum pH value for both free and immobilized amylase was at pH 5 but immobilized amylase exhibited more pH sensitivity at the pH values 6, 7 compared with free amylase and there were no differences in pH profiles for amylase that immobilized in room temperature or 4°C.

Keywords: α-amylase, Magnetic nanoparticles, Immobilization, Carbodiimide.

1. Introduction

Immobilization is one of the efficient methods to improve enzyme stability [1]. There are various methods for immobilization of enzymes on many different types of supports. It can be a chemical method in which ionic or covalent bond formations occur between the enzyme and the carrier, or it can be a physical method, such as adsorption or entrapment of the enzyme in or on a solid support material [2].

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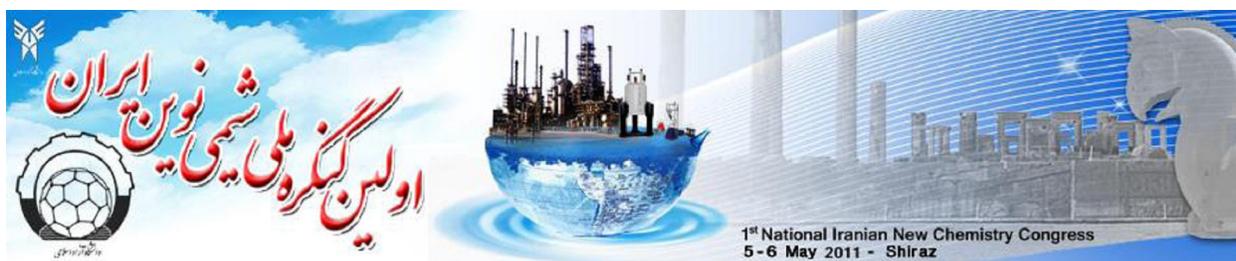
Magnetic nanoparticles (MNP) when are used as support of immobilized enzymes have the following advantages: 1) higher specific surface area was obtained for the binding of a larger amount of enzymes, 2) lower mass transfer resistance and less fouling, and 3) the immobilized enzymes can be selectively separated from a reaction mixture by application of a magnetic field [3].

Iron oxide nanoparticles ,Fe_3O_4, are one of the widely used type of magnetic nanoparticles and have great potential for applications in biology and medicine due to their strong magnetic properties and low toxicity [4]. Different types of biomolecules such as proteins, enzymes, antibodies, and anticancer agents can be immobilized on these nanopaticles[5]. Magnetic supports for immobilization purpose are either prepared by incorporating magnetic particles during the synthesis of the supporting polymer [6, 7] or magnetic particles itself be coated with common support materials such as dextran or agarose [8]. Recently, a new method for the direct binding of proteins on magnetic nanoparticles via carbodiimide activation was proposed [9]. This method was employed for the immobilization of a few bioactive substances such as bovine serum albumin (BSA) [9], streptokinase [10], and yeast alcohol dehydrogenase (YADH) [11]. Amylases belong to an enzyme group which is very commonly used in food and fermentation industry. They are use for the hydrolysis of starch and production of maltose [2].

The immobilization of α-amylase on various supports has been investigated by several researchers [12-16], however, there are not any reports about its immobilization on magnetite nanoparticles. In the present work, the immobilization of α-amylase onto the magnetic nanoparticles Fe_3O_4 via 1-[3-(dimethylamino)propyl]-3 ethylcarbodiimide methiodide activation was done. The immobilization procedure was done at two different temperatures and different mass ratios of carbodiimide: α-amylase: magnetic nanoparticles. The efficiency of immobilization and the residual activity was investigated and pH dependence of hydrolytic activity of the immobilized α-amylase was studied and compared with those of the free enzymes.

2. Experimental

2.1. Materials

α-amylase (EC 3.2.1.1 from *B. subtilis* with an activity of 50 U/mg), 1-Ethyl-3-(3 dimethylaminopropyl) carbodiimide methiodide(CDI), 3,5- Dinitrosalicylic acid (DNS) were purchased from Sigma-Aldrich. Coomassie Brilliant Blue G250 and Bovine Serum Albumin (BSA) were purchased from Merck.

2.2. α-Amylase immobilization

Magnetic Fe_3O_4 nanoparticles used for immobilization of α-amylase were prepared by co-precipitation method [17]. For the immobilization of α-amylase, magnetic nanoparticles: CDI: α amylase were mixed in the ratios of 4:1:1, 3:0.5:1 and 4:0.25:1. The reaction mixture was shaken at 100 rpm at room temperature (RT) and 4°C for 24 h. After that the α-amylase immobilized magnetic nanoparticles were recovered from the reaction mixture by application of an external magnetic field [9]. Then, they were washed with phosphate buffer (3mM, pH 6) for several times until no free α-amylase was detected in the supernatant.

The supernatant was used for protein determination according to the method of Bradford by using BSA as a standard [18]. The immobilized amount of enzyme was calculated by subtracting the amount of non-immobilized enzyme from the total amount of the amylase used for immobilization [19].

2.3. Characterization

The KBr pellet technique was used for determining the Fourier transform infrared (FTIR) spectra of magnetic nano-particles, free amylase, and amylase bound nanoparticles and the spectra confirm the binding of amylase to the magnetic nanoparticles.

2.4. Enzyme activity assay

The activities of free and immobilized α-amylase were determined in the presence of 1% (w:v) soluble starch as the substrate in phosphate buffer (3mM, pH 6) at room temperature, according to the Bernfeld method [20]. For free α-amylase, a sample of 1 ml was incubated for 12 min at room temperature with 1 ml of the substrate solution and the enzymatic reaction was interrupted by the addition of 1 ml of DNS reagent. The mixture was heated for 15 min in boiling water and then cooled at room temperature. After addition of 9 ml of distilled water, the absorbance of the digested products was measured spectrophotometrically at wavelength 540 nm. A blank was prepared in the same manner without free α-amylase. A calibration curve established with maltose, 0.2– 2 mg in 1 ml of deionized water. For immobilized α-amylase, the tube containing 10 mg α-amylase immobilized magnetic nanoparticles were suspended in 1ml phosphate buffer (3mM, pH 6) and then this mixture was incubated for 12 min at room temperature with 1 ml of the substrate solution after that α-amylase immobilized magnetic nanoparticles were separated by application of an external magnetic field and then 1ml of DNS reagent was added to its supernatant. The subsequent procedures were similar to those of free α-amylase. All activity measurement experiments were carried out at least three times.

2.5. Effects of pH

To investigate the effect of pH on the activity of the immobilized α-amylase, free α-amylase, and the α-amylase immobilized magnetic nanoparticles were immersed into 3 mM phosphate buffer solutions at different pH values for 2 h and the activity was determined. The pH values of the buffer solution were varied in the range between 2 and 8 [4].

3. Results and Discussion

The immobilization of enzymes onto nanoparticles usually depends on various physical and chemical factors such as the immobilization time, quantity of nanoparticles, immobilization temperature, buffer solution, and etc. [21]. In this work, the efficiency of enzyme immobilization was studied with respect to immobilization temperature and mass ratio of MNP: CDI: amylase. The specific activity of free and immobilized α-amylase was calculated according to the following equation [22]: Carbodiimide is used to activate a hydroxyl group on the surface of magnetic particles and thereby couples the amino group on the enzymes to the hydroxyl group through an amide linkage (Fig. 1).

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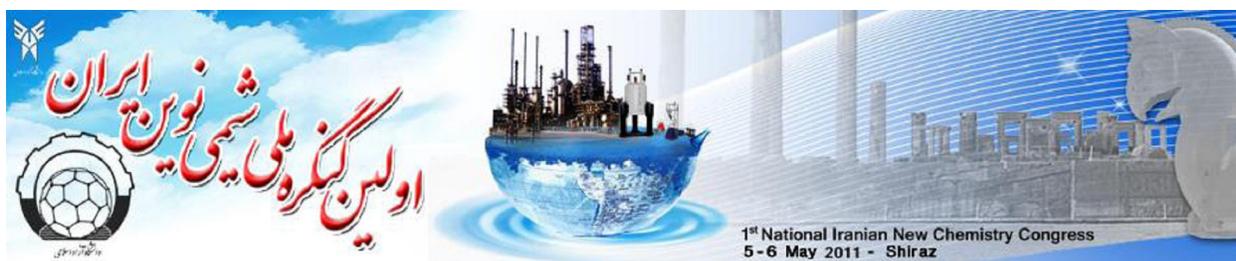
The coupling procedure is too simple to perform and has the advantage of occurring under very mild conditions and of conjugating the enzymes directly to magnetic particles without interposing the additional groups between them [23].
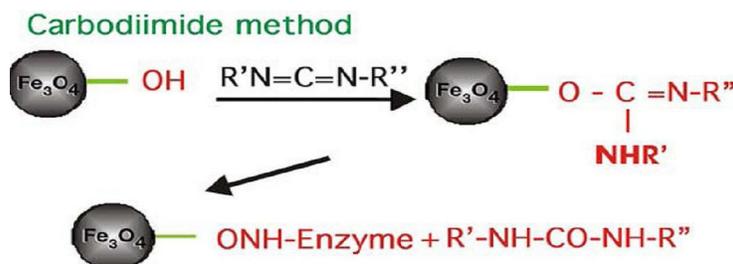

Carbodiimide method

Fig. 1. Scheme of immobilization of enzyme protein to magnetic support via carbodiimide coupling agent.

As table 1 shows, the immobilization efficiencies at room temperature were higher than those of at 4°C for different mass ratios. the residual activity of immobilized amylase at 4°C was better than in the case of room temperature. The immobilization of α-amylase in the 3:0.5:1 and 4:0.25:1ratios was found more efficient than 4:1:1 ratio but this ratio displayed the best percentage of residual activity especially at 4°C.

Table.1. Efficiency of immobilization and the residual activity

MNP:CDI:E (Mass ratio)	Immobilization at RT		Immobilization at 4° C	
	% Immobilized Enzyme	% Residual Activity	% Immobilized Enzyme	% Residual Activity
4:1:1	93.3	6.7	48	13.4
3:0.5:1	100	7.1	100	8.3
4:0.25:1	100	5.5	93	4.6

MNP, Magnetic nanoparticles; CDI,Carbodiimide; E, Enzyme (amylase); RT, Room Temperature.

For immobilization at 4° C, the residual activities were between 4.6 and 13.8%, whereas it was 5.5-7.1% for immobilization at RT. The differences in the retained activities are quiet large, however, it appears that the retention of functional activity for an immobilized enzyme depends on its nature and the size of the support matrix. In this work, greater loss of α- amylase activity immobilized at RT could be attributed to the exposure of enzyme at RT for 24 h whereas other samples were immobilized at very lower temperature (4°C). Additionally, the covalent linking of the protein may result (i) in a change in conformation of the enzyme

where the active site is partly or totally obscured by the immobilization matrix [24] (ii) Covalent bond formation could take place through residues which though not actually present at the active site may be essential for maintaining the optimal conformation of the active site [10]. The results show that the optimum conditions for immobilization was 4:1:1 ratio and 4°C.

The binding of α-amylase to magnetic nanoparticles was confirmed by FTIR analysis. The characteristic bands of proteins are at 1656 and 1531 cm-1, and 1654 and 1531 cm-1for Fe3O4-α-amylase, and α-amylase, respectively. It shows that α-amylase effectively present in the samples confirming the binding of α-amylase to Fe3O4 nanoparticles.

The pH dependence of the activity for the free and immobilized α-amylase was investigated for both α-amylase immobilized at RT and 4°C. As illustrated in Fig. 2, the maximum activity was observed at pH 5 for both free and immobilized α-amylase. This indicates that the optimal pH value for enzyme activity has no evident changes after the α- amylase is immobilized to the magnetic nanoparticles but the immobilized amylase exhibites more pH sensitivity at the pH values 6, 7 compared with free amylase. At the pH range between 3 and 5 the activities of the free and immobilized amylase were quite similar and reached a maximum at pH 5. The similarities in these activities in the pH range of 3 to 5 indicate that in these conditions, amylase did not suffer from any major activity constraint. The activity then decreased from pH 6 to 8, in this rang the immobilized amylase exhibited more pH sensitivity respect free amylase. It is well known that the ability of the amino acids at the active sites of the enzyme to interact with the substrate depends on their electrostatic state [25]. The pH profiles of the amylase immobilized at RT was similar to those amylase immobilized at 4°C.

4. Conclusions

α-amylase was successfully immobilized to magnetic Fe3O4 nanoparticles using carbodiimide activation. Confirmation of the binding was demonstrated by FTIR.
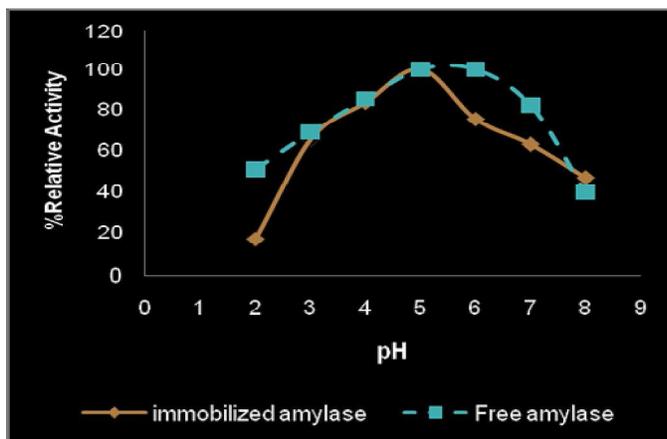

Fig. 2. Effect of pH on the activity of the free and immobilized amylase. The maximum was defined as 100% activity. (Each data point was the average of three measurements).

spectroscopy. At room temperature immobilized amylase displayed more binding efficiency and less residual activity with respect to 4°C, hence 4°C was optimum immobilization temperature. Because of the 4:1:1 ratio displayed maximum residual activity, it was chosen a best ratio for immobilization in spite of high binding efficiency for other ratios. The optimum pH value for both free and immobilized amylase was 5 but immobilized amylase exhibited more pH sensitivity at the pH values 6, 7with respect to the free amylase. The pH profiles of the amylase immobilized at RT was similar to those amylase immobilized at 4° C.

In summary, the direct binding method for immobilization protein onto magnetic nanoparticles is simple, quick, and inexpensive. Such immobilized preparations can simplify the operation, reduce procedure time and allow repeated use of expensive enzymes and proteins [26]. It can be used for creating bioconjugates for a variety of applications in health and food safety [27].

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