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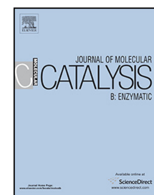

Effects of surface modification and activation of magnetic nanoparticles on the formation of amylase immobilization bonds under different ionic strength conditions

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ABSTRACT

In this research, the effects of coating layers and their activation level on the mechanisms of amylase immobilization onto the surface of magnetic ferric oxide nanoparticles (MNPs) were investigated. Two MNPs with two different coating conditions were subjected to surface treatment using the amino groups, and then were activated with different concentrations of glutaraldehyde (GA). Immobilization level and activity of the immobilized enzyme were studied for low, moderate, and high ionic strengths of buffer solutions. Furthermore, the type of the bond developed between the enzyme and carrier was identified using the amount of immobilized enzyme released under different desorption conditions, and also through adsorption kinetic studies and FTIR analysis. The results indicated that for most cases, the mechanisms for amylase immobilization on MNPs activated with GA are first physical adsorption, followed by forming covalent bond between the enzyme and the coated MNPs. The maximum amount and activity of the immobilized amylase were 700 mg/g_{MNP} and 30 $U/mg_{immobilizedenz.}$, respectively, related to low GA-activated silica-coated MNPs (0.5%) and low concentration of buffer (5 mM).

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1. Introduction

Enzymes are biological catalysts that facilitate complicated chemical processes under optimum ambient and laboratory conditions [1–3]. However, the large scale industrial usages of enzymes have not yet been achieved considerably due to their high costs, difficulty in recovery and recycling, and low stability. There have been different strategies to overcome these challenges, including chemical (e.g. covalent bond) and physical immobilization of the enzymes on supports [4,5]. For catalytic applications, immobilization through the covalent bond between the enzyme and carrier has been proposed, because the amount of enzyme released from the carrier within the chemical process would be minimum under this condition [6]. However, controlling the immobilization conditions is necessary for reaching the optimum immobilization value and activity of biocatalysts, as the formation of a chemical bond between the enzyme and support, in covalent immobilization method, can potentially cause conformational changes in the structure of the enzyme and variations in its catalytic activity [7].

For having unique characteristics such as nanometric scale, high specific surface area, biocompatibility and low toxicity, magnetic ferric oxide ($Fe_3O_4$and γ-Fe_2O_3) nanoparticles have found different applications over the past decade [8,9]. They are recently used as support for enzyme immobilization as well [10]. Because of their superparamagnetic properties, the performance of the enzyme immobilized on these carriers can be easily controlled, and recycling of the enzyme would be possible through creation of a magnetic field [10,11].

Depending on their applications, especially for biological purposes, the surface of MNPs must be treated and activated with appropriate molecules. Coating of MNPs with silica is among the common techniques applied for this purpose; this is carried out through various methods like the frequently used sol-gel technique, which is conducted based on the polycondensation of alkoxysilanes including tetraethyl orthosilicate (TEOS) [12]. Silica formed on the surface of MNPs develops an appropriate distribution of MNPs in an aqueous medium. It also forms a chemically inactive surface. In addition, presence of silanol groups on the surface of MNPs facilitates treating their surface with active groups such as amines [13]. Using active amino silane is another common method applied for silica coating and aminization of MNPs [14]. In these two cases, the high density of amino groups on the surface of MNPs is a useful tool for chemical surface treatment of MNPs by, for

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example, GA, which is majorly used for applications like covalent enzyme immobilization on MNPs [15].

By now, numerous studies have been conducted about enzyme immobilization on MNPs using silica-coating treated by amino groups with the use of conventional chemicals for synthesis of amino groups, like 3-(2-(amino ethylamino) propyl methyldimethoxy silane (APMS) [16] and 3-aminopropyltriethoxysilane (APTES) [9,10,13,17]. Since, the amino groups existing on the surface of nanoparticles provide a suitable environment for surface treatment through cross-linking with enzyme, the aminated MNPs are activated with GA molecules before immobilization [18,19]. Despite these large number of studies, lack of investigations on the effect of silica coating and density of amino groups on the amount and activity of immobilized enzyme at different ionic strength conditions can be noted. Moreover, no studies on the effect of surface activation level of aminated MNPs with GA have yet been reported.

Monsan et al. [20] investigated the effect of using various concentrations of GA on the immobilization level and activity of trypsin on porous silica. Besides, they reported that activation level can be controlled by adjusting GA concentration and duration of activation reaction; hence, it is possible to produce supports, which are activated by one or two GA molecules (i.e. monomer and dimer) for each amino group on the surface [20,21]. The GA activated supports can participate in enzyme immobilization by various mechanisms: hydrophobic adsorption through the GA chain; adsorption through anion exchange using the amino groups; and covalent immobilization using the reactive GA groups [21]. Since each of these mechanisms becomes predominant at various ionic strength conditions, enzyme immobilization mechanism can be controlled by changing the ionic strength of immobilization medium.

It is to be noted that evaluation of the effects of GA and media ionic concentrations and interactions have not yet been fully pictured. Moreover, covalent immobilization of amylase as one of the most commonly used industrial hydrolytic enzymes on MNPs is reported in few studies [22,23].

In the present work, mechanisms of amylase immobilization on Fe_3O_4 MNPs were investigated in different conditions. Surface treatment of MNPs was carried out through two coating methods with different densities of amino groups on the surface of MNPs. In the first method, the surface was treated by creating amine coating via direct incorporation of APTES on the surface of MNPs, while in the second method, a silica coating was produced using TEOS on the surface of MNPs followed by treatment by APTES. In addition, the effect of surface activation level of MNPs was investigated using different concentrations of GA and times of activation reaction. It was assured that the reaction took place under fully controlled conditions, which allowed us to dictate the number of GA molecules bond on each amino group. The effect of surface bonding strategies on the amount and activity of immobilized amylase was investigated at three different buffer concentrations.

Next, the effects of these methods on the dominant immobilization mechanism were confirmed via the release level of immobilized enzyme in a salt solution and medium pH changes, and also through studying enzyme adsorption kinetics and FTIR analysis.

2. Materials and methods

2.1. Materials

All chemicals used had analytical grade, and were used without further purification. Ferrous chloride ($FeCl_2{\cdot}4H_2O$), ferric chloride ($FeCl_3.6H_2O$), ethanol, and aqueous ammonia (25%, v/v aqueous solution) were products of the Merck. Crude amylase from *Aspergillus oryzae* (EC 3.2.1.15, activity 35.7 U/mg), GA, maltose, starch and 3,5dinitro-salicylic acid (DNS) were purchased from Sigma–Aldrich. Deionized water (DI-water) was used in the preparation of all solutions.

2.2. Synthesis, coating and activation of MNPs

2.2.1. Synthesis of Fe_3O_4MNPs

Fe_3O_4MNPs were synthesized using chemical co-precipitation method. For this, 0.146 g of $FeCl_2{\cdot}4H_2O$ and 0.4 g of $FeCl_3{\cdot}6H_2O$ were dissolved with 2:1 molar ratio in 60 ml of heated DI-water. Next the solution was placed in a 70 °C water bath with stirring under nitrogen atmosphere for 10 min. By adding 2 ml of 25% NH_4OH solution to the obtained solution, a black deposit of MNPs was formed. After 1 h, this dark sediment was collected by a magnet, and then washed and rinsed with DI-water and ethanol for several times.

2.2.2. Preparation of silica-coated MNPs

To prepare silica-coated MNPs, the procedure developed by Stober et al. [13], with slight modification, was employed. To do so, 0.5 g of MNPs synthesized in the previous step was dispersed in 20 ml of heated DI water, 80 ml of ethanol and 2.5 ml of 25% ammonium, and then sonicated with a power of 14 W and frequency of 20 kHz for 2 min. In the next step, 1.5 ml of TEOS was added to the nitrified solution. The reaction was preceded for 4 h at ambient temperature while stirring the solution. The final silica-coated MNPs were collected from the solution with an external magnet and rinsed with water and ethanol for several times.

2.2.3. Surface treatment of MNPs using amino groups

The silica-coated (MNP2) and uncoated (MNP1) nanoparticles were functionalized through the silanization reaction with aminosilane function using the APTES. In this procedure, 0.5 g of each type of MNP was dispersed in 50 ml of the solution containing 35 ml of DI-water and 15 ml of ethanol followed by sonicating for 2 min. Next, 3 ml of APTES was added to each solution and stirred for 2 h at 40 °C. After surface treatment using the APTES, MNPs (type 1 and 2) were rinsed with DI-water and ethanol for several times, and then collected [9,16].

2.2.4. Surface activation of MNPs using GA

The surface of aminated MNPs was activated by GA. To prepare aminated MNPs activated by monomer (MMNP) or dimer (DMNP) GA for each amino group, the method proposed by Betancor et al. was applied [21]. Based on this method, the particles were dispersed in 0.5 and 10% GA and 0.2 M citrate-phosphate buffer with pH 7, and then shaken for 1 and 16 h, respectively. Finally, they were washed with DI-water. The surface treatment and activation procedure of MNPs are shown in Fig. 1.

2.3. Enzyme immobilization

Immobilization of amylase was performed on both monolayer and bilayer coated surface supports, either by one GA molecule (MMNP) or two GA molecules (DMNP). In all cases, amylase was immobilized in different citrate-phosphate buffer concentrations at pH 6. The levels of GA and buffer concentrations and type of surface treatment of nanoparticles on amylase immobilization process are shown in Table 1.

For enzyme immobilization, 2 mg of each support was added to 2 ml of 5, 100 and 500 mM citrate-phosphate buffer. Then 2 ml of 1000 ppm amylase in DI-water was mixed to the support suspension, and put in a shaker incubator under the rotation speed of 200 rpm at 30 °C.

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