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IMMOBILIZING ALCALASE® ENZYME ONTO MAGNETIC NANOPARTICLES

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Abstract. *In recent years, magnetic nanoparticles (MNPs) have been applied to numerous biological systems. The nanoparticles are particularly useful in separating biological molecules due to its low price, scalable ability and very little interference. Here, MNPs, which can efficiently separate biocatalysts from reaction media by external magnet, was used to immobilize an alkaline protease (Alcalase®). Covalent attachment of the enzyme to MNPs began with the functionalization of the MNPs' surface with amines (APTES). Then, glutaraldehyde was introduced to link the MNP surface amines with enzyme surface amine residues, typically lysine. Successful covalent bonds were checked by FT-IR. Our results showed the attached enzyme did not affect superparamagnetic property of MNPs, therefore the MNPs-attached enzyme was easily recovered after the reaction. The immobilized enzyme maintained its activities after 10 times of recycle uses.*

Keywords: magnetic nanoparticles, alkaline protease, enzyme immobilization, glutaraldehyde.

I. INTRODUCTION

In recent years, magnetic nanoparticles (MNPs) have been applied to numerous biological systems due to their high saturation magnetization, their biocompatibility, their stability under hard condition, and the simplicity of their preparation process [1,2]. MNPs have been used in drug delivery system, protein separation, enzyme and protein immobilization [3] and diagnostics [4]. In those applications, the surface of MNPs needs to be functionalized and attach to various molecules such as enzyme, antibodies, drug moieties, biopolymers.

Enzymes immobilized on superparamagnetic nanomaterials can be easily separated from the reaction media due to a strong interaction between external magnet and nanoparticles in solution. Furthermore, those immobilized enzymes with very small sizes can penetrate into solid substances; thus eliminating one of main limitations of commercial immobilized enzymes. Bare MNPs are hydrophobic, so they tend to aggregate in aqueous solutions and are difficult to link to other molecules. To improve the hydrophilicity of MNPs, a layer of silica derivatives is often made onto the surface of MNPs. 3-amino propyltrethoxysilane (APTES) containing an amino group is preferred to use to activate this silica layer to react with various compounds. With this functional

group, MNPs are able to bind to biological molecules, such as enzymes. However, to increase the efficiency of binding, glutaraldehyde (GA) with two aldehyde groups in their molecules is used as a bridge between amino group on MNPs surface and amino group from residues of enzyme, typically lysine [5].

Alcalase® is an alkali protease used widely in many applications, for example in detergent formulations. The immobilized Alcalase® can offer several advantages of reusability and high hydrolytic activity [6]. However, obstacles still exist to realize those advantages. Therefore, the aim of the present study was to immobilize the enzyme onto MNPs in order to achieve complete enzyme reusability through the unique features of the MNPs located in a magnetic field. The structure and characterization of the MNPs used were examined while the stability and activity of the bound Alcalase® were assessed.

II. MATERIALS AND METHODS

II.1. Materials

Alcalase® 2.4L FG was purchased from Novozyme, Danmark. MNPs coated with an ionic coating ($Fe_3O_4.SiO_2$) were kindly provided by a laboratory of Hanoi University of Natural Sciences, Hanoi National University. The protein assay standard was bovine serum albumin (BSA) obtained from Bio-Rad, USA; 3-aminopropyl triethoxysilane and glutaraldehyde (GA) were purchased from Sigma-Aldrich, USA. Other chemicals were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

II.2. Methods

Preparation of immobilized enzyme

The purchased MNPs were washed in distilled water and ethanol 50%. Amine-derived SiO_2-MNP was prepared by modifying the MNP (coated with SiO_2) with APTES to introduce amine groups on the particles' surface. Ten milligrams of SiO_2-MNP was treated in 940μl ethanol 96% and 940μl distilled water with an ultrasonic processor at 37Hz, 30 minutes. And then 20μl 3-APTES and 100μl glycerol was added. The solution was shaken at 0^oC, 150 rpm in an incubator for 5 hours, continued ultrasound in 10 minutes. The modified MNPs were recovered with a magnet after washing five times with ethanol 50%. After washing with ethanol, they were washed three more times with phosphate buffer of 1M. The washed solid was mixed well in 0.5ml aqueous GA wt. 10% solution. The mixture was incubated in an hour with a light shaking and washed five times with the above phosphate buffer to remove the redundant GA. At the final washing step, the mixture was centrifuged at 4000xg for 10 minutes and then the pellet was rewashed with a 0.1 M borate buffer of pH 7.4. The obtained solid was mixed with 64 μl enzyme and 436 μl of the borate buffer and incubated overnight with a light shaking. Then the mixture was added with 500 μl of NaCNBH3 1% (wt.) in 0.2M Tris buffer and incubated for an hour. The particles were washed four times, each time used 500 μl of the borate buffer plus Tween 20 0.05% in a cool centrifuge (4^oC) at 4000xg for 10 minutes. The resulted immobilized enzymes on MNPs were stored in the borate buffer solution containing 0.1% Tween 20 and 0.05% wt. NaN_3.

Determination of enzyme concentration

The concentration of enzyme was measured by A280 assay, using SmartSpec™Plus Spectrophotometer BioRad [7].

Determination of enzyme activity

The activity of Alcalase® was determined by Anson assay with a small modification [8]. Briefly, the reaction mixture was prepared by following method: 1ml casein 2% and enzyme at 55^{o}C was mixed in 15 minutes, 55^{o}C, adding 0.5ml TCA to 0.5ml above solution, centrifuged to remove the solid. Adding 0.8ml Na_2CO_3 6% and 0.2ml folin 5X onto 0.2ml supernatant in 15 minutes, measured absorbance of sample at 750nm. The same process also was performed without reaction between enzyme and casein. This sample is control sample.

III. RESULTS AND DISCUSSION

III.1. Characterization of the support and immobilized protease

In this work, APTES was used as a reactive agent to attach –NH_2 on the surface of MNPs. This process improved the MNPs' capacity to immobilize enzymes owing to the increase of the density of -NH_2 in these beads. These amine groups were linked with other amino groups on the enzyme surface via GA bridge. The whole immobilization process were illustrated in Fig. 1.

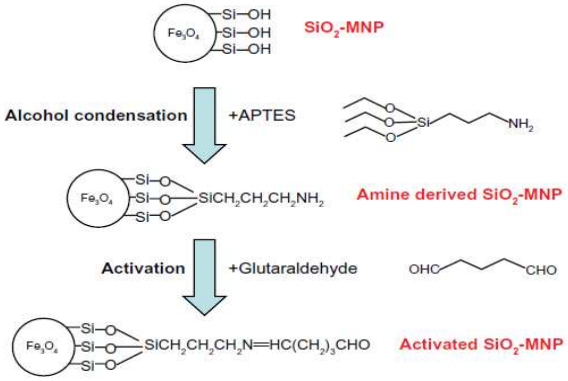

Fig. 1. The schemes of the immobilization process

Reacting with APTES is an important step to activate MNPs. FT-IR spectroscopy was used to confirm the establishment of silanizated layer onto MNPs surface. Fig. 2 shows the FT-IR spectra of the SiO_2-coated Fe_3O_4 nanoparticles and APTES-nanoparticles. For the SiO_2-coated Fe_3O_4 nanoparticles, the characteristic absorption peaks at 464.76 cm^{-1} are attributed to the Fe-O structure [9]. APTES is resided on the magnetite nanoparticles surfaces by Fe-O-Si bands, because of the absorption band corresponding to this band appears at around 500 cm^{-1} and therefore overlaps with the Fe-O band, the latest band cannot be seen in the FT-IR spectrum. The band at 1096.33 cm^{-1}, which dues to the stretching vibration of Si-O linkage, can be seen on both of the FT-IR spectra. However, after the silanization of APTES on the MNPs' surface, the transmission at this band increase a lot in the case of the APTES-linked MNPs. This indicates the success of silanization reaction. The two bands at 3305.6 and 1639.1 cm^{-1} due to the N-H stretching vibration and -NH_2 bending mode of free -NH_2 groups again confirm the existence of APTES.

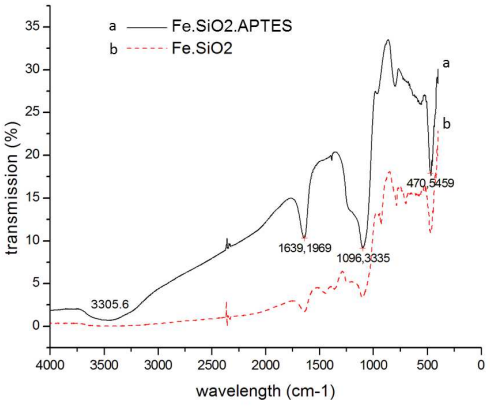

Fig. 2. FTIR spectra of (a) SiO_2-coated Fe_3O_4 nanoparticle, (b) APTES-linked nanoparticle

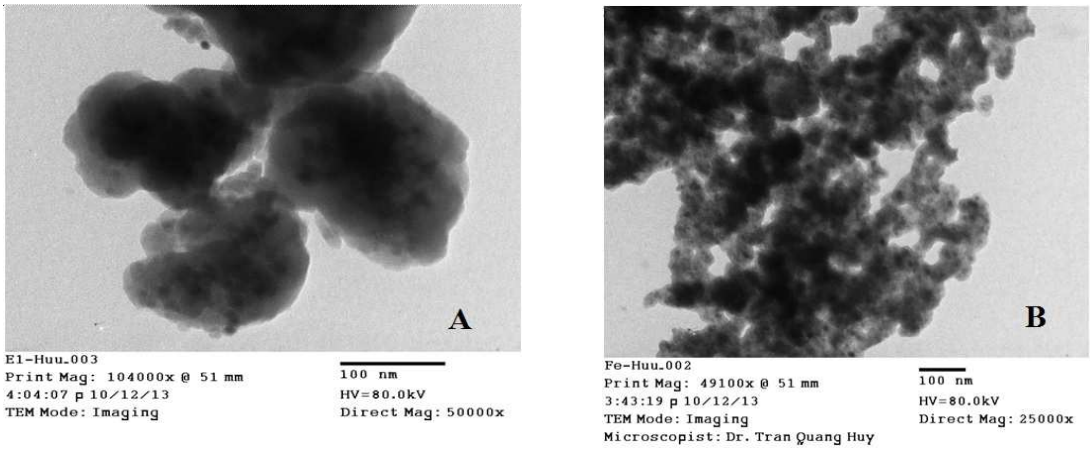

Fig. 3. TEM images in $Fe_3O_4.SiO_2$-Alcalase (A) and $Fe_3O_4.SiO_2$ (B)

Overlap bands at 3305.6 cm^{-1} for N-H stretching is a crucial reason for not to use FT-IR spectra for confirmation of covalent binding between GA and APTES, or between GA and enzyme. Transmission electronic microscopy (TEM) images of the particles were taken (Fig. 3). The diameter of the MNPs coated with enzyme (about 23.0 nm in Fig. 3A) increased compared to that of SiO_2-Fe_3O_4 nanoparticles (about 17.5 nm in Fig. 3B). The enzyme modification looks do not change the surface morphology of the MNPs. When the samples were examined by magnetic measurements, the superparamagnetic property of MNPs was conserved after APTES binding (Fig. 4). The reduction in magnetization of MNPs coated with enzyme is acceptably predicted by higher dissolubility of MNPs-enzyme ones in solution.

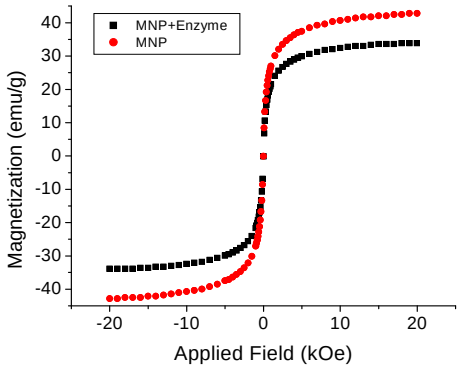

Fig. 4. Superparamagnetic property of samples MNP and MNP-binding APTES examined by vibrating sample magnetometer

III.2. The activity of the immobilized Alcalase®

From the FT-IR spectra (Fig. 2) and TEM images (Fig. 3), a covalent binding of enzyme on activated MNPs is suggested but cannot be firmly concluded. In a further experiment, a certain amount of enzyme Alcalase® was incubated with different kinds of MNPs, including of Fe-SiO_2, Fe-SiO_2-APTES, Fe-SiO_2-GA, Fe-SiO_2-APTES-GA, and a bead of Fe-SiO_2-APTES-GA which not incubated with enzyme as a control at the same condition, and then the beads were separated by external field and washed several times.

After that, the proteolytic activities of these nanoparticles were assessed. The results are shown in the Table 1. The proteolytic activities of absorbed enzyme on $Fe_3O_4.SiO_2$ with GA (0.0062 units/mg enzyme) or APTES (0.0208 units/mg enzyme) was low. This is because the

Table 1. The proteolytic activity of the immobilized Alcalase of five different conditions.

Samples	$Fe_3O_4.SiO_2$-GA-E	$Fe_3O_4.SiO_2$-APTES-E	$Fe_3O_4.SiO_2$-E	$Fe_3O_4.SiO_2$-APTES-GA	$Fe_3O_4.SiO_2$-APTES-GA-E
Proteolytic activity (units/mg enzyme)	0.0062	0.0208	0.0102	0	0.3686

physically absorbing processes allow very limited amounts of protease stay on the MNPs after washing. The same result happened for $Fe_3O_4.SiO_2$ without covered by APTES and GA (0.0102 units/mg enzyme). The other experiments proved that the $Fe_3O_4.SiO_2$ nanoparticles, which only were covered by APTES and GA, had no proteolytic activity, either. Meanwhile, under the same condition, the proteolytic activity of the enzyme on $Fe_3O_4.SiO_2$ nanoparticles, which underwent the reactions with APTES and GA, was 0.3686 units/mg enzyme. It can be seen clearly from these results that Alcalase® on $Fe_3O_4.SiO_2$-APTES-GA nanoparticles has been immobilized on the MNPs more stably than in the normal absorption. This only can be the result of the covalent attachment of the enzyme on the MNPs.

III.3. Effect of pH and temperature to activity of immobilized Alcalase®

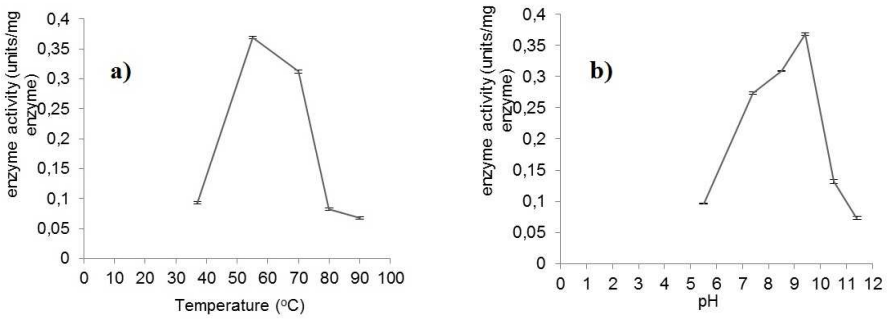

Fig. 5. Effect of environmental conditions to activity of immobilized alcalase: a) effect of temperature; b) effect of pH.

The activity of the immobilized enzyme is affected by environmental conditions, such as pH, temperature. When the Alcalase® was immobilized on $Fe_3O_4.SiO_2$ nanoparticles by bonds between groups amino of enzymes and groups cacboxyl of GA, the bonds can change ionic of protein. Therefore, that can influent the pH of the proteolytic reaction. To determine that impact, we performed experiments to investigate the proteolytic activity of immobilized enzyme at five different pH: 5.5, 7.4, 8.5, 9.4, 10.5 at the same temperature of 55^oC. The activity of immobilized Alcalase® was increasing as pH increased, until it peaked at pH 9.4 (Fig. 5a). This is consistent with enzyme's characteristics, which is alkaline protease. Therefore, the enzymatic activity in acidic reaction pH was low and a steady rise to neutral pH. However, when pH increased to 10.5, the activity of enzyme dropped suddenly. Then, the optimum reaction temperature of the immobilized enzyme was tested. When the reaction temperature went up, kinetic energy of molecules also rises, thereby increasing the possibilities of collisions between the protease and casein and

the casein fitting into the enzyme. However, the temperature exceeding the optimum limit, the Alcalase® starts to degrade dues to the breaking of chemical bonds, that loss active sites.

To see this change, the hydrolysis reactions were performed in the range of 37^{o}C-90^{o}C and at pH 9.4. It can be seen clearly from the Fig. 5b, the activity of immobilized Alcalase® increased with greater temperature was 55^{o}C and then it slipped back. This may be due to high heat resistance of Alcalase® , which is limited at 55^{o}C.

III.4. Reusability assay

The reusability of immobilized enzyme is important advantages as it finds wide applications in the economical point of view. In this study, number of recycles was performed with casein in pH 9.4 and 55^{o}C. After that, immobilized Alcalase® was separated from the reaction solution by magnetic field and reached next reactions after it was washed with buffer clearly. For each cycle, the corresponding activity was determined. Fig 6 shows that there was a steady decreasing from the first to thirteenth times. This loss of the proteolytic activity may be caused due to many reasons such as protein denaturation, lost enzyme during washing.

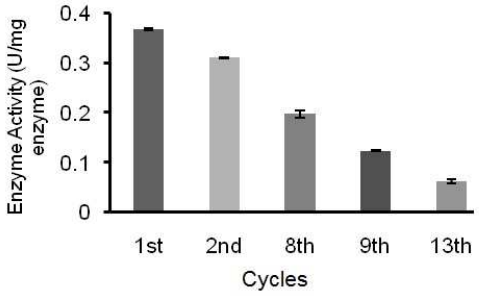

Fig. 6. Proteolytic activity of immobilized enzyme after number of recycles

IV. CONCLUSIONS

An easily recoverable and reusable support for the immobilization of Alcalase®, a broadly used protease, is synthesized by a covalent attachment of the enzyme to magnetite nanoparticles. The attachment reaction's conditions were optimized. The obtained particles are thoroughly characterized. They possess a higher enzyme immobilization efficiency and high superparamagnetism, which enable a fast enzyme recovery. The immobilized enzyme was more thermally stable than the free enzyme. The immobilized enzyme has also been efficiently recycled after more than ten cycles.

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