Conformational changes and bioactivity of lysozyme on binding to and desorption from magnetite nanoparticles

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ABSTRACT

A fundamental understanding of the conformational behaviors of lysozyme during the process of adsorption and desorption has been studied using spectrophotometric techniques, and interpreted in terms of the secondary structures in this work. FTIR data show an increase in α-helix and β-sheet content when lysozyme interaction with magnetite nanoparticles (Fe_3O_4 (PEG + CM-CTS) NPs) which indicates that the lysozyme would adopt a more compact conformation state. The mechanism of fluorescence quenching of lysozyme by magnetite nanoparticles is due to the formation of lysozyme–nanoparticles complex. High desorption of lysozyme from Fe_3O_4 (PEG + CM-CTS) NPs were achieved using phosphate buffer solution (PBS) (20 mM, pH 5.0, 0.2 M NaCl), PBS (20 mM, pH 5.0, 0.5 M NaCl) and acetic acid (0.2 M, pH 4.0) as eluents. The alterations of lysozyme secondary structure on desorption from nanoparticles were confirmed by circular dichroism and fluorescence spectroscopy. Lysozymes desorbed by PBS (20 mM, pH 5.0, 0.2 M NaCl) and PBS (20 mM, pH 5.0, 0.5 M NaCl) retain high fraction of its native structure with negligible effect on its activity, and about 92.4% and 89.5% activity were retained upon desorption from nanoparticles, however, lysozyme desorbed by acetic acid (0.2 M, pH 4.0) solution showed significant conformational changes. The stability of NPs-conjugated protein and retention of higher activity may find useful applications in biotechnology ranging from enzyme immobilization to protein purification.

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1. Introduction

Adsorption is one of the most important parts of separation. Proteins, because of containing both hydrophobic and hydrophilic functional groups which belong to amphoteric compounds, tend to adsorb at interface may lead to the conformational and functional changes during the process of adsorption and desorption [1–5]. Such change may occur due to both the intrinsic structural properties of proteins and physicochemical properties of the solid surfaces [6,7]. Nanoparticles (NPs), on account of their small size, have favorable properties to ensure highest level of conformational stabilization of protein compared to the larger particles of the same material [8]. Fe_3O_4 magnetite nanoparticles, with perfect magnetic responsively, larger specific surface area and easy manipulation of surface modification are potential nanomaterial in biotechnology, thus offering many new developments in the fields of biomedicine, biosensors and protein separation and purification.

Besides the higher purity and recovery consideration, evaluation of the bioactivities of the proteins obtained by magnetic separation is, therefore, important and necessary. Conformational changes of proteins may lead to the changes in bioactivity. Thus, a fundamental understanding of the conformational behavior of proteins during the process of adsorption and desorption is of critical importance. However, only a limited amount of work have been studied the conformational changes and activity retention of enzymes during the process of adsorption and desorption [9,10].

Chicken egg white lysozyme (EC 3.2.1.17, molecular weight (M_W) = 14.6 kDa) which often chosen as a model protein to understand the underlying principles of protein structure and function, is a small globular protein, consisting of 129 amino acid residues. X-ray crystal structure proved that lysozyme possesses a relatively rigid structure [11]. High natural abundance is also one of the important reasons for choosing lysozyme as a model protein for studying.

A variety of methods have been developed to characterize the protein conformational changes, and one of the most efficient methods is spectroscopic technology, including circular dichroic (CD) [5,12], Fourier transform infrared (FTIR) spectroscopy [13], fluorescence spectroscopy [14,15], and so on. A combination of different techniques is often necessary if one wants to obtain a comprehensive understanding of the protein conformational behavior.

The goal of this work is to study the conformation changes and bioactivity retention of lysozyme during the process of

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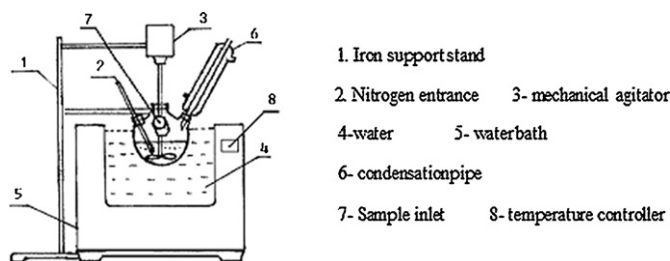yangyanjun2009@hotmail.com (Y. Yang).

Fig. 1. Chemical coprecipitating equipment of Fe_3O_4 (PEG + CM-CTS) NPs.

1. Iron support stand
2. Nitrogen entrance   3- mechanical agitator
4-water   5- waterbath
6- condensationpipe
7- Sample inlet   8- temperature controller

adsorption and desorption. Superparamagnetic nanoparticles (Fe_3O_4 (PEG + CM-CTS) NPs) which coated with carboxymethyl chitosan (CM-CTS) were used as the magnetic support for adsorption of lysozyme. In our previous paper, we show that lysozyme, when bound to Fe_3O_4 (PEG + CM-CTS) NPs of 15 nm diameter at pH 9, takes on a more compact structure in comparison to its native form. Phosphate buffer solution (PBS) (20 mM, pH 5.0, 0.2 M NaCl), PBS (20 mM, pH 5.0, 0.5 M NaCl) and acetic acid (0.2 M, pH 4.0) were used to desorb lysozyme from the magnetite nanoparticles. The secondary structure changes of lysozyme during the process of adsorption and desorption were studied by Fourier transform infrared spectroscopy (FTIR), circular dichroic (CD) and fluorescence spectroscopy. The Fe_3O_4 (PEG + CM-CTS) NPs which possess the virtue of better stability, good magnetic response, high adsorption capacity and retention of higher activity may find useful applications in biotechnology ranging from enzyme immobilization to protein purification.

2. Materials and methods

2.1. Materials

The sources of the chemicals are as follows: carboxymethyl chitosan (CM-CTS), lysozyme (chicken egg white, EC 3.2.1.17, activity 20,000 units/mg protein), lyophilized *Micrococcus lysopdeikticus* cells, iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), polyethylene glycol (PEG, M_W = 6000), 25% ammonia solution ($NH_3 \cdot H_2O$), sodium dihydrogen phosphate, acetic acid. All the chemicals were of analytical reagent grade used without further purification and the water used in all experiments was prepared in a three-stage purification system and had an electrical resistivity of 18.2 MΩ cm^{-1} (highly pure water).

2.2. Methods

2.2.1. Preparation of surface-modified superparamagnetic nanoparticles

Superparamagnetic carboxymethyl chitosan nanoparticles (abbreviated as Fe_3O_4 (PEG + CM-CTS) NPs) have been synthesized in our laboratory by chemical coprecipitating iron(II) ($FeSO_4 \cdot 7H_2O$) and iron(III) ($FeCl_3 \cdot 6H_2O$) in 25% ammonia solution and then conjugated with PEG 6000 and CM-CTS under hydrothermal conditions. The chemical coprecipitating equipment of Fe_3O_4 (PEG + CM-CTS) NPs is shown in Fig. 1. The reaction procedures of Fe_3O_4 (PEG + CM-CTS) NPs were shown point for point in our previous paper [16]. Superparamagnetic Fe_3O_4 (PEG + CM-CTS) NPs are spherical in shape with an average size of about 15 nm which measured by transmission electron microscope (TEM, JEOL JEM-2100(HR)).

2.2.2. Adsorption and desorption of lysozyme

Adding 4 mL of 1 mg/mL lysozyme in PBS (20 mM, pH 9.0) and 10 mg of Fe_3O_4 (PEG + CM-CTS) NPs into a centrifugal pipe (5 mL), the mixed suspension was shaken in a thermostated shaker (200 rpm) at 37 °C for 60 min which the adsorption had reached equilibrium. Longer incubation time did not alter the spectroscopic results and the adsorption capacity. The concentration of lysozyme was measured at 280 nm by using UV/Vis spectrophotometer (MAPADA, Shanghai, China, Model 1100). Each experiment was performed in three times for quality control and statistical purposes. The amount of lysozyme adsorbed onto the magnetite nanoparticles was calculated by mass balance.

Desorption of lysozyme from Fe_3O_4 (PEG + CM-CTS) NPs was investigated at 37 °C using PBS (20 mM, pH 5.0, 0.2 M NaCl), PBS (20 mM, pH 5.0, 0.5 M NaCl) and acetic acid (0.2 M, pH 4.0) as the desorbing agents. When equilibrium had been achieved for adsorption, the magnetic nanoparticles were recovered from the reaction mixture by permanent magnet. The lysozyme–Fe_3O_4 (PEG + CM-CTS) NPs conjugates which washing with highly pure water for five to six times then mixed with one of the above desorbing agents to desorb the lysozyme at a constant shaking rate of 200 rpm. After 60 min of desorption, permanent magnet was used to precipitate the magnetite nanoparticles and the supernatant was collected for further analyzed.

2.2.3. Fourier transform infrared (FTIR) spectroscopy

FTIR (Nicolte Nexus, Thermo Electrin Corporation) was used to determine the conformation of lysozyme in its native and absorbed state. KBr pellets were prepared by admixing about 1 mg of samples with 100 mg of spectroscopy-grade KBr and fully grinding then pressing the mixture under high pressure. The band assignments to lysozyme adsorption were collected in the range of 4000–400 cm^{-1}.

2.2.4. Circular dichroism (CD)

We measured the far-UV CD spectra to evaluate the structural changes of lysozyme before and after desorption from the Fe_3O_4 (PEG + CM-CTS) NPs. The UV-CD spectra of native and desorbed lysozyme which corrected by subtracting the buffer baseline were measured on MOS-450/AF-CD spectrometer (Bio-Logic, France). The far UV region was scanned between 200 and 250 nm with an average of three scans and also a bandwidth of 5 nm at 25 °C. The conformational change of lysozyme was evaluated by comparing the α-helix contents corresponding to the ellipticity of the bands at 208 nm. The protein concentration in the sample was in the range 0.25 mg/mL.

2.2.5. Fluorescence spectroscopy

Fluorescence spectroscopy was also used to monitor the secondary structure change in lysozyme which desorbed from Fe_3O_4 (PEG + CM-CTS) NPs. All measurements were carried out using a Hitachi F-7000 spectrofluorimeter with 0.2 mg/mL lysozyme concentration. The slits were 10 nm for excitation and emission scans. Fluorescence was measured by excitation at 295 nm and emission at 310–400 nm.

Fluorescence quenching measurements: A series amount of Fe_3O_4 (PEG + CM-CTS) NPs was added to the 50 μM lysozyme stock solution, and the fluorescence intensity was measured.

2.2.6. Enzymatic activity

The lysis rate of *Micrococcus lysodeikticus* by lysozyme was measured as reported [17]. The activity of lysozyme was monitored at 450 nm at pH 7.0 and 30 °C. Lysozyme solution (50 μL) was added into a 1 mL suspension of *M. lysodeikticus* in 0.1 mM of sodium phosphate buffer (pH 7.0). One unit is equal to a decrease in turbidity of 0.001 per minute at 450 nm under the specified conditions. The activity was calculated with following equation:
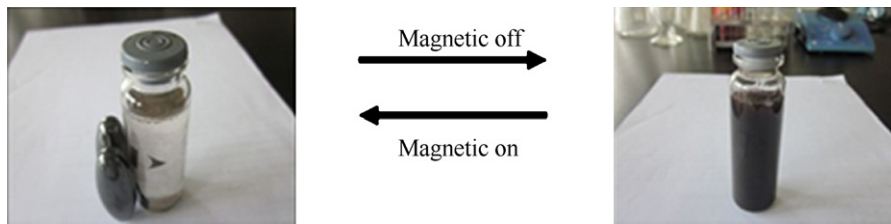
$$U = \frac{A_{450}}{0.001 \times m} \tag{1}$$

Fig. 2. Magnetic-field-assisted separation for Fe_3O_4 (PEG + CM-CTS) NPs in water solution.

where U (U/mg) is the activity units contained in 1 mg lysozyme, A_{450} is the decrease of absorbance at 450 nm per minute, and m is the mass of lysozyme (mg) added in the reaction solution.

3. Results and discussion

The choice of lysozyme and Fe_3O_4 (PEG + CM-CTS) NPs for this study were depended on the following considerations. First, lysozyme, which has bactericidal and bacteriostatic properties, is often used in food technology and pharmacological technology [18]. Second, besides the stringent purity and recovery consideration, evaluation of the biological activities of protein obtained by magnetic separation is, therefore, important and necessary. Third, the values of pI for Fe_3O_4 (PEG + CM-CTS) NPs and lysozyme are 5.1 [16] and 11.2 [19], respectively; therefore, strong electrostatic interaction could be anticipated between the negatively charged Fe_3O_4 (PEG + CM-CTS) NPs and positively charged lysozyme over the wide pH range. Finally, Fe_3O_4 (PEG + CM-CTS) NPs possess the advantages of high stability, strong magnetic responsiveness, cost-effectiveness and excellent binding of a larger amount of lysozyme and easier separation from the reaction system.

3.1. Adsorption on magnetite nanoparticles

The superparamagnetic properties of the Fe_3O_4 (PEG + CM-CTS) NPs is critical for their application in biomedical and bioengineering field, which prevents them from aggregation and enables them to re-disperse rapidly when the magnetic field is removed (Fig. 2). Due to the uniform, small sizes and excellent dispersibility in water provide them with a larger surface area-to-volume ratio (S/V), rapid response to magnetic field, and large density of binding sites for adsorption of protein, which is of interest to their application. In this paper, adsorption of lysozyme on Fe_3O_4 (PEG + CM-CTS) NPs and subsequent its conformational changes during the process of adsorption/desorption were studied.

The biocompatible CM-CTS coating not only endowed the magnetic nanoparticles water-soluble properties but also allowed the Fe_3O_4 (PEG + CM-CTS) NPs to be bioconjugated with protein molecules by its functional group. FTIR analysis provides information regarding the change of Fe_3O_4 (PEG + CM-CTS) NPs after absorption of lysozyme at pH 9.0 (Fig. 3). After conjugation with lysozyme, the spectrum of the resultant nanoparticles (lysozyme–Fe_3O_4 (PEG + CM-CTS) NPs conjugates, Fig. 3c) shows not only the characteristic bands of the original Fe_3O_4 (PEG + CM-CTS) NPs at 584, 1079, and 1408 cm^{-1} (Fig. 3b) but also the characteristic peaks of the native lysozyme at 1662 cm^{-1} (CONH amide band II) and 1522 cm^{-1} (NH amide band II) which is consistent with Uddin's research [10]. It indicates that lysozyme was successfully attached on the surface of Fe_3O_4 (PEG + CM-CTS) NPs. The maximum amount of lysozyme adsorbed on the Fe_3O_4 (PEG + CM-CTS) NPs in the present work was 236.2 ± 2.8 mg/g at pH of 9, which higher than some reported values in the literature [20–22].

3.1.1. Secondary structure of lysozyme in the presence of magnetite nanoparticles

FTIR spectroscopy could offer valuable method to monitor the changes in secondary structure of lysozyme before and after absorbed on the superparamagnetic Fe_3O_4 (PEG + CM-CTS) NPs. Among the different bands of lysozyme, whereas the absorbance intensity of the amide II band in the region 1500–1600 cm^{-1} (C–N stretch coupled with N–H bending mode) has been reported to be proportional to the amount of the lysozyme absorbed on a surface [23], but it is believed not to be very sensitive to the conformation of the lysozyme. As shown in Fig. 4, a shift in the amide II band from 1527 to 1531 cm^{-1} indicates absorption of lysozyme on the Fe_3O_4 (PEG + CM-CTS) NPs surface. The amide I band in the region 1600–1700 cm^{-1} has a tight relationship with the secondary structure of protein. Different regions of the amide I band are contributed by different secondary structural elements: 1600–1639 cm^{-1} by β-sheet, 1640–1650 cm^{-1} by γ-random coil, 1651–1660 cm^{-1} by α-helix, and 1661–1700 cm^{-1} by T-turns [24]. As compared with the native lysozyme, the band of lysozyme after absorption on the Fe_3O_4 (PEG + CM-CTS) NPs showed obvious changes in both shape and peak position, which indicated that distinct conformational change of lysozyme may be caused during absorbed on the surface of the magnetite nanoparticles. Software of PeakFit v4.12 was used to further analyze the secondary structural of lysozyme before and after absorption on the Fe_3O_4 (PEG + CM-CTS) NPs and calculate the content of secondary structural elements (Fig. 5). Total β-sheet content of lysozyme rose from 25.42% to 27.49% before and after absorption on the surface of the magnetite nanoparticles, and α-helix content of lysozyme rose from 15.9% to 24.29%. On the contrary, the contents of γ-random coil and T-turns showed a clear drop of 3.24% and 7.26%, respectively. These changes in
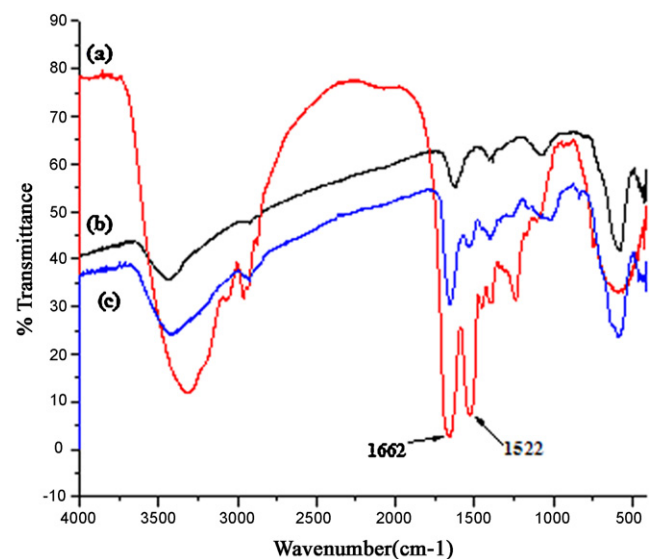

Fig. 3. FTIR spectra of (a) lysozyme, (b) Fe_3O_4 (PEG + CM-CTS) NPs, (c) lysozyme–Fe_3O_4 (PEG + CM-CTS) NPs conjugates.
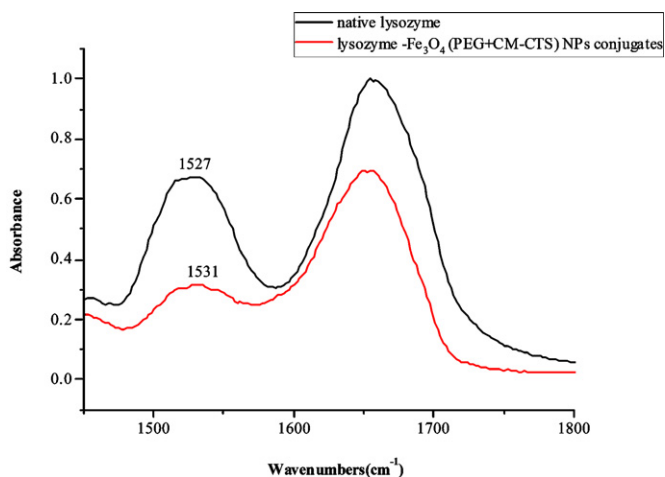
Fig. 4. FTIR spectra of native lysozyme and lysozyme–Fe_3O_4 (PEG + CM-CTS) NPs conjugates (1400–1800 cm^{-1}).

the FTIR spectra of lysozyme in native state and after conjugated with Fe_3O_4 (PEG + CM-CTS) NPs indicated that the secondary structure of lysozyme underwent obvious changes when bio-conjugated with Fe_3O_4 (PEG + CM-CTS) NPs. Increase of the β-sheet and α-helix structures, whereas the content of unordered structures decreased implied that on the surface of Fe_3O_4 (PEG + CM-CTS) NPs, the lysozyme would adopt a more compact conformation state.

Interestingly, lysozyme has also been reported to bring about a very similar change in secondary structural when absorbed on the surface of naked Fe_3O_4 and ZnO nanoparticles [8,10]. However, TiO_2 nanoparticles resulted in a substantial inactivation of lysozyme [25]. Similarly, bovine serum albumin when conjugated to gold nanoparticles underwent substantial conformational changes which becoming more flexible [26]. It is likely that the hydrophilic/hydrophobic nature of the nanoparticles, the size, and the charge distribution on the protein would have significant effect on the binding site of the protein on nanoparticles and how the nanoparticles affects the structure of the protein. The discussion below provides some insight into the possible binding site of lysozyme on Fe_3O_4 (PEG + CM-CTS) NPs.

3.1.2. Quenching studies of lysozyme by magnetite nanoparticles

The fluorescence intensity of a compound can be decreased by a variety of molecular interactions, including excited-state reactions, molecular rearrangements, energy transfer, ground state complex
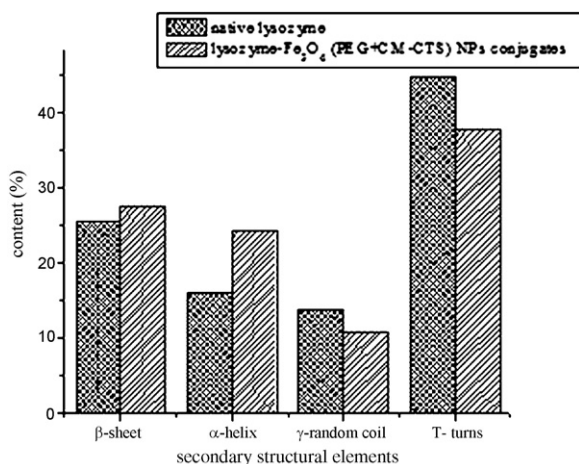

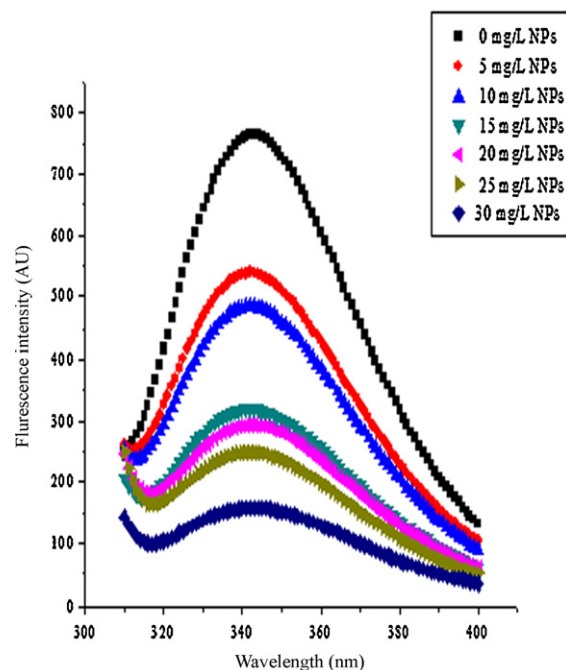**Fig. 5.** Secondary structural contents of lysozyme determined by FTIR spectroscopy.

Fig. 6. Quenching of Trp fluorescence of lysozyme in the presence of varying concentrations of Fe_3O_4 (PEG + CM-CTS) NPs.

formation and collisional quenching [27]. Such decrease in fluorescence intensity is called quenching. Lysozyme is a multi-tryptophan (Trp) protein that contains six Trp residues (Trp 28, Trp 62, Trp 63, Trp 108, Trp 111, and Trp 123) [28]. Among them, Trp 62 and Trp 108 are the most dominant fluorophores [29]. In order to investigate the proximity of the lysozyme binding sites on Fe_3O_4 (PEG + CM-CTS) NPs to the location of Trp residues in the protein structure, we analyzed the change with increasing Fe_3O_4 (PEG + CM-CTS) NPs concentrations (0–30 mg/L). The fluorescence emission spectra were recorded in the range of 310–400 nm upon excitation at 295 nm. The fluorescence quenching of lysozyme (Fig. 6) indicated that increasing concentration of Fe_3O_4 (PEG + CM-CTS) NPs on the fluorescence emission spectrum of lysozyme results in the quenching of lysozyme fluorescence emission. Mechanisms of fluorescence quenching are usually based on dynamic or static processes. Benefit from the similar studies [8], it could be deduced that the quenching of the intrinsic fluorescence of lysozyme results from a complex formation between lysozyme and the Fe_3O_4 (PEG + CM-CTS) NPs, and Trp is the possible site of binding of lysozyme to Fe_3O_4 (PEG + CM-CTS) NPs.

The active site of lysozyme contains two Trp residues that are important for substrate binding [29]. Our speculation of this is that the binding site of lysozyme to magnetic nanoparticles is also supported by the fluorescence quenching data (Fig. 6), as this position would bring Fe_3O_4 (PEG + CM-CTS) NPs in close proximity to the two Trp residues. Interestingly, the increase in the α-helix content of lysozyme brought about by the binding of Fe_3O_4 (PEG + CM-CTS) NPs at the active site has also been observed during the binding of lysozyme on ZnO nanoparticle which at the same active site [8].

3.2. Desorption from magnetite nanoparticles

The effects of pH and ionic strength on protein interaction with and desorption from the surfaces of adsorbing medium is very crucial which have been widely investigated for over several decades; the research proved that if the dominant force was electrostatic attraction between adsorbent and adsorbate, the ionic strength had a large effect on the adsorption capacity, because of increasing

Table 1
Adsorption and desorption of lysozyme from Fe_3O_4 (PEG + CM-CTS) NPs (adsorption at pH 9).

Initial lysozyme concentration (mg/mL) pH 9	Adsorbed lysozyme (mg/g)	Desorption agent		
		PBS (20 mM, pH 5.0, 0.2 M NaCl) elution ratio (%)	PBS (20 mM, pH 5.0, 0.5 M NaCl) elution ratio (%)	Acetic acid (0.2 M, pH 4.0) elution ratio (%)
1.0	236.2 ± 2.8	93.5% ± 2.0	99.0% ± 1.3	95.1% ± 3.8

electrolyte concentration in the medium the surface charges are screened. In the present study, PBS (20 mM, pH 5.0, 0.2 M NaCl), PBS (20 mM, pH 5.0, 0.5 M NaCl) and acetic acid (0.2 M, pH 4.0) were used to desorb lysozyme from Fe_3O_4 (PEG + CM-CTS) NPs at 37 °C for 60 min. The adsorbed and desorbed quantities of lysozyme when the initial concentration of lysozyme was 1.0 mg/mL are shown in Table 1. The result showed that high desorption of lysozyme from Fe_3O_4 (PEG + CM-CTS) NPs was achieved using PBS (20 mM, pH 5.0, 0.2 M NaCl), PBS (20 mM, pH 5.0, 0.5 M NaCl) and acetic acid (0.2 M, pH 4.0) as desorption agents, furthermore, increasing the ionic strength will be favorable for lysozyme desorption.

3.3. Conformational changes and activity assay

Conformational changes of protein may result in the changes in bioactivity. Thus, a fundamental understanding of the conformational behavior of protein during the process of adsorption and desorption is of critical importance. Circular dichroism (CD) and fluorescence spectroscopy were adopted to evaluate the conformational changes of lysozyme after desorbed from Fe_3O_4 (PEG + CM-CTS) NPs in this research.

3.3.1. Circular dichroism

Further evidence of conformational changes of lysozyme upon desorption from Fe_3O_4 (PEG + CM-CTS) NPs was provided by CD spectra. CD is a sensitive technique to monitor the conformational changes in protein. The CD spectra of native lysozyme and lysozyme desorbed from Fe_3O_4 (PEG + CM-CTS) NPs by PBS (20 mM, pH 5.0, 0.2 M NaCl), PBS (20 mM, pH 5.0, 0.5 M NaCl) and acetic acid (0.2 M, pH 4.0) are shown in Fig. 7. The native lysozyme shows two extreme valleys at 208 and 222 nm, characteristic of the α-helical structure of protein. The reasonable explanation is that the negative peaks between 208 and 209 nm and 222 and 223 nm are both contributed to $n \to \pi^*$ transition for the peptide bond of α-helix [30]. The eluent PBS (20 mM, pH 5.0, 0.2 M NaCl) and PBS (20 mM, pH 5.0, 0.5 M NaCl) caused only a decrease in band intensity without any significant shift of the peaks, clearly indicating that desorbed lysozyme occurred a slight decrease in the α-helical structure compare to the native lysozyme, however, as for the lysozyme which desorbed by acetic acid (0.2 M, pH 4.0) solution, the bands at 208 and 222 nm become less negative, indicating an decrease in the α-helical content of lysozyme at the expense of the coil region.

Quantitative analysis of the structural change could be evaluated by the content of α-helix in the lysozyme. The α-helix content is estimated from the molar ellipticity at 208 nm. It could be estimated from the following equation [31]:

$$[\theta]_{mrd} = \frac{[\theta]_d M}{10 \, CLN_r} \tag{2}$$

$$\% \ \alpha\text{-helix} = \frac{[\theta]_{mrd} - 4000}{33,000 - 4000} \tag{3}$$

where $[\theta]_{mrd}$ is the mean molar ellipticity per residue at 208 nm (deg cm^2/dmol), $[\theta]_d$ is the ellipticity in the unit of mdeg, M is the molecular weight of lysozyme (Dalton), C is the lysozyme

concentration (mg/mL), L is the path length (0.1 cm), and Nr is the number of amino acid residues for lysozyme, which consists of 129 amino acid residues.

As calculated from Eqs. (2) and (3), the percentage of α-helix in native lysozyme is 31.2% which is near the literature value of 32% [32]. The α-helix percentage for lysozyme desorbed by PBS (20 mM, pH 5.0, 0.2 M NaCl) and PBS (20 mM, pH 5.0, 0.5 M NaCl) are 30.07% and 28.7%, close to the native lysozyme, which clearly indicating that PBS (20 mM, pH 5.0, 0.2 M NaCl) and PBS (20 mM, pH 5.0, 0.5 M NaCl) could not destroy the protein hydrogen bonding networks and induce the secondary structure changes in lysozyme. However, when desorbed by acetic acid (0.2 M, pH 4.0) solution, α-helix percentage for lysozyme is 19.3% which is far away from the native one, it was apparent that acetic acid (0.2 M, pH 4.0) caused a secondary structure change of the lysozyme, with the loss of α-helix stability.

3.3.2. Fluorescence spectroscopy

Fluorescence spectroscopy analysis provides information regarding the changes in secondary structures of lysozyme in its native and desorbed state (Fig. 8). As X-ray crystal structure proved that lysozyme possesses a relatively rigid structure, it is not expected to detect a large change in the structure. As shown in Fig. 8, the native lysozyme (pH 9.0) has a λ_{max} at 342.4 nm, and the lysozyme which desorbed by PBS (20 mM, pH 5.0, 0.2 M NaCl) and PBS (20 mM, pH 5.0, 0.5 M NaCl) show λ_{max} at 341.8 nm and 341.4 nm respectively, which only 0.6 nm and 1.0 nm band shift. However, the λ_{max} of lysozyme moves to 339.4 nm when desorbed by acetic acid (0.2 M, pH 4.0) solution. As is well known, 80% of the intrinsic fluorescence of lysozyme originated from Trp
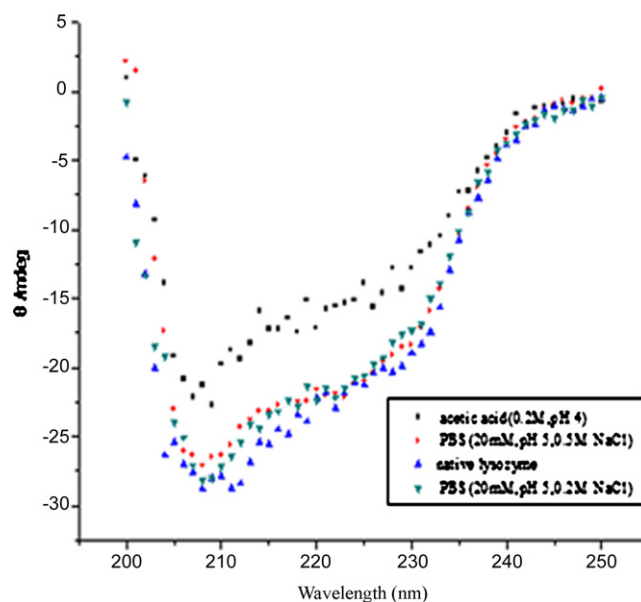

Fig. 7. Comparison of CD spectra of native lysozyme (pH 9) and lysozyme desorbed by PBS (20 mM, pH 5.0, 0.2 M NaCl), PBS (20 mM, pH 5.0, 0.5 M NaCl) and acetic acid (0.2 M, pH 4.0) solutions from Fe_3O_4 (PEG + CM-CTS) NPs.
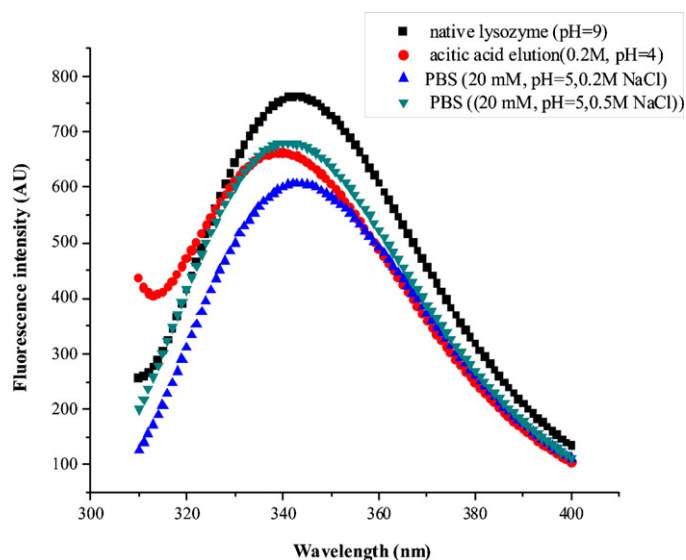
Fig. 8. Fluorescence emission spectra of native lysozyme (pH 9) and desorbed lysozyme by PBS (20 mM, pH 5.0, 0.2 M NaCl), PBS (20 mM, pH 5.0, 0.5 M NaCl) and acetic acid (0.2 M, pH 4.0) solutions from Fe_3O_4 (PEG + CM-CTS) NPs.

62 and 108 which at the active site [29]. From this information it can be stated that the apparent hydrophobicity of the active site in lysozyme has not changed after desorption, therefore, it indicates that lysozyme which desorbed by PBS (20 mM, pH 5.0, 0.2 M NaCl) and PBS (20 mM, pH 5.0, 0.5 M NaCl) could keep most of its original structure. Therefore, according to the results of CD and fluorescence spectroscopy, we can come to a proposal that PBS (20 mM, pH 5.0, 0.2 M NaCl) and PBS (20 mM, pH 5.0, 0.5 M NaCl) are the optimal desorption agents to maintain the conformational stability of lysozyme when desorbed from Fe_3O_4 (PEG + CM-CTS) NPs.

3.3.3. Lysozyme bioactivity

To test whether desorption of lysozyme from Fe_3O_4 (PEG + CM-CTS) NPs have any role in enzymatic activity; we examined the activity of the desorbed lysozyme relative to that of the native lysozyme. It was observed that after desorption from Fe_3O_4 (PEG + CM-CTS) NPs by PBS (20 mM, pH 5.0, 0.2 M NaCl) and PBS (20 mM, pH 5.0, 0.5 M NaCl) that about 92.4% and 89.5% lysozyme activities were retained compared to native lysozyme. As conformation of lysozyme desorbed by acetic acid (0.2 M, pH 4.0) solution changed clearly as demonstrated by CD and fluorescence results, so we did not check the bioactivity of lysozyme which desorbed from Fe_3O_4 (PEG + CM-CTS) NPs by acetic acid (0.2 M, pH 4.0) solution.

4. Conclusions

In conclusion, using lysozyme as a model protein, Fe_3O_4 (PEG + CM-CTS) NPs are capable of preventing the lysozyme unfolding, and help it in a more compact conformation state. Desorption of lysozyme by PBS (20 mM, pH 5.0, 0.2 M NaCl), PBS (20 mM, pH 5.0, 0.5 M NaCl) and acetic acid (0.2 M, pH 4.0) solutions was carried out and then compared with the native

state for conformation and bioactivity research. The recovered lysozyme which desorbed by PBS (20 mM, pH 5.0, 0.2 M NaCl) and PBS (20 mM, pH 5.0, 0.5 M NaCl) preserved both its structure and its enzymatic activity well, about 92.4% and 89.5% activities of lysozyme were retained compared to native lysozyme, which suggests that Fe_3O_4 (PEG + CM-CTS) NPs is capable of purifying lysozyme efficiently in terms of high adsorption capacity and preserving the structure and enzymatic activity well. Moreover, the stabilizing influence of Fe_3O_4 (PEG + CM-CTS) NPs on lysozyme in this article would be useful for the fruitful application of Fe_3O_4 (PEG + CM-CTS) NPs to serve as a superior support for the immobilization and separation of lysozyme.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.09.009.

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