Adsorption and desorption of lysozyme on thermosensitive nano-sized magnetic particles and its conformational changes

N. Shamim, L. Hong, K. Hidajat, M. S. Uddin* Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4 , Singapore 117576.

Introduction

In recent years, there has been a great interest in the fabrication of stimuli responsive polymer microspheres for technological application and fundamental studies. These stimuli responsive smart and intelligent polymeric materials can respond in shape or volume changes to small external stimuli such as temperature, pH and ionic strength etc [1, 2]. Among these stimuli responsive polymers N-isopropylacrylamdie (PNIPAM) is the most widely studied thermosensitive polymer. It has a Lower Critical Solution Temperature (LCST) of 32^{o}C in water. It collapses and shrinks above the LCST and swells and expands below the LCST. Due to its well defined and reversible low critical solubility temperature, poly (N-isopropylacrylamide) (PNIPAM) has long been investigated as a versatile tool in biology. The technology can be applied to various aspects of biotechnology, for example enzyme immobilization [3, 4], cell sorting [5], drug delivery [6], and protein separation and purification [7, 8].

Many researchers used reversible thermosensitivity of N-isopropylacrylamide as adsorption desorption tool. Elaissari and Bourrel [9] reported adsorption of human serum albumin (HSA) on polystyrene core and PNIPAM shell by controlling pH and temperature as adsorption parameters. They observed the maximum amount of protein was adsorbed at the isoelectric point of the protein and the main driving force involved was hydrophobic interaction. However, no work on conformational changes was conducted. Desorption of proteins from interfaces depends essentially on the conditions under which they have been adsorbed. Norde and Favier [10] reported adsorption of BSA and LYZ on finely dispersed silica particles and they observed subsequent desorption of about (80-100)% in morpholine solution of pH 8.5. The result showed structural alternation of both BSA and LYZ upon adsorption on silica surface. Desorption of protein from the magnetic particles could be achieved by controlling various factors such as pH, temperature and ionic strength.

It has been generally recognized that protein molecules which are interacting with solid surfaces will undergo some conformational changes. The structural change may occur due to the intrinsic properties of protein. One of the most commonly used methods to study protein conformation in solution or adsorbed on to colloidal substrate is circular dichroism (CD) spectroscopy [11]. The CD spectrum provides a global measure of the secondary structural features present in a molecule. Haynes and Norde [12] adsorbed lysozyme on negatively charged polystyrene microspheres and suggested that lysozyme denatures significantly on the polystyrene surface. However, no detail work was reported on adsorption, desorption and conformational change of lysozyme during processes of extraction by thermosensitive nano magnetic particles.

In this present work, nano sized magnetic particles coated with thermosensitive polymer (N-isopropylacrylamide) were used to adsorb lysozyme at different temperature and pH. Desorption was carried out using NaH_2PO_4 (pH 4), and NaSCN (pH 6) as the desorption agent. The conformational changes of lysozyme during the process of adsorption and desorption were studied by CD and fluorescence intrinsic spectroscopy.

Experimental

Magnetic fluids were prepared by chemical co-precipitation method under inert environment. A complete precipitation of Fe_30_4 was achieved under alkaline condition and the molar ratio was maintained at Fe^{2+}: Fe^{3+} = 1:2. In a typical synthesis to obtain 1 g of Fe_30_4 precipitate, 0.86 g of $FeCl_2$. $4H_2O$ and 2.35 g of $FeCl_3$. $6H_2O$ were dissolved under N_2 atmosphere in 40 ml of deaerated Mili-Q water with vigorous stirring at a speed of 1000 rpm[13]. As solution being heated to 80^{o}C, 100 mg of thiodiglycolic acid (TDGA) was added, followed by 5 ml of NH_4OH addition. Further, TDGA was added to the suspension in five 0.2 g amounts over 5 minutes. The experiment was continued for 30 minutes at 80^{o}C. The stable water based suspension was then cooled at room temperature and washed using Mili-Q water. The precipitates were isolated from the solvent by magnetic decantation. 4-vinylaniline was used as secondary surfactant. 1 g of the fresh precipitate obtained from the previous preparation was combined with 20 ml mili-Q water and the mixture was heated to about 45^{o}C under vigorous stirring. About 0.2 ml of 4-vinylanile was added drop wise using a syringe. Bilayer coated magnetic particles were first cleaned to eliminate any free electrolyte and adsorbed surfactants. 1 g of the seed magnetic particles were purged with nitrogen for 30 minutes at a temperature of 70^{o}C , then a mixture of NIPAM (0.3g), MBA (20mg), KPS (5mg) was added. Polymerization was carried out for 6 hours. The polymerization conversion was gravimetrically determined and the final particles were washed at least three times before adsorption.

Adsorptions of lysozyme on thermosensitive magnetic particles were carried out by mixing 5 ml of LYZ solution of certain concentration and 137 mg of wet solid magnetic particles. The mixture was incubated at 40^{o}C in 10 mM phosphate buffer solutions at 11.1 pH. After 2 hours of incubation the protein adsorption was determined from the supernatant analysis by UV at a wavelength of 280 nm. The solid content of the wet magnetic particles were measured to be 15.59%. Desorption experiments were performed as follows. First, protein adsorbed on thermosensitive magnetic particles at 40^{o}C were separated from the medium and redispersed in 0.5M NaH_2PO_4 (pH 4), and NaSCN (pH 6) solution. Then desorption was carried out at 20^{o}C for 2 hours under constant shaking. Finally, the desorbed amount of protein was determined by measuring the free protein concentration in the supernatant.

Circular dichroism (CD) spectroscopy (Jacob J810 spectrometer) was used to measure the conformation change of desorbed lysozyme with respect to native lysozyme. Solutions of the native and desorbed lysozyme were diluted in the range of (0.05-0.1) mg ml $^{-1}$ and scanned over the wavelength range 200-260 nm, using 5 mm quartz cylindrical cell. The secondary structures of native and desorbed lysozyme were evaluated by comparing the α-helix content, corresponding to the ellipticity of the bands at 208 nm.

Results and Discussions

Adsorption isotherm

Typical adsorption isotherms obtained at 40^{o}C and 25^{o}C are shown in Figure 1. The experimental results indicate that temperature dependent adsorption of LYZ on thermosensitive magnetic particles is mainly attributed to the change in the properties of the particles' surfaces. More protein was adsorbed at a higher temperature compared to lower temperature due to the hydrophobic effect above the lower critical solution temperature of

poly (NIPAM). The shapes of the isotherms were Langmuir, therefore, all isotherms were fitted to the Langmuir isotherms (Eq 1) using nonlinear regression method.

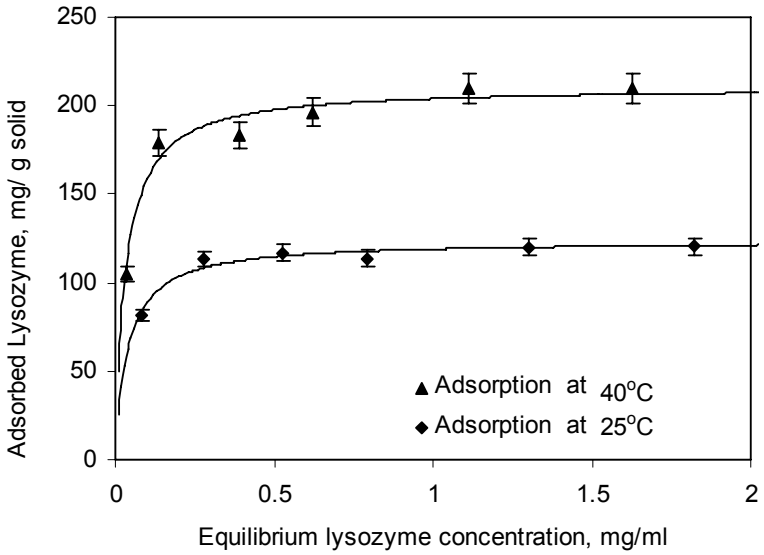

Figure1. Adsorption equilibrium isotherm of LYZ at different temperature (Ionic strength 0.01M and pH 11)

$$C_s = C_m \frac{KC_b}{1 + KC_b} \qquad [1]$$

Where, C_b (mg/ml) and C_s (mg/ g solid) are lysozyme concentration in the aqueous solution and the absorbed lysozyme on the solid at equilibrium, respectively. C_m in the maximum adsorption amount, and K is the adsorption constant. Experimental data are fitted to the Langmuir equation using non linear regression.

Desorption of lysozyme from thermosensitive nanomagentic particles

The Desorption of LYZ from thermosensitive nanomagnetic particles were carried out by 0.5M NaH_2PO_4 (pH 4), and 1.5 M NaSCHN (pH 6)solution. It was observed that no significant desorption was measured with the buffer of NaH_2PO_4 and NaSCN.

Circular dichroism (CD) measurements

The CD spectra of native LYZ and desorbed LYZ by 0.5M NaH_2PO_4 (pH 4), and 1.5M NaSCN solution are shown in Figure 2. From the figure it could be observed that there are two extreme valleys at 208 and 222nm for native lysozyme. However, the CD spectrums of desorbed protein by NaH_2PO_4 shows extreme valleys at 208 and 222nm. That means there is no structural change in the desorbed proteins. On the other hand desorbed LYZ by NaSCN shows a very different spectrum from the native one. This leads to the conclusion that this desorption agent can not retain the native structure of the native lysozyme.

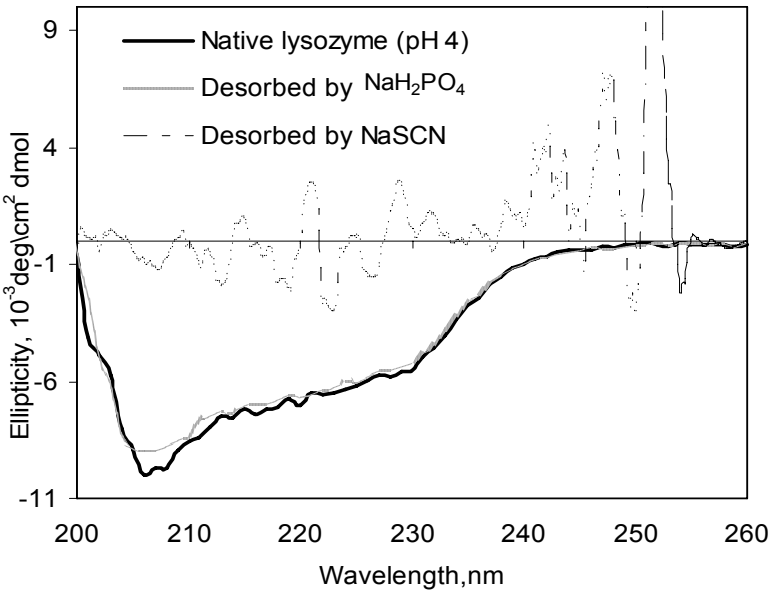

Figure 2. Comparison of CD spectra of native lysozyme (pH 4) and lysozyme desorbed by NaH_2PO_4 (pH 4) and NaSCN (pH 6.0) from thermosensitive nanomagnetic particles

Protein has different level of structures, such as primary, secondary, tertiary and quaternary structures. α – helix is one of the elements of secondary structure therefore the quantitative analysis of the structural change of lysozyme could be evaluated by the content of α –helix content preserved. Moreover, the CD spectrum data provide quantitative information about the change in the content in secondary structure. The α –helix content is estimated from the molar ellipticity at 208 nm $[\theta]_{208}$ according to the following equation.

$$\% \text{ of } \alpha\text{-helix} = \frac{[\theta]_{208} - 4000}{33000 - 4000} \times 100\% \quad (2)$$

Table 2. Estimated percentage of α – helix of lysozyme from circular dichroism spectrum

% α – helix		
Native lysozyme	Desorbed lysozyme	
pH 4.0	NaH_2PO_4 pH4.0	NaSCN pH 6.0
20.7	17.28	-

Table 2 shows percentage of α – helix in native lysozyme is 20.7% at pH 4 which is near the literature value of 29% [11] . The α – helix percentage for lysozyme desorbed by NaH_2PO_4 is 17.24% which is very close to the native lysozyme.

Reference

1. Kim, J.J. and K. Park, Smart hydrogels for bioseparation. Bioseparation. 1998; 7: 177-184.
2. Kawaguchi, H. and K. Fujimoto, Smart latexes for bioseparation. Bioseparation. 1998; 7: 253-258.
3. Dekker , R.F., Immobilization of a lactase onto a magnetic support by covalent attachment to polyethyleneimine-glutaraldehyde-activated magnetite. Applied Biochemistry And Biotechnology. 1989; 22: 289-310.
4. Kondo, A. and H. Fukuda, Preparation of thermo-sensitive magnetic hydrogel microspheres and application to enzyme immobilization. Journal of Fermentation and Bioengineering. 1997; 84: 337-341.
5. Haik , Y., V. Pai, and C.-J. Chen, Development of magnetic device for cell separation. Journal of Magnetism and Magnetic Materials. 1999; 194: 254-261.
6. Rusetski, A.N. and E.K. Ruuge, Magnetic fluid as a possible drug carrier for thrombosis treatment. Journal of Magnetism and Magnetic Materials. 1990; 85: 299-302.
7. Abudiab , T. and R.R. Beitle, Jr, Preparation of magnetic immobilized metal affinity separation media and its use in the isolation of proteins. Journal Of Chromatography. A. 1998; 795: 211-217.
8. OBrien, S.M., O.R.T. Thomas, and P. Dunnill, Non-porous magnetic chelator supports for protein recovery by immobilised metal affinity adsorption. Journal of Biotechnology. 1996; 50: 13-25.
9. Elaissari, A. and V. Bourrel, Thermosensitive magnetic latex particles for controlling protein adsorption and desorption. Journal of Magnetism and Magnetic Materials. 2001; 225: 151-155.
10. Norde, W. and J.P. Favier, Structure of Adsorbed and Desorbed Proteins. Colloids and Surfaces. 1992; 64: 87-93.
11. Greenfield, N. and G.D. Fasman, Computed circular dichroism spectra for the evaluation of protein conformation. Biochemistry. 1969; 8: 4108-4116.
12. Haynes, C.A. and W. Norde, Structures and Stabilities of Adsorbed Proteins. Journal of Colloid and Interface Science. 1995; 169: 313-328.
13. Shen, L.F., P.E. Laibinis, and T.A. Hatton, Bilayer surfactant stabilized magnetic fluids: Synthesis and interactions at interfaces. Langmuir. 1999; 15: 447-453.