Modern Physics Letters B
Vol. 27, No. 19 (2013) 1341028 (6 pages)

DOI: 10.1142/S0217984913410285

PURIFICATION OF LYSOZYME BY INTRINSICALLY SHIELDED HYDROGEL BEADS

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Published 19 July 2013

Macro-sized intrinsically shielded hydrogel beads have been prepared from BSA and CM-dextran grafted with CB using a technique based on freeze-thawing gelation method. The size of the beads lies in around 500 μm. Isothemal titration calorimetry (ITC) showed that the relative binding affinities of the lysozyme for CB, compared with BSA, at pH 3.0 was stronger than that at pH 7.4. They were employed for the affinity separation of lysozyme using chromatography column. Their adsorption capacity for lysozyme at pH 3.0 is higher than that at pH 9. In a binary mixture of lysozyme and ovalbumin, the beads showed very high selectivity toward lysozyme. Lysozyme of very high purity ($> 93\%$) was obtained from a mixture of lysozyme and ovalbumin, and 85% from egg white solution. The results indicate that the macro-sized bead can be used for the separation, purification, and recovery of lysozyme in a chromatograph column.

Keywords: Intrinsically shielded hydrogel beads; freeze-thawing gelation method; lysozyme; affinity, purification.

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1. Introduction

Lysozyme is a commercially valuable protein. A large quantity of pure lysozyme is widely used in research laboratories for biochemical, biophysical and medical studies.[1] Commercially available purified lysozyme is manufactured from hen egg white using a combination of conventional processes such as crystallization,[2] precipitation,[3] ultrafiltration.[4] However, the low content (about 3.5 wt.%) of lysozyme in hen egg white makes its purification a technical challenge because substantial amount of protein has to be processed to get a small amount of lysozyme.

More recently, the development of dye-ligands as affinity adsorbents has allowed the use of affinity chromatography in the recovery of lysozyme. The affinity based separations allow production of higher purity lysozyme with shorter operation times. The dye Cibacron blue (CB) is widely used in affinity purification of lysozyme as it is cheap, stable and selectively binds a number of proteins. It carries a number of strongly acidic groups, which will be ionized at neutral pH hence and so can also act as an ion exchange group.[5]

We introduced the concept of "an intrinsic shielding approach", whereby Bovine serum albumin (BSA), a protein known to show a weak interaction with CB, is co-immobilized to the support. The intention is that the co-immobilized components undergo weak binding, which is only displaced by a strongly bound adsorbate. Macro-sized beads as support matrices for chromatography offer several potential advantages over membrane. They have a large internal surface area that is densely covered in reactive groups, to which ligands are easily attached. What is more, by regenerating the columns they could be used many times with only a gradual decline in performance.

In the present study, we propose a procedure for fabricate bead with BSA as gel backbone by cryo-gelation. Its lysozyme adsorption, stability and separation ability to recover lysozyme from protein mixture and egg white solution were studied.

2. Materials and Methods

2.1. *Materials*

480KD Dextran, Lysozyme, Ovalbumin, Cibacron blue F3GA, BSA used here were obtained from Sigma-Aldrich UK. EDC and NHS were obtained from Lancaster Synthesis Ltd., UK. Eggs are purchased from local supermarket. The proteins solutions were prepared by dissolving the powder in pH 7.4 phosphate buffer (0.1 M). The egg white solutions were prepared by dissolving egg white in pH 7.4 phosphate buffer.

2.2. *Hydrogel beads preparation*

CM-dextran was prepared according to the previous report.[6] Hydrogel beads consisting of a CB-CM-dextran backbone cross-linked to BSA were mixed using a syringe pump to inject the solutions into a chamber with a magnetic stirrer.

The beads were transferred to a −20°C freezer and left overnight for the slow gelation of the hydrogel beads. Following freezing, the beads were rinsed several times in 0.02 M phosphate buffer, pH 7.4 containing 0.1 M NaCl and 1 mM NaN_3. After washing in phosphate buffer, the beads were freezing dried. After drying, uniform beads of similar size were obtained by sieving. Above steps were repeated with hemoglobin instead of BSA with a similar protocol to make control beads.

2.3. *Adsorption and desorption of the hydrogel beads*

20 mg hydrogel beads were immersed in 5 ml of the test protein solution in phosphate buffer 0.1 M pH 7.4 with agitation at 25°C for 24 h. Unbound protein was then determined from absorbance measurements at 280 nm. After adsorption experiments, the beads were removed from the protein solution and rinsed in pH 7.4 phosphate buffer (I = 0.1) for at least 24 h to remove residual nonaffinity bound protein. The desorption of protein from beads was achieved using 5 ml phosphate buffer containing 1 M NaCl for 24 h. Desorbed protein concentration was calculated using calibration data for lysozyme determined at the 280 nm wavelength. All experiments were conducted at 25°C.

2.4. *Column experiments*

These experiments were conducted with 2 g dry hydrogel beads (0.5 mm) packed into 1.5 × 25 cm column. A mixture containing 1 mg/mL lysozyme and 1 mg/mL ovalbumin was loaded through the column at the flow rate of 1 mL/min for 30 min. This was followed by washing with pH 7.4 0.1 M phosphate buffer for 1 h, elution with 1 M NaCl phosphate buffer for 1 h and regeneration with pH 7.4 0.1 M phosphate buffer for 30 min. All elution samples were collected.

The selectivity of adsorption was assessed by using size exclusion chromatography to determine the ratio of lysozyme concentrations to the pooled ovalumin+conalbumin concentration in the protein elution. Chromatography was conducted on a Sephdex G-25 column (1.5 × 25 cm) using a Shimadzu HPLC system and UV detector. The mobile phase used was 20 mM sodium phosphate pH 7.4 (I = 0.1 M). A mobile-phase velocity of 0.06 cm min^{-1} was used to and a 0.1 ml sample loop was employed for sample injection. All protein samples were filtered through a 0.2 μM membrane filter before injection. Data was collected on computer using Shimadzu Class VP software, version 5.02.

3. Results and Discussion

3.1. *Adsorption and desorption of the hydrogel beads*

The beads of BSA-CB-CM-dextran hydrogel prepared as described above were analyzed by scanning electron microscopy (SEM) and around 500 μm. The SEM structure illustrates the pores and affinity bound of CB-CM-Dextran with BSA.

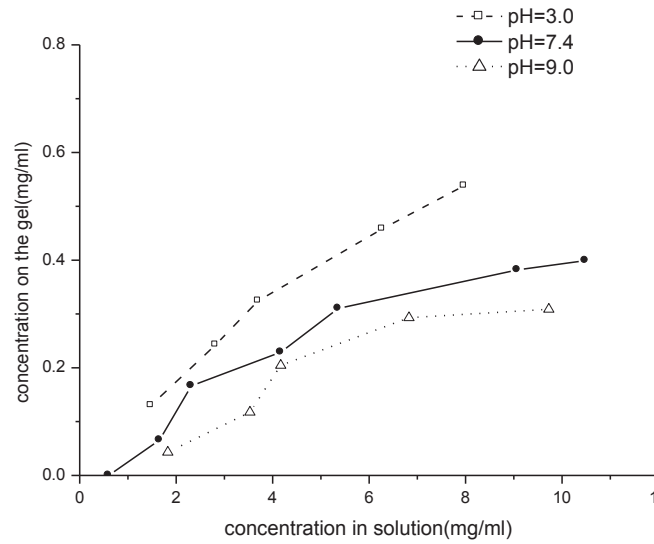

Fig. 1. Adsorption isotherms of BSA backbone beads under different pH value.

The relative binding affinities of the lysozyme for CB, compared with BSA, at pH 3.0 and 7.4 were tested by isothemal titration calorimetry (ITC). The adsorption isotherms of BSA backbone beads under different pH value were showed in Fig. 1. It shows the effect of pH on the amount lysozyme adsorbed on the beads. The maximum lysozyme adsorption was obtained at pH 3.0. Significantly lower adsorption capacities were obtained with the affinity membrane at pH 9.0. The possible explanations for this behavior are as follows: (i) chemical interaction between the hydrogel bead and the lysozyme molecules increased with increasing pH; (ii) compared with BSA, the relative CB binding capacity with lysozyme at pH 3.0 is much stronger than that at pH 7.4.

3.2. *Column experiments*

The selectivity of the beads for lysozyme was investigated by using a mixture containing 1 mg/mL lysozyme and 1 mg/mL ovalbumin. The results are presented in Fig. 2. A high purity lysozyme is obtained in pH 3.0. Ovalbumin electric charge effect with lysozyme leads to low lysozyme purity as pH increase.

The beads as support matrices for chromatography were packed onto column to investigate the purification efficiency of lysozyme from the mixture protein and egg white. Because the hen egg white is a major source of lysozyme, it is important to investigate the separation of lysozyme from that mixture. Being highly viscous, the egg white was first diluted with phosphate buffer (9 volume buffer to 1 volume egg white). Lysozyme purified with BSA -CB-CM-dextran beads has higher purity from both protein mixture and egg white solution compared with that of Hemoglobin-

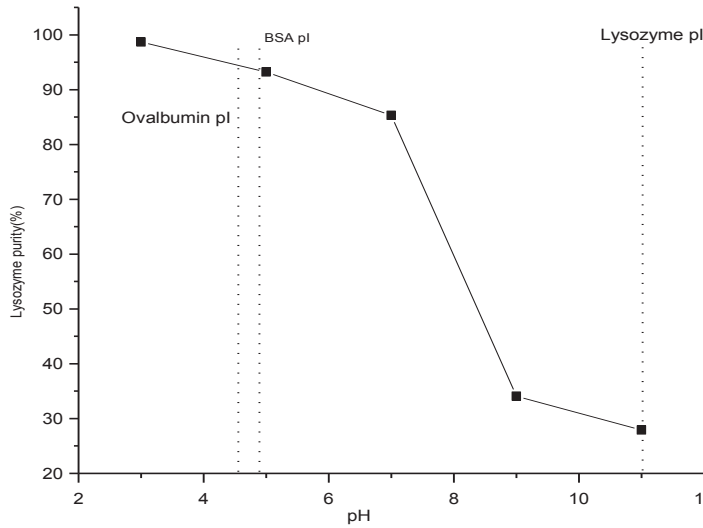

Fig. 2. Lysozyme separation from protein mixture with medium hydrogel beads (around 500 μm) under different pH value.

CB-CM-dextran bead. BSA successfully protected ligand CB from binding with weakly binding contaminant.

The stability of the hydrogel beads were performed. After 6 cycle of adsorption/washing/desorption/regeneration, the hydrogel beads maintained their adsorption capacity at an almost constant value. It indicates that the hydrogel beads have a good stablility.

4. Conclusion

This research describes an affinity hydrogel beads grafted with both a CB ligand and a BSA protein receptor. Lysozyme adsorption results at different pH show that pH effects significantly the lysozyme adsorption capacity of the hydrogel beads partly due to the charge effect between bead and protein and also result from the relative affinity binding capacity of lysozyme and BSA with CB at various pH conditions. The results from the column experiments showed these beads have the same separation and purification efficiency as the hydrogel membrane.[7,8] The result of this study indicates that the BSA-CM-dxtran-CB hydrogel beads is very stable and suitable for the economical recovery of lysozyme. The clear advantage of the use of an immobilized shielding agent is that it is retained on the adsorbent and does not require subsequent removal from the product.

Acknowledgments

We gratefully acknowledge financial support from the BBSRC Grant No 86/E12129 and NSFC (National Nature Science Foundation of China) Grant No: 20975079.

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