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Tris(hydroxymethyl)aminomethane-modified magnetic microspheres for rapid affinity purification of lysozyme

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ABSTRACT

A novel affinity purification method for lysozyme (LZM) based on functionalized magnetic microspheres was developed. Tris(hydroxymethyl)aminomethane (Tris)-modified magnetic microspheres with specific affinity toward LZM were prepared using Tris as ligand and silica-coated magnetic microshperes as support. Transmission electron microscopy and magnetic property measurement results showed that the Tris-modified magnetic microspheres have a very good core-shell structure and high magnetization.The maximum binding capacity of LZM was about 108.6 mg/g magnetic microspheres. LZM purified from chicken egg white had high purity and well-maintained activity of 8140 U/mg. This magnetic-mediated LZM purification strategy has advantages of high efficiency, low cost and easy operation.

1. Introduction

Functionalized magnetic microspheres have been extensively applied in various fields, such as separation and purification [1–4], immunoassay [5], targeted drug delivery [6] and magnetic resonance imaging [7]. Functionalized magnetic microspheres are ideal biomacromolecule carriers in separation and purification process with several unique advantages: various functional groups can be immobilized on the surfaces of magnetic microspheres according to different target analytes, and the process of functionalization is generally simple; magnetically driven separation is much easier and faster in liquid medium than filtration and centrifugation; and the functionalized magnetic microspheres can be regenerated by releasing the target analytes from the microsphere surface. In magnetic affinity purification, however, the classical affinity ligands, including antibodies, dyes, metal ions, etc., are expensive, toxic or unstable. Therefore, there is always a need to develop novel affinity ligands.

Protein purification is vital for the characterizations of the function, structure, physico-chemical properties and industrial application of the proteins of interest. Lysozyme (LZM) is a commercially valuable enzyme and has widespread application [8,9] as cell-disrupting reagent, antibacterial agent, food additive, etc. Practically, LZM is vastly obtained from chicken egg white in which the content of LZM is about 0.34%. A large number of coexisting proteins make it challenging to purify LZM from chicken egg white. LZM purification methods have been developed based on various techniques including ultrafiltration [10], ion-exchange [11,12], crystallization [13,14], and affinity precipitation [15]. However, these separation and purification methods are usually complicated and time-consuming. A simple, rapid and efficient purification method for LZM is demanded both in laboratory and industry.

This work aims at combining the advantages of affinity ligand and easy operation of magnetic microspheres to develop an inexpensive, simple and rapid LZM purification method. The specific interaction between tris(hydroxylmethyl)aminomethane and LZM was proved in our previous work [16,17]. Inspired by former studies, novel magnetic microspheres with high magnetization and specific affinity toward LZM were prepared, using Tris as the affinity ligand to functionalize magnetic microspheres synthesized by solvothermal method. The effect of pH and initial LZM concentration on the binding capacity, the repeatability and reproducibility of Tris-modified magnetic microspheres are investigated. Subse-

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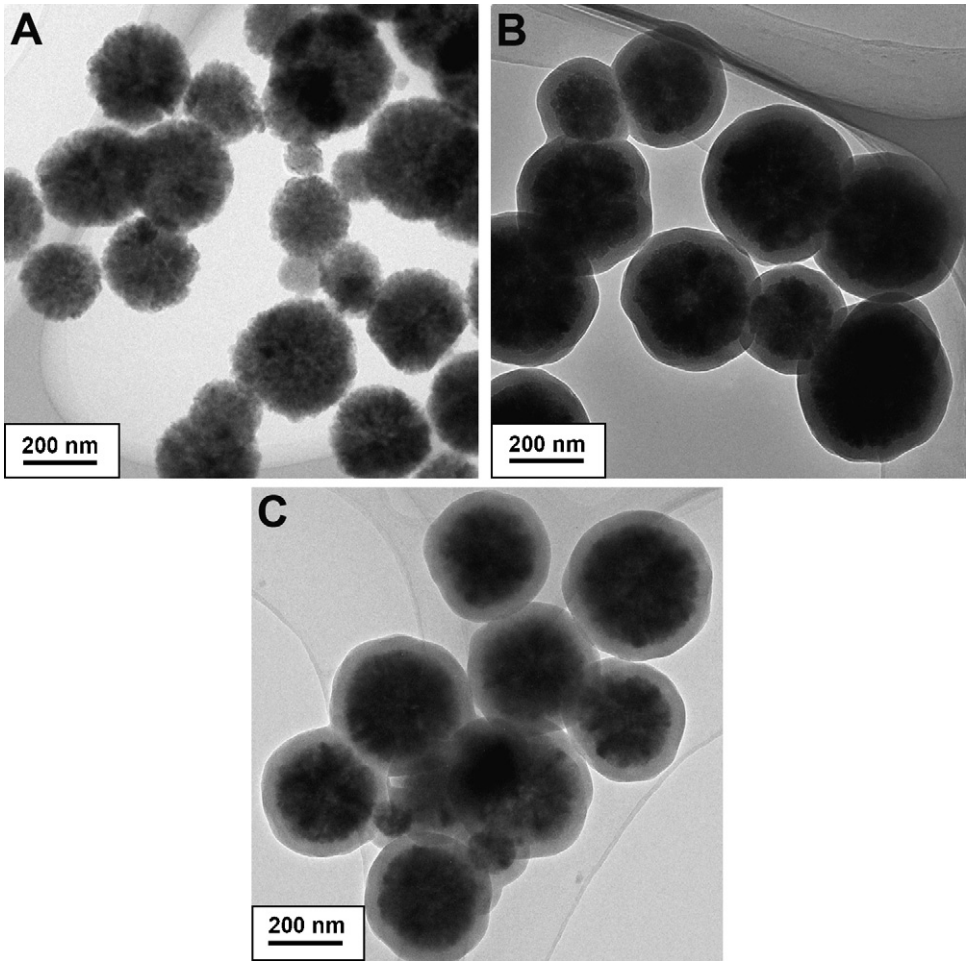


Fig. 1. TEM images of (A) Fe_3O_4, (B) Fe_3O_4@SiO_2 and (C) Fe_3O_4@SiO_2@GPS@Tris.

quently, such microspheres were used to purify LZM from chicken egg white.

2. Experimental

2.1. Chemicals

Tris(hydroxylmethyl)aminomethane (Tris) and protein marker were bought from Amresco (Solon, OH, USA). Chicken egg white lysozyme, oval albumin (OVA) and 3-glycidoxypropyltrimethoxysilane (GPS) were obtained from Sigma (St. Louis, MO, USA). $FeCl_3{\cdot}6H_2O$ was bought from Shanghai Chemical Reagents Company (Shanghai, China). Tetraethoxysilane (TEOS), $NH_3{\cdot}H_2O$ (25 wt%), methanol, ethanol, toluene and glycol were purchased from Beijing Chemicals Plant (Beijing, China). The LZM activity assay kit was acquired from Jiancheng Institute of Bioengineering (Nanjing, China). All other chemicals were of analytical grade, and deionized water was used for aqueous solution preparation.

2.2. Preparation of submicron-size Fe_3O_4 particles

Submicron-size Fe_3O_4 particles were prepared by a solvothermal method [18]. In a typical synthesis, 1.35 g of $FeCl_3{\cdot}6H_2O$ was added into 50 mL of glycol, and the mixture was stirred vigorously to acquire a transparent solution. The solution was sealed in a stainless-steel autoclave and heated at 200 °C for 8 h. This reaction led to the formation of submicron-size Fe_3O_4particles. Fe_3O_4microspheres were separated with a magnet and washed 3 times with ethanol and hot water, sequentially. The obtained Fe_3O_4microspheres were dried at 40 °C under vacuum for 24 h.

2.3. Preparation of Tris-modified Fe_3O_4microspheres

100 mg of submicro-size Fe_3O_4particles were firstly treated by 100 mL of 2 M HCl aqueous solution under ultrasonic vibration for 5 min, and then washed with deionized water. The microspheres were transferred into a solution consisting of 200 mL ethanol, 50 mL deionized water and 10 mL concentrated ammonia (25 wt%). A stable dispersion was obtained after ultrasonic vibration for 20 min. Subsequently, 0.3 mL of TEOS was added and the reaction mixture was stirred for 8 h at 40 °C. The resulted silica-coated magnetic particles with core-shell structure were expressed as Fe_3O_4@SiO_2.

0.2 g of dried Fe_3O_4@SiO_2 was redispersed into a mixture of 30 mL of anhydrous toluene and 1.5 mL GPS. This suspension was refluxed for 8 h to synthesize particles Fe_3O_4@SiO_2@GPS. The obtained microspheres were separated from the mixture, and washed with methanol for three times. Then the epoxy-activated Fe_3O_4@SiO_2@GPS reacted with 0.2 g Tris in 10 mL of potassium phosphate buffer (2.5 M, pH 7.9) at 60 °C for 48 h. Finally, 10 mL of Tris-HCl (1.0 M, pH 8.0) was used to block the residual reacting sites for 3 h, the obtained Tris-modified magnetic microspheres were denoted as Fe_3O_4@SiO_2@GPS@Tris.

2.4. Characterization of magnetic microspheres

The morphology and sizes of Fe_3O_4, Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2@GPS@Tris were observed by Tecnai F30 high resolution transmission electron microscopy (TEM) (FEI, Holland). The saturation magnetization curves were measured with a MPMS XL5 SQUID system (Quantum Design, San Diego, USA). Power X-ray diffraction (XRD) patterns were recorded on a D/max-2400 X-ray diffractometer (Rigaku, Japan).

2.5. Effect of initial LZM concentration on amount of bound LZM

The effect of initial LZM concentration on binding capacity of Tris-modified magnetic microspheres was studied in 0.1 M potassium phosphate buffer with pH 7.0. 5.0 mg of Fe_3O_4@SiO_2@GPS@Tris was added to 3 mL LZM solutions with different initial concentration of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL, respectively. After vibrated for 1 h, microspheres loaded with LZM were collected from the solutions by a magnet. The concentration of LZM was measured spectrophotometrically at 280 nm using a U-3010 UV–vis spectrophotometer (Hitachi, Tokyo, Japan). The amount of bound LZM was calculated by subtracting the concentration of LZM in the supernatant from the initial concentration.

2.6. Effect of pH on amount of bound LZM

The effect of pH on amount of LZM bound on Tris-modified magnetic microspheres was investigated in acetate buffer (0.1 M, pH 5.0) and phosphate buffer (0.1 M, pH 6.0–8.0). 5.0 mg of Fe_3O_4@SiO_2@GPS@Tris was added to 3 mL of 0.4 mg/mL LZM with different pH. The same procedure as that in Section 2.5 was followed to obtain the binding capacity.

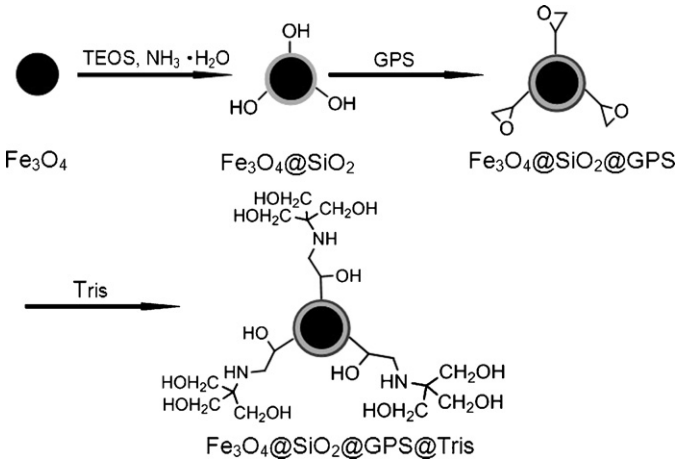
2.7. Purification of LZM from chicken egg white

Chicken egg white was separated from fresh egg and diluted by 2-fold with phosphate buffer containing 0.1 M NaCl (0.1 M, pH 7.0). The diluted egg white was homogenized in an ice bath and centrifuged at 4 °C, 10,000 rpm for 30 min. 30 mg of Tris-modified magnetic microspheres was incubated with 5 mL of diluted egg white for 20 min under vibration. The magnetic microspheres were collected with a magnet from the suspension and washed to remove unbound proteins. Subsequently, 1 mL of phosphate buffer containing 0.6 M NaCl (0.1 M, pH 7.0) was used to desorb the LZM from the microspheres.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using 15% acrylamide separation gel and 5% stacking gel to determine the purity of the separated LZM, with a Biorad Mini Protein II system (Richmond, CA, USA) with a constant current of 8 mA. The desorbed LZM from the microspheres was desalted and 10-fold concentrated by ultrafiltration membrane (molecular weight cut-off 3000) before electrophoresis. 0.05% Coomassie Blue R250 was used to stain the separated bands and protein markers from 97.4 to 14.4 kDa.

2.8. Activity assays of purified LZM

The activity assays of standard and purified LZM from chicken egg white were performed using the commercial reagent kit based on Shugar method [19]. The solvent offered in the reagent kit was used to dilute the substrate solution containing 5 mg of *Micrococcus luteus* to 20 mL. Subsequently, 0.5 mL of 5 μg/mL LZM was added into a 2.5 mL substrate solution. After vigorous vibration for several seconds, the decrease of absorbance at 450 nm was immediately recorded for 4 min. And the curve of decrease of the absorbance of the *Micrococcus luteus* solution at 450 nm versus time was recorded via adding 0.5 mL of 50 μg/mL LZM to a 2.5 mL substrate solution.



Scheme 1. The procedures for preparing Tris-modified magnetic microspheres.

3. Results and discussion

3.1. Preparation of Tris-modified magnetic microspheres

The procedures for preparing Tris-modified magnetic microspheres, Fe_3O_4@SiO_2@GPS@Tris, were shown in Scheme 1.

The preparation of Fe_3O_4 particles through solvothermal method is realized by reduction reactions between $FeCl_3$ and ethylene glycol [18] with modification. To improve the stability and dispersion of Fe_3O_4 microspheres, the Stöber and Fink method [20] was applied to coat the Fe_3O_4 microspheres with a SiO_2 shell through the hydrolysis and condensation of TEOS under basic condition. After the reaction was finished, composite magnetic microspheres designated as Fe_3O_4@SiO_2 with core-shell structure was obtained. This uniform inert silica coating on the surface of magnetite microspheres prevents their aggregation in liquid medium, improves their chemical stability, and provides better protection against toxicity. Additionally, the silica layer facilitates further functionalization due to the presence of surface silanol groups that can easily attach specific ligands to these magnetic microspheres. In this study, GPS was used as a coupling agent to react with Fe_3O_4@SiO_2 to prepare Fe_3O_4@SiO_2@GPS, whose surface possessed epoxy groups.

Chemical activity of epoxy groups enables easy attachment of specific ligands. The flexible chains of GPS provide suitable length of the binding arm, which allows the immobilized ligands to circumrotate freely to make it easy for the biomacromolecules to have access to specific binding sites on the microsphere surface. Through the formation of covalent bond between Tris and epoxy groups of the Fe_3O_4@SiO_2@GPS microspheres, Tris as ligand was immobilized on the surface of the Fe_3O_4@SiO_2@GPS, and affinity magnetic microspheres, Fe_3O_4@SiO_2@GPS@Tris, were obtained. Tris–HCl buffer (1.0 M, pH 8.0) as capping agent was used to block the residual epoxy groups reacting sites in order to minimize the nonspecific binding of coexisting proteins.

3.2. Characterization of magnetic microspheres

The transmission electronic microscopy images showed in Fig. 1 that all of microspheres were spherical and Fe_3O_4@SiO_2, Fe_3O_4@SiO_2@GPS@Tris possessed obvious core-shell structure and that the shells were uniform in thickness (Fig. 1B and C). The diame-

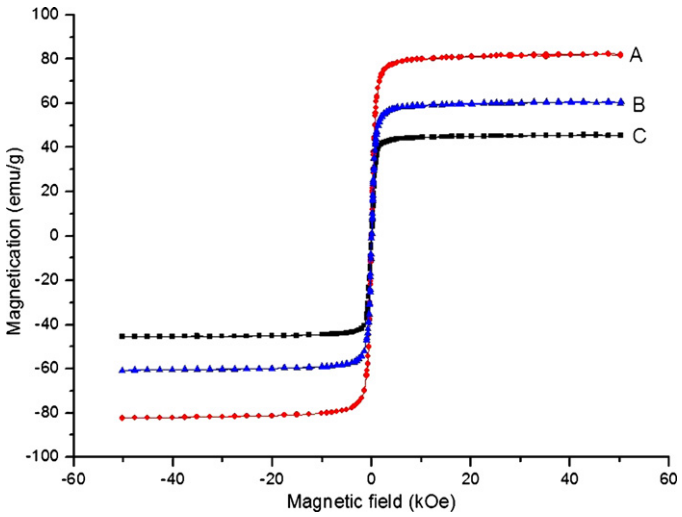


Fig. 2. Magnetization curves of (A) Fe_3O_4, (B) Fe_3O_4@SiO_2 and (C) Fe_3O_4@SiO_2@GPS@Tris.

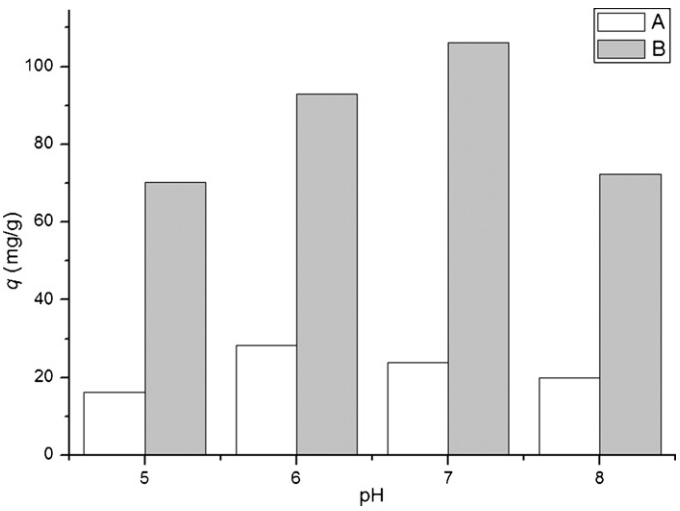


Fig. 4. Effect of pH on binding amount of (A) Fe_3O_4@SiO_2 and (B) Fe_3O_4@SiO_2@GPS@Tris.

ters of Fe_3O_4@SiO_2@GPS@Tris ranged from 350 nm to 430 nm with the thickness of the shell about 40 nm.

The magnetic properties of magnetic microspheres were analyzed by a magnetic Quantum Design MPMS XL5 SQUID system. The saturation magnetic moments of Fe_3O_4, Fe_3O_4@SiO_2, and Fe_3O_4@SiO_2@GPS@Tris, as shown in Fig. 2 were 82.4, 60.7 and 45.9 emu/g, respectively, which suggested that the prepared magnetic microspheres possessed high magnetization. When external magnetic field was removed, there was no hysteresis for all the three kinds of microspheres, demonstrating that such microspheres were superparamagnetic. High magnetization means quick response to external magnetic field, thus a better performance in separation. And superparamagnetism can make magnetic microspheres form a stable, homogeneous suspension in liquid media, which is vital for the application of magnetic microspheres in bioseparation.

XRD was performed to obtain the crystalline structure of Fe_3O_4 and Fe_3O_4@SiO_2 (Fig. 3). The X-ray diffraction pattern for the standard Fe_3O_4 crystal has six diffraction peaks, (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1) and (4 4 0). The XRD patterns of prepared microspheres agreed well with that of the standard magnetite, which demonstrated that the magnetic particles coated with SiO_2 shell

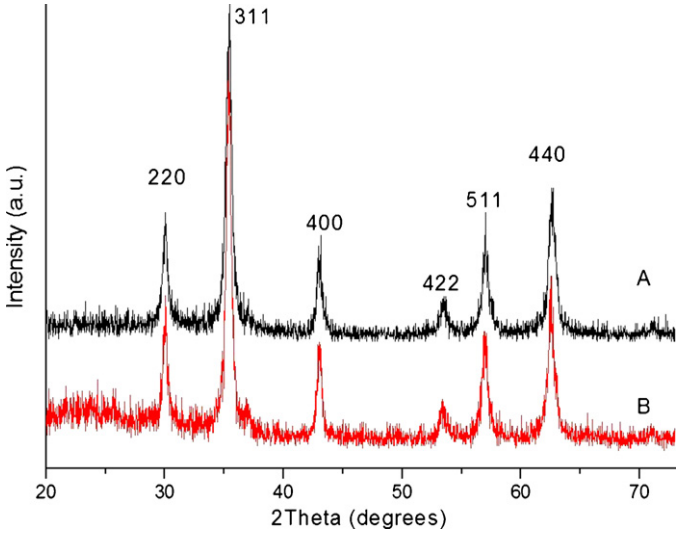


Fig. 3. XRD diffraction patterns of (A) Fe_3O_4 and (B) Fe_3O_4@SiO_2.

were also magnetite and the process of preparation SiO_2 shell did not affect the crystal structure of magnetite core.

3.3. Effect of pH on the amount of LZM bound on magnetic microspheres

pH had a significant effect on the binding amount of such prepared magnetic microspheres. The binding performances of Tris-modified and control magnetic microspheres were studied with batch adsorption experiments in the pH range of 5.0–8.0. The amount of bound LZM was calculated according to the following equation:

$$q = \frac{(C_0 - C)V}{W}$$

where q (mg/g) is the amount LZM bound on a unit mass of the magnetic microspheres, C_0 (mg/mL) and C (mg/mL) are the concentrations of LZM in the initial solution and in the supernatant after magnetic separation, respectively, V (mL) is the volume of the solution, W (g) is the mass of the magnetic microspheres used. The results are presented in Fig. 4.

The results showed that the maximum binding amount was 106.3 mg/g at pH 7.0. In the control experiment, the binding amount was only 23.8 mg/g at the same pH. Therefore, the following experiments were performed at pH 7.0. After the affinity ligand Tris was grafted onto the surface of magnetic microspheres, the binding amount of LZM increased significantly.

3.4. Effect of LZM concentration on binding amount

The effect of the initial concentration on the binding capacity of LZM on Tris-modified magnetic microspheres was investigated in the concentration range of 0.1–0.5 mg/mL (Fig. 5). When the concentration of initial LZM was below 0.3 mg/mL, the amount of bound LZM increased as the initial concentration of LZM increased. The isotherm curve reached a platform in the concentration range of 0.3–0.5 mg/mL, which meant saturation of the active bonding sites on the magnetic beads. The maximum binding capacity is about 108.6 mg/g, which is good enough for practical application.

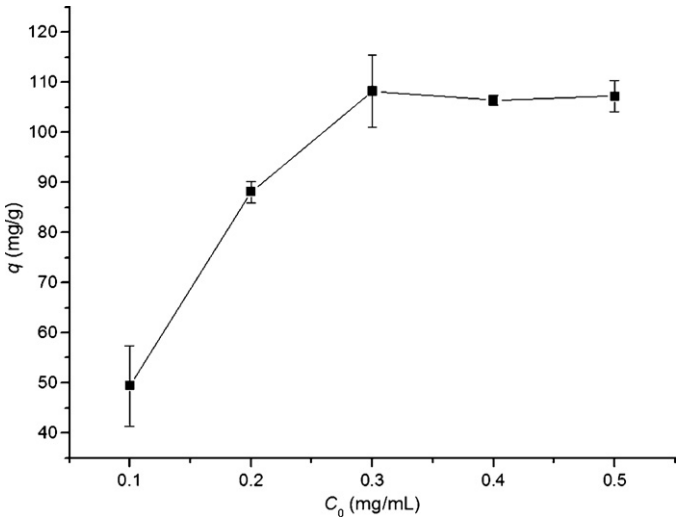


Fig. 5. Effect of initial concentration of LZM on binding amount.

3.5. Repeatability and reproducibility of the Tris-modified magnetic microspheres

The repeatability and reproducibility of the Tris-modified magnetic microspheres were also investigated by using three batches of the functionalized magnetic microspheres. 5 mg of Tris-modified magnetic microspheres was incubated in 0.4 mg/mL LZM solution in 3 mL phosphate buffer (0.1 M, pH 7.0). The results are listed in Table 1. The average of three replicates was used for each binding amount with the relative standard deviation (RSD). The RSD of each batch of Tris-magnetic microspheres was 7.1%, 11.2% and 9.8%, respectively. And the RSD of three different batches of microspheres was 6.7%. The reproducibility and the repeatability were satisfactory.

3.6. Purification of LZM from chicken egg white

In order to investigate the practical application of the Tris-modified magnetic microspheres, it was applied to extract LZM from chicken egg white. The purity of the LZM released from the Tris-modified magnetic microspheres was determined by SDS-PAGE. The electropherogram (Fig. 6) showed clearly that only one band belonging to LZM was detected, which meant that Tris-modified magnetic microspheres possessed specific binding ability toward LZM. The extracted LZM had similar purity with the commercial standard LZM.

3.7. Activity assays of purified LZM

Purity and activity of the target molecules are important to evaluate purification method for biomolecules. The activity of LZM was determined via Shugar method [19]. The activity of LZM was defined as the quantity that makes the absorbance of a certain concentration of *Micrococcus luteus* solution decreases 0.001/min at 450 nm. The activity was worked out according to the following

Table 1
Repeatability and reproducibility of the Tris-modified magnetic microspheres for LZM.

	Batch			Average
	1	2	3	
LZM binding amount (mg/g)	106.9	102.4	116.6	108.6
RSD (%, $n = 3$)	7.1	11.2	9.8	6.7

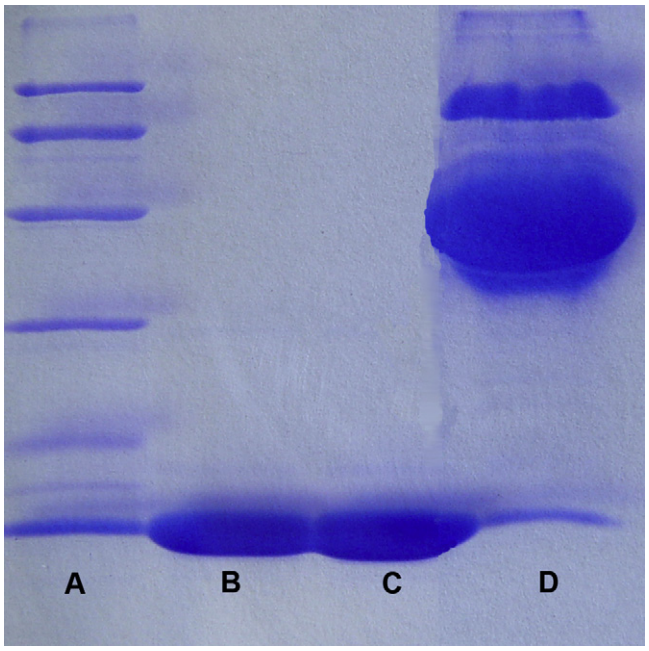


Fig. 6. SDS-PAGE analysis of (A) protein mark, (B) LZM purified from chicken egg white, (C) standard LZM, and (D) chicken egg white.

equation:

$$U = \frac{A_{450}}{0.001 \times m_{LZM}}$$

where U (U/mg) is the activity units contained in 1 mg LZM, A_{450} is the decrease of absorbance per minute, and m_{LZM} (mg) the mass of LZM added in the reaction solution. The values of the activities of purified and standard LZM were 8140 and 8400 U/mg, respectively. The decrease of absorbance of *Micrococcus luteus* solution was shown in Fig. 7. It was clear that the activity of purified LZM was similar to that of the standard, which meant that purified LZM's activity was well-maintained. The Tris-modified magnetic microspheres are excellent magnetic carrier for LZM separation and purification.

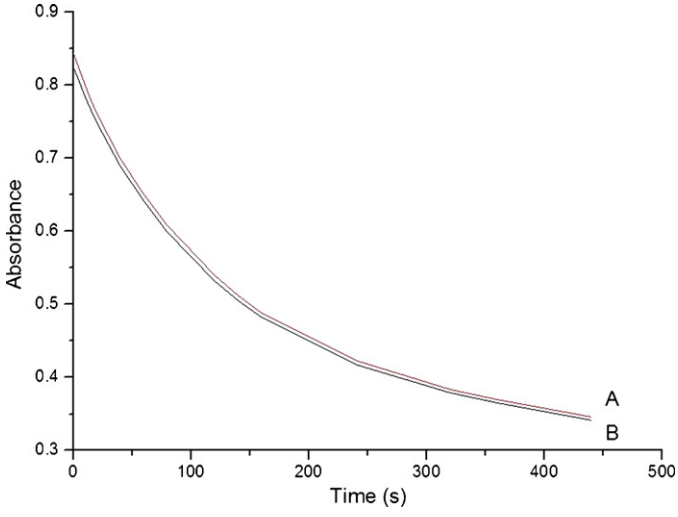


Fig. 7. Activity of (A) LZM purified from chicken egg white and (B) LZM standard.

4. Conclusion

A rapid affinity purification method for LZM based on Tris-modified magnetic microspheres was reported. This new functionalized magnetic microspheres had high specificity toward LZM with satisfactory repeatability and reproducibility, and were successfully used to purify LZM from chicken egg white. The separated LZM had high purity and well-maintained activity. This work provided a simple, inexpensive, and specific LZM purification strategy, and extended the application of functionalized magnetic microspheres in protein separation and purification.

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