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Separation of lysozyme from salted duck egg white by affinity precipitation using pH-responsive polymer with an L-thyroxin ligand

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ABSTRACT

Lysozyme could be efficiently purified using affinity precipitation by using a pH-responsive polymer P_{MMDN} with L-thyroxin as affinity ligand. A pH-responsive polymer P_{MMDN} was polymerized and subsequently coupled with L-thyroxin as the ligand. The pI of the affinity polymer was 4.65 and the recovery was 96.7% of its original amount after recycling three times. The Optimal adsorption condition was 0.02 M phosphate buffer (pH 5.5) with 1.0 mol/L NaCl, and the adsorption isotherm showed the maximum adsorption capacity as 22.76 mg/g polymer, the dissociation constant as 0.085 mg/ml, and the label-free detection data analyzed by ForteBio's Octet also verified the results. The recovery of total lysozyme by elution with 0.2 mol/L Gly-NaOH buffer (pH 10.0), and the maximum elution recoveries were 94.32% (protein) and 96.79% (activity). The surface morphologies of the samples in the whole process of affinity precipitation were obtained by scanning electron microscope (SEM).

1. Introduction

The salted duck egg is one of the most traditional and popular egg products. The salted duck egg yolks are much more demanded, while the salted duck egg white is by-product of manufacturing salted duck egg yolk. More than ten thousands tons of salted egg white is produced from salted duck egg yolk process in China. At present, salted duck egg white is wasted because of the high salt concentration in the egg white [1]. There are nearly 40 different proteins found in egg white, contributing to 10–15% of its total mass. The major proteins include ovalbumin, ovotransferrin, ovomucoid and ovomucin andlysozyme [2,3]. Lysozyme is one of the most important egg white proteins for pharmaceutical, biotechnology and food applications, which is consisting of a single polypeptide chain containing 129 amino acids with the ability to break down bacterial cell walls. It has a molecular weight of 14.3 kDa and an isoelectric point value of 10.7. As lysozyme is a small protein with a unique pI and has a well-studied structure, it is also widely used as a model protein for the characterization of systems [4].

The methods of ultrafiltration [5], ion exchange chromatography [6,7], membrane chromatography [8,9], precipitation [10] and affinity chromatography [11,12] have been reported to separate lysozyme from egg white. Although these methods are effective, they are usually complicated, expensive and time-consuming especially in purification of lysozyme from salt duck egg white in large volume.

Affinity precipitation could be achieved by using reversibly soluble-insoluble polymers coupled with an affinity ligand to purify proteins from dilute solution with large volume of material. The applications of the technology depend upon the design of efficient synthetic soluble-insoluble polymers, such as pH-, temperature- and light-responsive polymers. Since late 1960s, affinity purification methods have been developed and continuously improved. Affinity precipitation had been reported to purify lysozyme. Roy et al. [13] used metal-affinity precipitation to purify lysozyme from hen's-egg-white proteins. Shen and Cao [14] synthesized thermosensitive polyacrylamide derivatives for affinity precipitation of lysozyme in our group, and the purification yield was 80.0%. Dyes [11,15,16] and metal ions ligand [17,18] were generally used as affinity ligand to separate lysozyme from egg white. L-thyroxin was first reported as an affinity ligand by Ding and Cao [19] to purify human serum albumin. Compared with dye or metal ion ligands, it is a relatively safe affinity ligand. According to Litwack and Sears [20], L-thyroxin has strong interactions with lysozyme. The outer ring of L-thyroxin allows the phenolic hydroxyl to form hydrogen bonds with the side chains of lysozyme and the inner ring forms van der Waals contact with it. We thus speculated that L-thyroxin could be used as a novel affinity ligand for lysozyme purification.

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In this study, L-thyroxin was immobilized as an affinity ligand on a pH-responsive polymer comprising MMA, MAA, DMAEMA and N-MAM monomers to form an affinity polymer, which was used for lysozyme separation by affinity precipitation. The adsorption and elution of lysozyme from the affinity polymer were investigated and several interesting results were obtained. This paper is the first to report lysozyme purification by affinity precipitation with a pH-responsive polymer with L-thyroxin as a new ligand. Using this affinity polymer, lysozyme was purified from salted duck egg white to high purity in a single step.

2. Materials and methods

2.1. Materials

Azobisisobutyronitrile (AIBN), methyl acrylic acid (MAA), methyl methacrylate (MMA), methacrylic acid 2-(dimethylamino) ethylester (DMAEMA), N-Methylolacrylamide (N-MAM), were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). L-thyroxin and pure Lysozyme were purchased from Sigma. Salted duck eggs with the weight range of 65–75 g were bought from the supermarket. Other chemicals were of analytical reagent grade.

2.2. Methods

2.2.1. P_{MMDN} polymer polymerization

The synthesis procedure followed Ding's method [21]. The specified amount of four monomers MAA, MMA, DMAEMA and N-MAM, and AIBN as polymerization initiator and ethanol as solvent were poured into a flask with nitrogen maintained for 15 min. The reaction was carried out for 24 h at 60 °C bath and the polymer was obtained from the reaction solution. The polymer was washed three times with acetone and absolute ethanol to remove unreacted monomers. In the end, the polymer was dried under vacuum condition.

2.2.2. Immobilization of ligand on P_{MMDN} polymer [19]

Various amount of epichlorohydrine (ECH) and 1 g P_{MMDN} were dissolved in 100 ml NaOH (1 mol/L) in the conical flask, and the reaction was carried out for 2 h at 40 °C bath. The hydroxyl groups on the polymer were activated by ECH. L-thyroxin of different amount was added into the flask for the subsequent reaction. The reaction of binding L-thyroxin to the polymer through ECH was carried out for 24 h at 60 °C bath. After the reaction completed, HCl (1 mol/L) was used to adjust the pH of the solution to pI to precipitate the polymer. P_{MMDN}-T was obtained by filtration and vacuum drying. All the reaction solution was collected for determination of ligand density. The reaction formula of P_{MMDN} and immobilization of ligand on P_{MMDN} polymer was shown in Fig. 1.

2.2.3. Affinity precipitation of lysozyme

2.2.3.1. Adsorption of lysozyme. P_{MMDN}-T was dissolved in aqueous solution up to 8.0% (wt/vol). Meanwhile 2.5 ml lysozyme solution of varied concentration was mixed with 2.5 ml above P_{MMDN}-T solution together. All the samples were kept for 2.0 h at 25.0 °C with in a rotation shaker 50 rpm. At last, the complex precipitate was collected by adjusting the pH to pI by HCl (0.1 mol/L) and centrifugation at 4000 rpm at 25.0 °C for 10 min. The adsorption capacity of P_{MMDN}-T was investigated by varying the time, pH (3.0–8.0 buffers), ionic strength (0.0–1.0 mol/L NaCl) and ligand density (20.0–80.0 μmol/g). All tests were repeated in triplicate.

2.2.3.2. Desorption. The collected precipitate was dissolved in 5.0 ml various eluant and precipitated again in the same method as the adsorption step. If necessary, this step of desorption could be repeated again with different elution solutions. The target enzyme was obtained, and then the activity and protein amount of lysozyme were measured.

2.2.3.3. Recycle of affinity polymer. After desorption of the lysozyme finished, the affinity polymer was recovered and regenerated with the suitable adsorption solution and eluent. The regenerated polymer was reused in the next cycle of purification experiments.

2.2.4. Affinity precipitation of lysozyme from salted duck egg white [11]

Salted duck egg white was 10-fold diluted with 0.02 mol/L pH 7.0 phosphate buffer followed by gently stirring to form a homogeneous solution. Then, the supernatant was collected by centrifugation at 8000 rpm for 20 min at 4 °C. The lysozyme was separated from the supernatant by P_{MMDN}-T by the optimal adsorption and desorption conditions above. The components of proteins in the source salted duck egg white solution and the desorbed solution were examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

2.2.5. Analytic methods

2.2.5.1. Fourier transform infrared spectra. Fourier transform infrared(FT-IR) spectra were recorded on a Nicolet-6700 FT-IR spectrophotometer (Thremo, USA) from 400 to 4000 cm^{-1} and P_{MMDN} and P_{MMDN}-T were prepared by the KBr pellet methods.

2.2.5.2. X-ray photoelectron spectra. The X-ray photoelectron spectra (XPS, Thermo Scientific, ESCALAB 250Xi) were used to determinate the elements of P_{MMDN} and P_{MMDN}-T.

2.2.5.3. Test of the isoelectric point (pI) and recovery of polymer. Determination of zeta potential was used to detect the isoelectric point (pI) of the polymer. Different pH values of the polymer solution (10 ppm) were adjusted and then the zeta potentials were measured using Zetasizer Nano ZEN3600 (Malvern, UK). The pH-responsive polymer could be precipitated and dissolved by adjusting pH of the polymer solution and the recovery was determined by weight of the polymer after pH-precipitation. The recovery was calculated as the ratio of the dried weight of the precipitated polymer to the initial weight. 1.0 g polymer was dissolved in a centrifuge tube, and then HCl (0.1 mol/L) was added slowly to adjust pH of the solution to pI. The precipitate was separated from solution by centrifugation at 4000 rpm for 15 min and dried to constant mass. The recovery of the polymer was measured in three trials.

2.2.5.4. Determination of ligand density by high-performance liquid chromatography. The high-performance liquid chromatographic (HPLC) system consisted of two LC-20 AD pumps and a SPD-20 A ultraviolet detector (Shimadzu, Japan). Separations were achieved on an analytical reversed-phase ZORBAX C18 column (4.6 × 150 mm) at a flow rate of 1.0 ml/min at room temperature. The UV detector was operated at 212 nm. The two mobile phases used were as follows: phase A: 0.1% phosphoric acid in methanol; phase B: 0.1% phosphoric acid in water.

2.2.5.5. CD spectra assay. CD spectral data can be used to reveal secondary structure information for a protein to monitor conformational changes. This method [22] was used to analyze the interactions between ligand and lysozyme.

2.2.5.6. ForteBio Octet system assay. The interaction between different ligand and lysozyme was tested by the ForteBio Octet System (ForteBio Inc., USA). The P_{MMDN}-T was immobilized onto the biosensors, and then the processes of adsorption and desorption of