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# Highly efficient and low-cost purification of lysozyme: A novel tris(hydroxymethyl)aminomethane immobilized affinity column

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ABSTRACT

A highly efficient and low-cost affinity chromatography strategy for lysozyme (LZM) purification is reported. Using tris(hydroxymethyl)aminomethane (Tris) as ligand and macroporous silica spheres as matrix, a novel affinity column was prepared. The high specificity, stability and repeatability of this Tris immobilized affinity column were proved by LZM separations from protein mixture solutions for 20 circles and 6 months test. LZM purified from chicken egg white on the Tris affinity column had even higher purity than the commercial standard and well-maintained activity of 8287 U/mg (activity of commercial LZM was 8171 U/mg). The efficient affinity process avoiding expensive or fragile ligand would bring advantages to the routine production of LZM from chicken egg white.



## 1. Introduction

Lysozyme (LZM), an enzyme that hydrolyzes polysaccharide chains, lyses certain bacteria by hydrolyzing the β-linkages between the muramic acid and *N*-acetylglucosamine of the mucopolysac-charides which are present in the bacterial cell wall [1]. Nowadays lysozyme is widely used and greatly demanded in clinical medicine, pharmacology, bioengineering and food industry [1,2]. Practically applied LZM is mostly separated from chicken egg white (CEW). However, a large number of interferential proteins make the massive LZM purification and separation from chicken egg white challenging, since the content of LZM is about 3.4% of total protein in chicken egg white [3,4].

High performance affinity chromatography (HPAC) is one of the most effective methods for the purification of biological macromolecules [5–7]. Compared with other techniques for LZM purification, such as ion-exchange process [8], salting out-crystallization [9,10], organic reagent precipitation [11], and ultrafiltration [12], the prominent advantages of HPAC are the continuous online separation, specific selection and real time detection of the target molecules. But currently, in the industrial production of LZM, affinity chromatography is not equally employed as the other techniques. This may be due to the high cost of HPAC. The classical affinity ligands for the target protein, including antibodies [5,13,14], drugs [5], metal ions [15–18] and dyes [19–21], are either expensive or instable. The affinity columns synthesized with the corresponding affinity ligands are therefore high in cost and low in stability and repeatability. These disadvantages limit the application of affinity chromatography in industrial production of some proteins, including LZM. In order to expand the application of affinity chromatography in industrial production of LZM, novel ligands are required for developing low-cost, stable and repeatable affinity columns.

In our recent research, the interaction between tris(hydroxymethyl)aminomethane (Tris) and LZM was proved with a dissociation constant ($K_D$) of $10^{-5}$ M [22]. That strength of specific interaction between ligand and target protein is enough to retain LZM on the Tris immobilized affinity column from other interferential proteins. Meanwhile, the interaction is a weak specific one, which enables the elution of LZM under a mild elution condition maintaining the natural structure of enzyme to avoid the loss of activity. It was revealed that Tris was a good candidate of high affinity ligand to LZM.

In this paper, Tris was immobilized on macroporous silica spheres to obtain an affinity column. The specificity, stability and repeatability of this Tris immobilized affinity column were evaluated with artificial sample and chicken egg white. Then the purity and activity of the LZM separated by the Tris immobilized column was characterized. The stability of the column was also proved by testing the performance in 6 months period. Potential application of the Tris immobilized affinity column in LZM purification from chicken egg white was discussed.

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## 2. Experimental

### 2.1. Materials

Tris was analytical reagent supplied by Amresco (Solon, OH, USA). Chicken egg white lysozyme (LZM), human serum albumin (HSA), oval albumin (OVA), chicken IgG (C IgG), hemoglobin, transferrin, myoglobin, γ-globin, pepsin, trypsin, and RNase A were bought from Sigma (St. Louis, MO, USA). 3-Glycidoxypropyl tremethoxysilane (GPS) was from Aldrich (St. Louis, MO, USA). The LZM activity assay kit was obtained from Jiancheng Institute of Bioengineering (Nanjing, China). Fresh chicken eggs were bought from local market. All other chemicals were of analytical reagent grade, and deionized water was used for aqueous solution preparation. Prior to use, all solutions prepared were filtered through membrane (0.45 μm).

### 2.2. Methods

An Agilent 1100 series liquid chromatography system (Palo Alto, CA, USA) coupled with an analytical workstation was used. The liquid chromatography system consisted of a quaternary solvent delivery system, a manual sampler, and an Agilent 1100 VWD detector.

#### 2.2.1. Salt-induced immobilization of Tris on the silica matrix of HPAC

Two grams of Uetikon (Uetikon, CH) macroporous silica spheres (5 μm particle diameter, 300 Å pore size, 40 $m^2$/g specific surface area) were refluxed with 10% HCl solution for 8 h. Then the acidified silica was washed by deionized water thoroughly and dried. 1.5 mL of GPS was mixed with 30 mL of distilled toluene (dried with anhydrous calcium chloride before used). The toluene solution of silane was added to the silica, and the reaction mixture was refluxed for 8 h. After the reaction, silica was washed with methanol for three times. 0.2 g Tris reacted with epoxy-activated silica 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 (pH 7.9, 1.0 M) was used to block the residual reacting sites for 3 h. 0.8 g modified silica spheres were packed into stainless steel columns (70 mm × 4.6 mm i.d.) under 16 MPa pressure [22].

#### 2.2.2. Affinity chromatography evaluation conditions

The protein solutions were prepared with 50 mM pH 7.0 potassium phosphate buffer containing 100 mM NaCl, and filtered through 0.45 μm membrane. All the protein solutions were prepared with the concentration of each protein of 0.5 mg/mL, respectively. The injection volume was 50 μL with a standard 100-μL loop and a 50-μL syringe. The detection wavelength was 280 nm. 50 mM pH 7.0 potassium phosphate buffer containing 100 mM NaCl was used as loading buffer for all the chromatographic experiments. All experiments were performed at room temperature. After injection of the protein solution, all separations without special description were carried out by rinsing with the loading solution for 8 min, the elution solution (50 mM pH 7.0 potassium phosphate buffer containing 600 mM NaCl) for 12 min, and then loading solution for 10 min, all at a flow rate of 0.5 mL/min.

#### 2.2.3. Separation of LZM from chicken egg white

Chicken egg white (CEW) was separated from fresh eggs and diluted to 5% (v/v) with loading buffer. The diluted CEW was homogenized and centrifuged at 4 °C, at 12,000 rpm for 30 min. The solution was filtered through a 0.45 μm membrane and used as an LZM source. 100 μL of the treated CEW solution was injected into the affinity column. The column was washed with 50 mM pH 7.0 potassium phosphate buffer containing 100 mM NaCl (0–8 min) and 200 mM NaCl (8–12 min). The bound LZM was then eluted with 50 mM pH 7.0 potassium phosphate buffer containing 600 mM NaCl (12–30 min). After elution, the column was equilibrated with the loading buffer to obtain a baseline for the next run.

#### 2.2.4. Purity determination of the separated LZM

Purity determination of LZM separated from CEW was performed on a Bio-Rad electrophoresis system (Richmond, CA, USA) with a Mini-Protein II electrophoresis cell (gel size 7–8 cm) and PAC 300 power. The eluates were desalted and 10-fold concentrated by ultra filtration membrane (molecular weight cut-off 3000) and then 10 μL of each were determined by SDS-PAGE analysis using 12.5% polyacrylamide MiniGels (Bio-Rad, Miniprotein-3). Coomassie Blue R-250 (Amresco, Solon, OH, USA) was used to stain the separated bands and protein markers from 97.4 to 14.4 kg/mol.

#### 2.2.5. Activity assay of the purified LZM

The LZM activity was determined with Shugar method [23] by using a reagent kit. The substrate solution containing 5 mg *Micrococcus luteus* was diluted to 20 mL by the solvent offered in the kit. 0.5 mL LZM solution with a concentration of 5 μg/mL was added into 2.5 mL substrate solution and the decrease of absorbance at 450 nm was immediately detected for 3 min. The curve of lysozyme hydrolyzed the *M. luteus* versus time was monitored by adding 0.5 mL LZM solution at a concentration of 50 μg/mL into 2.5 mL substrate solution and recording the absorbance decrease at 450 nm for 30 min.

## 3. Results and discussion

### 3.1. Preparation of the Tris immobilized affinity column

Macroporous silica spheres were chosen as the packing matrix. The granule diameter of the spheres and the diameter of the macropores provided good hydrodynamics. The compression resistance of the inorganic silica spheres made silica suitable for online analysis and purification using HPLC. The surface area of the macroporous silica spheres (40 $m^2$/g) provided sufficient space of immobilization of Tris ligand and thus ensured the binding capacity of the affinity column.

The surface of macroporous silica spheres was epoxy-functionalized with GPS. Tris was directly linked to the modified silica spheres with the amino group, as a ligand of the affinity chromatography. Also as a classical blocking agent, Tris was used to block the residual activated site on the surface of the silica spheres. Therefore, the proteins injected into the affinity column could only bind to Tris but no other ligand. With the covalent bond between the macroporous silica spheres and Tris, stable immobilization of ligand were achieved, which is profitable for the purification of protein from real sample.

In the synthesis process of the Tris immobilized affinity column, the macroporous silica spheres were epoxy-functionalized with GPS. The suitable length of the binding arm allowed the free circumrotation of the immobilized Tris that decreased the steric hindrance of macromolecules and provided efficient binding to LZM. And the Tris immobilization on the epoxy modified silica could be accomplished in a weak alkaline solution (pH 7.9) that did not disrupt the structure of silica spheres which is very important for providing a good hydrodynamics for HPLC.

The frontal analysis had been carried out in our previous work to determine the apparent dissociation constant between Tris and LZM. According to Fig. 7 [22], the amount of immobilized Tris ligand is about 3.75 μmol/g silica and the binding capacity of the affinity column for LZM is about 2.48 μmol/g silica.

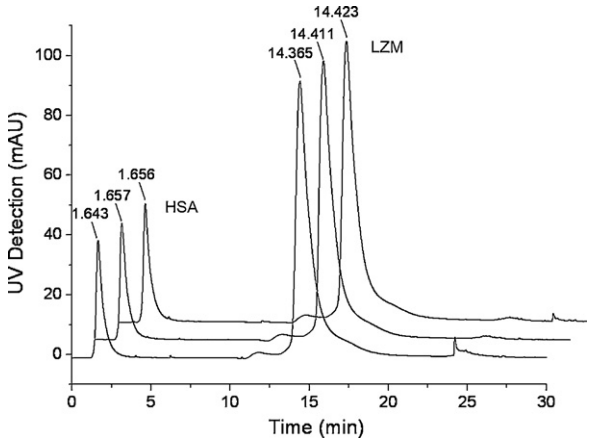


**Fig. 1.** Specificity of the Tris immobilized affinity column for LZM and HSA separation. Chromatographic conditions: Protein concentration, 0.5 mg/mL; injection volume, 50 μL; flow rate, 0.5 mL/min; UV detection, 280 nm; buffer A, 50 mM pH 7.0 potassium phosphate buffer containing 100 mM NaCl; buffer B, 50 mM pH 7.0 potassium phosphate buffer containing 600 mM NaCl; eluted condition, 0–8 min buffer A, 8–20 min buffer B, 20–30 min buffer A.

### 3.2. Evaluation of the Tris immobilized affinity column

#### 3.2.1. The specificity of the Tris immobilized affinity column

The specific binding between LZM and Tris has been proved by QCM screening in our previous work [22]. In present work, the specific separability of the Tris immobilized affinity column was detected by separating the binary mixture of LZM and HSA or the mixture of LZM and 10 interferential proteins (HSA, OVA, C IgG, hemoglobin, transferrin, myoglobin, γ-globin, pepsin, trypsin, and RNase A, concentration of each protein was 0.5 mg/mL, respectively). From the result shown in Fig. 1, the resolution was 11.9 ($n$ = 3, with the relative standard deviation, RSD, of 1.4%) with the retention time of HSA and LZM were 1.65 min ($n$ = 3, RSD = 0.47%) and 14.40 min ($n$ = 3, RSD = 0.21%), respectively. A good baseline separation of HSA and LZM was obtained on the Tris immobilized affinity column. Chromatograms of the 11-protein mixture were shown in Fig. 2. The resolution of LZM and its nearest peak was 13.7 ($n$ = 3, RSD = 0.93%), and the retention time of LZM on the affinity column was 10 min longer than that of interferential proteins. For all these standard samples and artificial samples, the nonspecific adsorption was not observed as the void time is very short. These results

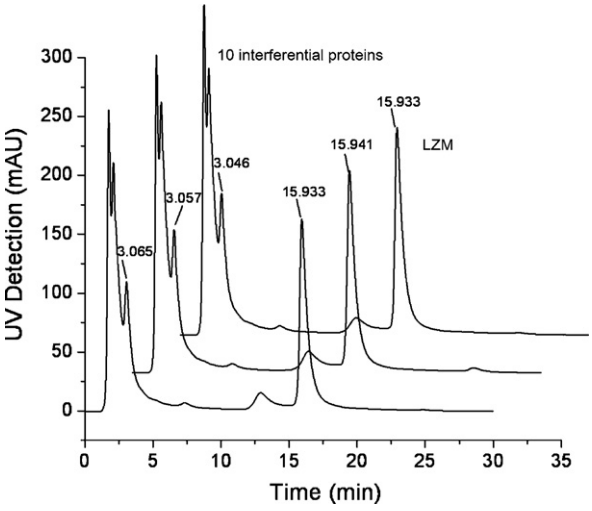


**Fig. 2.** Specificity of the Tris immobilized affinity column for LZM and various proteins mixed solutions. Chromatographic conditions refer to Fig. 1.

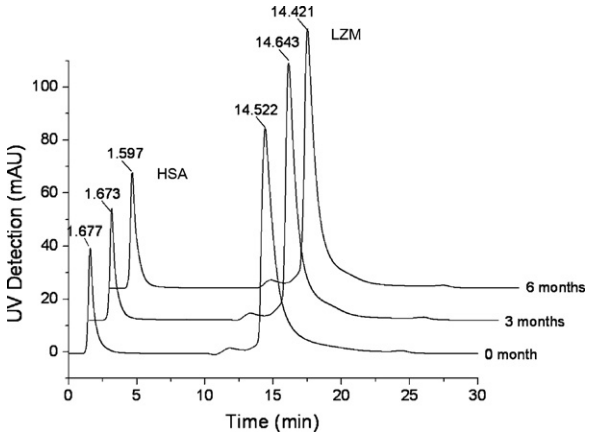


**Fig. 3.** Stability of Tris immobilized affinity column in 6 months on separation of the LZM and HSA mixed solution. Chromatographic conditions refer to Fig. 1.

were good indicators of the excellent specific separability of the Tris immobilized affinity column.

#### 3.2.2. Stability and repeatability of the affinity column

For the sake of the commercial usage, affinity column must be stable and repeatable. The stability of the Tris immobilized affinity column were characterized by separating the artificial sample contained 0.5 mg/mL HSA and 0.5 mg/mL LZM after 3 and 6 months usage of the affinity column. The performance was compared with that tested immediately after the preparation of the column. As shown in Fig. 3 the resolution of HSA and LZM peaks was 12.80 ($n$ = 3, RSD = 5.2%). The retention time of HSA and LZM were 1.65 min ($n$ = 3, RSD = 2.7%) and 14.53 min ($n$ = 3, RSD = 0.77%), respectively. These results indicated that the column did not have any detectable change in the separation of proteins after usage under room temperature for at least 6 months. The Tris immobilized affinity column was highly stable and suitable for routine applications.

The repeatability of the column was characterized by 20 consecutive injection and elution of 50 μL artificial sample mixed with HSA and LZM (0.5 mg/mL, respectively) on the affinity column for 20 times. As shown in Fig. 4 and Table 1, the resolution was 12.14 ($n$ = 20, RSD = 4.5%). The chromatograms and resolution results proved that Tris immobilized affinity column had a good repeatability for at least 20 circles binding and eluting.

The high stability and repeatability of the column could be mainly due to the adoption of the novel ligand, Tris. As a small organic molecule, Tris has a simple and stable chemical structure, which would greatly facilitate the synthesis, usage and storage of

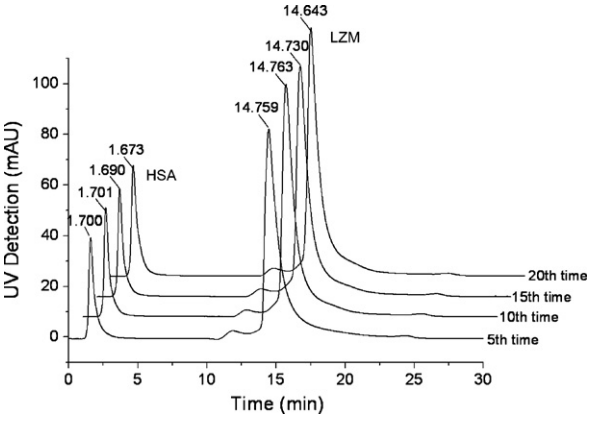


**Fig. 4.** Repeatability of LZM and HSA mixed solution on the Tris immobilized affinity column. Chromatographic conditions refer to Fig. 1.

**Table 1**
Retention time and resolution of LZM and HSA mixed solution (0.5 mg/mL) on the Tris immobilized affinity column for 20 circles. Chromatographic conditions refer to Fig. 1.

| No. | $t_{HSA}$ (min) | $t_{LZM}$ (min) | $R_s$ |
|---|---|---|---|
| 1 | 1.70 | 14.68 | 11.70 |
| 2 | 1.67 | 14.03 | 12.50 |
| 3 | 1.67 | 14.08 | 11.70 |
| 4 | 1.67 | 14.09 | 11.50 |
| 5 | 1.70 | 14.76 | 12.80 |
| 6 | 1.59 | 14.48 | 11.50 |
| 7 | 1.60 | 14.42 | 11.80 |
| 8 | 1.60 | 14.45 | 11.70 |
| 9 | 1.60 | 14.43 | 12.00 |
| 10 | 1.70 | 14.76 | 12.50 |
| 11 | 1.63 | 14.32 | 11.90 |
| 12 | 1.64 | 14.37 | 12.00 |
| 13 | 1.66 | 14.41 | 11.70 |
| 14 | 1.66 | 14.42 | 11.90 |
| 15 | 1.69 | 14.73 | 12.80 |
| 16 | 1.67 | 13.69 | 12.50 |
| 17 | 1.67 | 13.75 | 12.30 |
| 18 | 1.68 | 13.75 | 12.40 |
| 19 | 1.66 | 14.48 | 11.90 |
| 20 | 1.67 | 14.64 | 13.70 |
| Average | 1.66 | 14.34 | 12.14 |
| RSD | 2.1% | 2.3% | 4.5% |

the affinity column. For biomacromolecule ligands such as proteins, the covalent binding would usually induce the deactivation of the ligands. But for small molecule ligand, some strong conditions such as high pH or ionic strength could be used without considering the inactivation of the ligands, which would greatly simplify the design and operation of synthesis process. In our work, the immobilization of Tris was under a high ionic strength in order to form a covalent bond between the epoxy groups on the functional silica sphere matrix and the amino groups of Tris. The covalent binding promised a stable immobilization of the ligands on the matrix.

The affinity column with this small molecule as ligand could also be used and stored without special protection, such as special solution or low temperature. Meanwhile, the regeneration of the column after a purification process could be easily and satisfactorily accomplished by elution with a proper buffer without disrupting the performance of the column. All these merits generating from the stability of the ligand provided high stability and repeatability of the affinity column under the purification condition of LZM and the store condition.

Moreover, compared with the classical ligand, such as antibodies, drugs, metal ions or dyes, Tris is much more economical with a price of less than 100 $/kg.

### 3.3. HPAC purification of LZM from CEW using Tris immobilized column

A rapid and efficient separation and purification of LZM from CEW solution was achieved with the Tris immobilized affinity column. After introducing 100 μL of the pre-treated CEW solution into the affinity column with loading buffer, the column was equilibrated with 50 mM potassium phosphate buffer 200 mM NaCl for 4 min to pre-elute the adsorption of other proteins in CEW. The bound LZM was eluted thereafter with 50 mM pH 7.0 potassium phosphate buffer containing 600 mM NaCl (12–30 min). Potassium phosphate buffer (pH 7.0) was chosen as the eluent because this pH is close to the *in vivo* pH and the activity of LZM may be the least damaged. 600 mM NaCl was adopted because a good separation and purification can be achieved at this ionic strength and the relatively low concentration of salt would help maintain the enzyme activity.

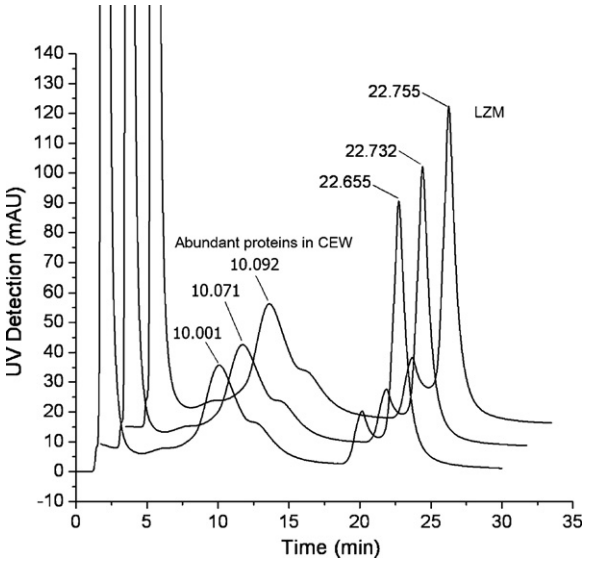


**Fig. 5.** Chromatograms of LZM purified from CEW on Tris immobilized affinity column. Chromatographic conditions: CEW solution (5%, v/v) injected, 100 μL; flow rate, 0.5 mL/min; UV detection, 280 nm; buffer A, 50 mM pH 7.0 potassium phosphate buffer containing 100 mM NaCl; buffer B, 50 mM pH 7.0 potassium phosphate buffer containing 200 mM NaCl; buffer C, 50 mM pH 7.0 potassium phosphate buffer containing 600 mM NaCl; eluted condition, 0–8 min buffer A, 8–12 min buffer B, 12–30 min buffer C.

Profiles of CEW proteins eluted from the column were shown in Fig. 5. The large peak before 15 min was composed of other components (abundant proteins) in CEW, while the retention time of LZM was 22.71 min ($n$ = 3, RSD = 2.3%). Therefore, a good baseline separation of LZM from CEW could be obtained. After the elution of LZM, the signal returned to baseline. These results indicated that the purification of LZM from CEW was satisfactorily performed and the Tris immobilized affinity column could be reused after elution under the condition of separating of LZM from chicken egg white.

### 3.4. Characterization of the LZM purified from CEW

#### 3.4.1. The purity of the LZM

The central fraction of the elution peak was collected, dialyzed, concentrated, and then analyzed by SDS-PAGE. The electropherogram in Fig. 6 showed that eluted fractions had high purity since only one band was detected, while a slight impure band could be observed in the commercial standard LZM. The purity of LZM purified from CEW was even higher.

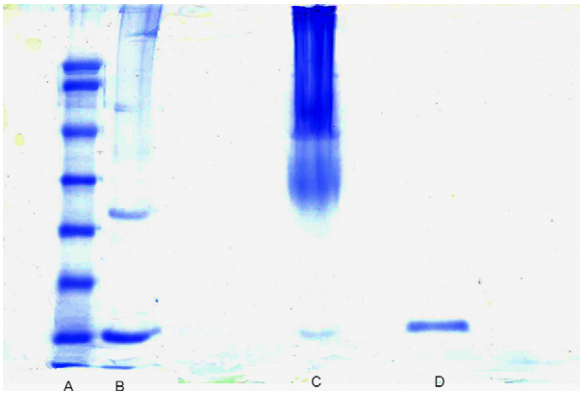


**Fig. 6.** SDS-PAGE analysis of fractions obtained from the purification of LZM from CEW. (A) Protein marker; (B) commercial standard LZM; (C) chicken egg white; D: purified LZM from CEW by using Tris immobilized affinity column.

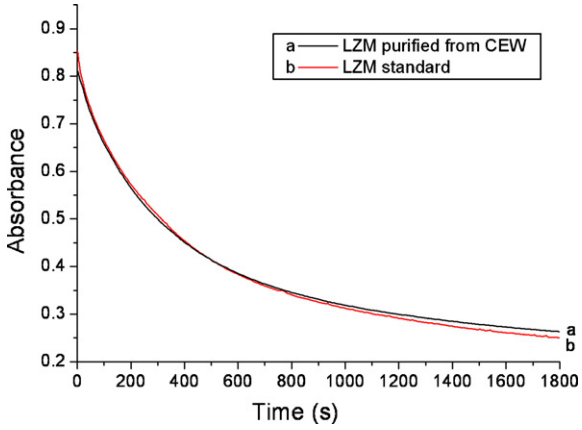


**Fig. 7.** Activity of the LZM purified from CEW by the affinity chromatography.

*3.4.2. The activity of the LZM*

Shugar method [23] was used to detect the activity of LZM. The definition of the LZM activity was the quantity that makes the absorbance of a certain concentration of *M. luteus* solution at 450 nm decreases 0.001/min. The activity was calculated with Eq. (1):

$$U = \frac{A_{450}}{0.001 \times m_{\mathrm{LZM}}} \quad (1)$$

$U$ (U/mg) is the activity units contained in 1 mg LZM, $A_{450}$ is the decrease of absorbance at 450 nm per minute, and $m_{\mathrm{LZM}}$ is the mass of LZM (mg) added in the reaction solution.

The activities of standard and purified LZM were 8171 and 8287 U/mg, respectively. And the curves that LZM destroyed the cell walls of *M. luteus* were shown in Fig. 7. These results showed that the LZM activity was well maintained after the affinity chromatography purification from chicken egg white.

The high efficient and activity maintaining purification of LZM on Tris immobilized affinity chromatography column was resulted from the specific Tris–LZM interactions, which allowed the high specificity and mildness of the affinity column. The apparent dissociation constant of the specific interaction between Tris and LZM was about $10^{-5}$ M [22]. This strength of the specific interaction was enough for LZM purification from a complicated sample matrix on affinity chromatography, and the bound LZM could be eluted under a mild salt concentration. Moreover, the vivo-like property of Tris ligand provided a friendly environment on the affinity column to maintain the structure and activity of proteins.

## 4. Conclusion

Using a novel ligand, Tris, we have developed a highly efficient and low-cost affinity purification strategy for LZM. The Tris immobilized column had preeminent specificity, stability and repeatability. The purified LZM on Tris immobilized affinity column had satisfying activity and purity. The purification process avoiding expensive ligand makes routine LZM production feasible. The adoption of Tris as a novel affinity ligand will make LZM purification much more convenient and cost effective. The development of this affinity chromatography method is also inspirational to discover more simple and inexpensive HPAC ligands.

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