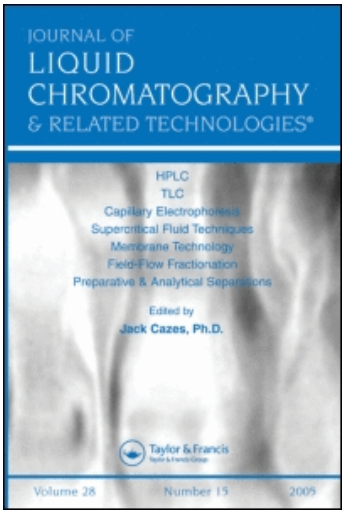

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Abstract: High-speed countercurrent chromatography (HSCCC) was employed for the first time to separate lysozyme from chicken egg-white using a reverse micellar system with 50 mM bis-(2-ethylhexyl) sulfosuccinate sodium (AOT) dissolved in isooctane and aqueous buffer. Lysozyme is separated from other main proteins through one step separation, and relatively high activity recovery (over 80%) can be achieved when applied to the purification of a preseparated sample, while a low recovery problem exists when applied to separate lysozyme from crude chicken egg-white samples due to the interference of a large amount of other proteins. However, this method has advantages over traditional reverse micellar extraction (RME) for its simple continuous elution mode and high separation efficiency, and it is promising for scale-up.

Keywords: High-speed countercurrent chromatography (HSCCC), Lysozyme, Reverse micellar extraction (RME)

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INTRODUCTION

Lysozyme is a commercially valuable enzyme and is currently used as an antimicrobial agent in various foods, either as a preservative or to control microbial processes in cheese, beer, and wine production, and in antibacterial food packaging films.[1] Lysozyme is also used as a cell disrupting agent for extraction of bacterial intracellular products, as a component of ophthalmologic preparations, and as a drug for treatment of ulcers and infections. Lysozyme occurs naturally in chicken egg-white (CEW),[2] which is a mixture of proteins (see Table 1). The low content of lysozyme in CEW (about 3.4%) makes its purification a challenge. The separation and purification of lysozyme from CEW has been traditionally done by a combination of conventional processes, such as crystallization, precipitation, centrifugation, and adsorption,[3] chromatographic methods including ion exchange chromatography, affinity chromatography, etc.,[4–8] ultra-filtration,[9,10] and reverse micellar extraction (RME).[11–16] Among them, RME presents a great potential for the industrial purification of proteins. Reverse micelles are aggregates of surfactant molecules containing polar cores of solubilized water, dispersed in a continuous solvent medium. Traditionally, the RME process includes two procedures: a target protein is selectively solubilized into organic phase (forward extraction) and, subsequently, is stripped into the aqueous phase (back extraction) by the addition of fresh aqueous buffer.

In this paper, high-speed countercurrent chromatography (HSCCC) was employed for the first time to process the reverse micellar extraction of lysozyme from chicken egg-white using a two-phase solvent system of bis-(2-ethylhexyl) sulfosuccinate sodium (AOT) dissolved in isooctane and aqueous buffer. The preliminary results of our studies were reported here.

EXPERIMENTAL

Materials

Chicken eggs were obtained from a market in Beijing. The egg-white was carefully collected after removal of chalazae and freeze dried egg-white

Table 1. Properties of major proteins in chicken egg-white (CEW)

Protein	Total protein (%)	Molecular weight (kDa)	Isoelectric point (p*I*)
Ovalbumin	<54–57	45	4.5
Conalbumin	<12–15	80	6.1
Ovomucin	<3–4	$5.5–8.3 \times 10^3$	4.7
Lysozyme	<3.4	14.3	11.0

powder was prepared. Standard egg-white lysozyme (EC3.2.1.7, 95%), Conalbumin (98%), and Albumin (Grade V), was purchased from Sigma-Aldrich Co. (St. Louis, USA). *Micrococcus lysodeikticus* were obtained from Institute of Microbiology, Chinese Academy of Sciences, Beijing. Bis-(2-ethylhexyl) sulfosuccinate sodium (AOT) was obtained from India Laxa Chemical Reagents Company.

Reagents used for electrophoresis include acrylamide, N′N′-ethylene-bis-acrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate, bromophenol blue, β-mercaptoethanol (Sigma-Aldrich Co., St. Luis, USA).

All other chemicals were of analytical grade; isooctane was from Tianjin Zhiyuan Chemical Reagent Company, and others were from Beijing Chemical Reagent Company.

Apparatus

HSCCC separation was performed with a model GS10A3 multilayer coil planet centrifuge fabricated at Beijing Institute of New Technology Application, Beijing China. The apparatus holds a column holder and a counterweight symmetrically, at a distance of 8 cm from the central axis of the centrifuge. The coil column was prepared from 1.6 mm I.D. PTFE (polytetrafluoroethylene) tubing with a total capacity of 220 mL. Beta values of the coiled column vary from 0.5 at the internal terminal to 0.75 at the outer terminal. The revolution speed is adjustable between 0–1000 rpm with a speed controller.

The UV2300 spectrometer was from Tianmei Instrument Co. (Shanghai, China). DYCZ-28B electrophoresis cell and DYY-6C electrophoresis power supply was from Beijing Liuyi Instrument Factory (Beijing, China).

Traditional RME Procedures

The extraction was carried out in a 1000 mL stoppered glass flask. A 5 g amount of freeze dried egg-white powder was solubilized in 200 mL sodium borate buffer (50 mM, pH = 9.00) containing 0.1 M KCl. The forward extraction was performed by mixing with an equal volume of isooctane containing 50 mM AOT in a shaking incubator for 45 min at 10°C, at a shaking speed 200 rpm. Then the resulting mixture was subjected to centrifugation at 4000 rpm for 20 min, and the upper phase was collected for subsequent backward extraction described below.

An equal volume (200 mL) of the sodium phosphate buffer (50 mM, pH = 11.8) containing 1 M KCl was mixed with the upper phase for 45 min at 30°C in the same manner. After centrifugation, the lower aqueous phase was collected and dialyzed to removed salt, and finally freeze dried.

Totally, 924 mg lysozyme sample was obtained from 15 g egg-white through the above RME procedures.

HSCCC Separation Procedures using RM System

The coiled column was first entirely filled with the stationary organic phase composed of isooctane containing 50 mM AOT, and then the lower aqueous buffer (50 mM sodium borate, pH = 9.00) containing 0.1 M KCl was pumped into the column as a mobile phase at a flow rate of 2 mL/min under 800 rpm. After the mobile phase front emerged, the sample solution (sample dissolved in the same aqueous buffer) was injected through the sample loop to start a forward extraction. After the other proteins were almost flushed out, the mobile phase was changed to another aqueous buffer (50 mM sodium phosphate, pH = 11.00) containing 1 M KCl to start a backward extraction, until the lysozyme peak was eluted out. Peak fractions were collected according to the recorded UV elution profile.

In order to test the feasibility of using HSCCC to process RME, a lysozyme sample derived from traditional RME was further purified through the above HSCCC procedures. Also, a 1 g crude egg-white sample was separated for lysozyme through the same manner.

Scale-Up of HSCCC Separation

In the scale-up separation, the same coiled column was first entirely filled with the same organic phase composed of isooctane containing 50 mM AOT, and then a certain volume of sample solution containing crude egg-white was pumped into the column at a flow rate of 2 mL/min under 800 rpm. Afterwards, another 200 mL of fresh aqueous buffer (50 mM sodium borate, pH = 9.00) containing 0.1 M KCl was pumped into the column to clean the residual of other proteins. Finally, the column was eluted with 50 mM sodium phosphate aqueous buffer (pH = 11.00) containing 1 M KCl until the lysozyme peak was collected.

Crude egg-white sample (3 g/200 mL, 6 g/300 mL, and 8 g/400 mL) were loaded and separated under the same condition as above.

Protein Content Determination

The protein concentration of the samples before and after separation was analyzed by the Bradford dye-binding method.[17] The sample solution was prepared by dissolving 1 mg dry sample into 1 mL pure water. Protein assay dye reagent, 5 mL, (Coomassie Brilliant Blue G 250) was mixed with 0.1 mL of the protein solution. The optical density of the mixture at 595 nm was observed spectrophotometrically to determine the protein concentration using a standard curve obtained by recording the OD_{595nm} of pure bovine serum albumin (BSA) solutions.

Table 2. Analytical results of the samples before and after separation

Samples	Specific activity (U/mg protein)	Purification-fold	Recovery (%)
Lysozyme standard (Sigma)	44300 U/mg protein	—	—
Crude egg-white	3257 U/mg protein	—	—
Lysozyme derived from traditional RME	34049 U/mg protein	10.45	84.2
Lysozyme further purified by HSCCC using RM system	52985 U/mg protein	1.56	82.4
Lysozyme obtained from 1 g egg-white separated direct by HSCCC using RM system	30076 U/mg protein	9.23	13.2
Lysozyme obtained from 3 g egg-white by HSCCC	43093 U/mg protein	18.2	25.8
Lysozyme obtained from 6 g egg-white by HSCCC	31441 U/mg protein	15.8	24.9
Lysozyme obtained from 8 g egg-white by HSCCC	33790 U/mg protein	13.8	17.8

SDS-Page Analysis

Protein profiles were determined by SDS-PAGE which was carried out on 15% separating and 4% stacking gel.[18] The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R 250 in 24% (v/v) ethanol containing 8% glacial acetic acid, and destained with 25% ethanol containing 8% glacial acetic acid.

Lysozyme Activity Determination

The lysozyme activity of the sample solution before and after extraction was determined by turbidimetric assay.[16] *Micrococcus lysodeikticus* cells were suspended in 0.067 M phosphate buffer (pH 6.24), and the rate of decrease in absorbance (450 nm) of cell suspension was used to determine the units of lysozyme activity. The concentration of the cell was adjusted to give an initial absorbance reading at 450 nm of 0.6–0.7. Of the cell suspension, 2.98 mL were mixed with 20 μL of sample and changes of absorbance at

450 nm were determined by spectrophotometer up to 90 s with 10 s intervals. A decrease in absorbance at 450 nm of 0.001/min was taken as one unit of enzyme activity (U) and results were calculated using the following equations:

$$\text{Activity (U/mL)} = (\geq A_{450}/\text{min})/(0.001/\text{min} \times 0.02\,\text{mL})$$

$$\text{Specific activity (U/mg)} = (\text{U/mL})/(\text{protein mg/mL})$$

$$\text{Lysozyme recovery} = \frac{\text{Total activity of lysozyme in the sample after separation}}{\text{Total activity of lysozyme in the sample before separation}} \times 100\%$$

$$\text{Purification (fold)} = \frac{\text{Specific activity of the sample after separation}}{\text{Specific activity of the sample before separation}}$$

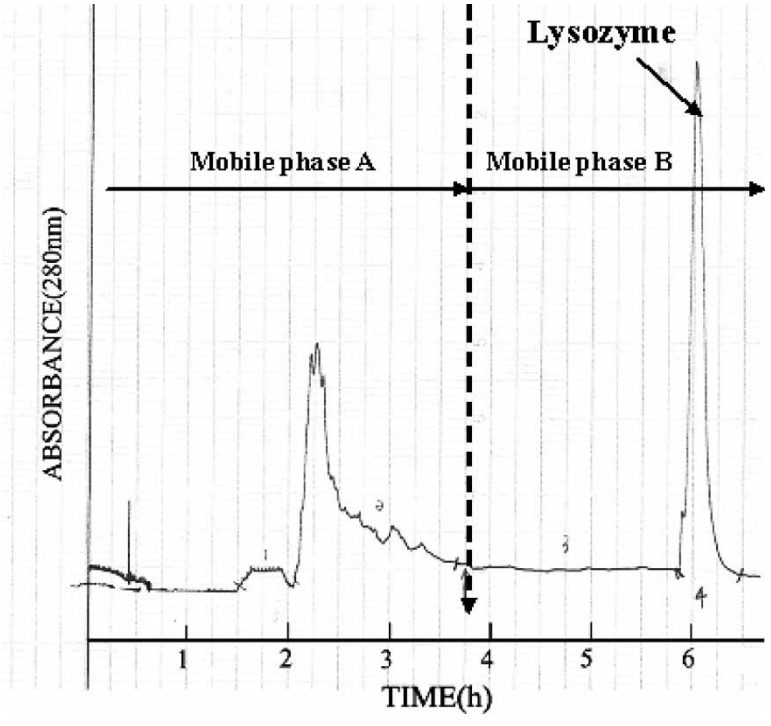

Figure 1. Purification of a lysozyme sample derived from traditional RME through HSCCC using the RM system. Sample weight: 0.9 g/50 mL; Mobile phase: A. Sodium borate buffer (50 mM, pH = 9.00) containing 0.1 M KCl, B. Sodium phosphate buffer (50 mM, pH = 11.8) containing 1 M KCl; Flow rate: 2 mL/min; Detection: 280 nm.

RESULTS AND DISCUSSION

Feasibility of Purification of Lysozyme by HSCCC using RM system

The separation of lysozyme from crude chicken egg-white by traditional RME can yield relatively high activity recovery of lysozyme (84.2%, see Table 2), but with relatively low purity as analyzed by SDS-PAGE (Figure 3). The complicated multiple procedures of traditional RME, including forward extraction, backward extraction, and two-phase separation made us think about the application of HSCCC to fulfill the same task. Thus, a lysozyme sample (0.9 g/50 mL) derived from traditional RME was first tested to purify through HSCCC, using the same RM system as shown in Figure 1. Although the retention of the stationary phase is about 22%, the lysozyme can be further purified at an activity recovery of 82.4% (see Table 2 and Figure 3). These

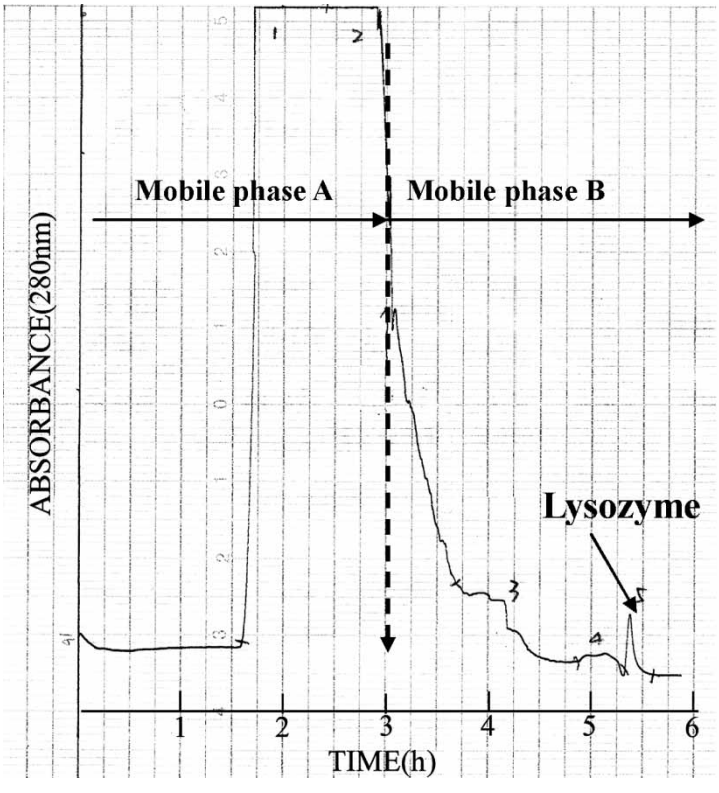

Figure 2. Separation of lysozyme from crude egg-white sample through HSCCC using the RM system. Sample weight: 1 g/50 mL. The other condition is the same as Figure 1.

results indicate that the purification of lysozyme by HSCCC using the RM system is feasible.

Accordingly, a crude chicken egg-white sample (1 g/50 mL) was separated through the same procedure as above. The separation profile is presented in Figure 2. The lysozyme can be separated completely with other two major proteins, ovalbumin and conalbumin, through a one step separation, although there was a minor unknown band coexisting with it in SDS-PAGE profile (Fig. 3). The purification fold is 9.2. However, the activity recovery of lysozyme was extremely low about 13% (see Table 2). This may be due to the interference of the large amount of ovalbumin and conalbumin in crude egg-white, and the low concentration of lysozyme in the sample.

Scale-Up Prospect of HSCCC Separation

Large scale preparative separations were performed with 3 g/200 mL, 6 g/300 mL, and 8 g/400 mL of crude egg-white samples by HSCCC in a different way, as mentioned in experimental section, to test the possibility of scale-up. Figure 4 gives the separation profile of 8 g/400 mL sample. Better results were obtained with a slightly higher activity recovery of lysozyme (25%) and purification fold (15.8) (see Table 2), where

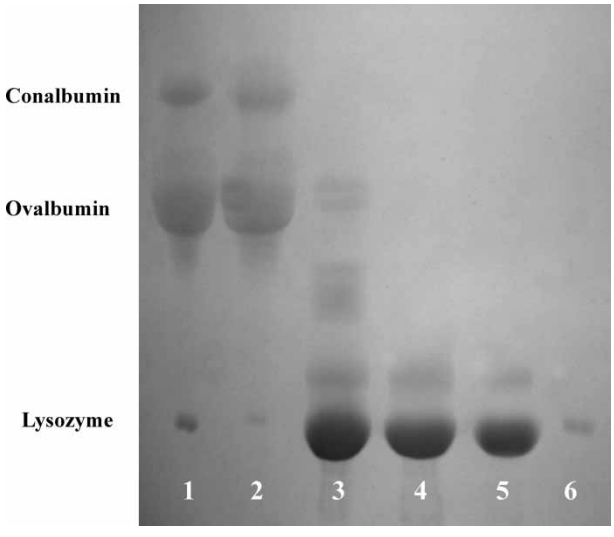

Figure 3. SDS-PAGE profile of egg-white proteins before and after separation. 1. Crude egg-white; 2. Aqueous phase after forward extraction; 3. Lysozyme derived from traditional RME; 4. Lysozyme derived from further purification by HSCCC using RM system; 5. Lysozyme derived from crude egg-white by direct HSCCC separation using RM system; 6. Standard Lysozyme.

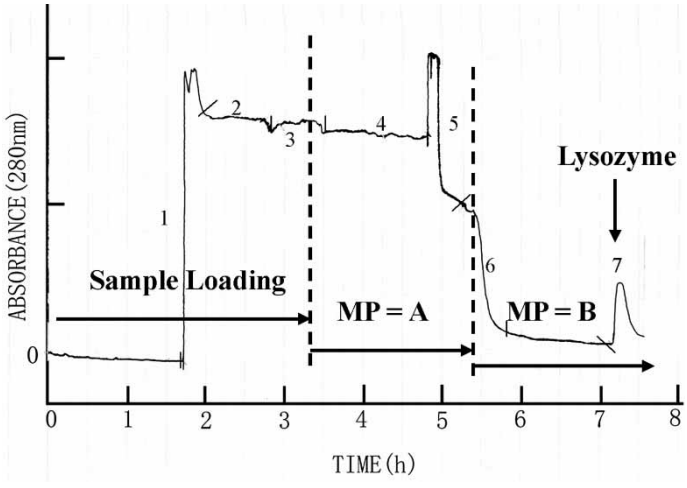

Figure 4. Scale-up separation of lysozyme from crude egg-white sample through HSCCC using RM system. Sample weight: 8 g/400 mL; Except for the sample loading way, the other condition is the same as Figure 1.

SDS-PAGE profile is shown in Figure 5. It was noticed, that lysozyme was co-eluting out with other proteins during the forward extraction. This situation was also observed in other two scale-up separations, but not in the 1 g/50 mL separation. It may rule out the possibility that the saturation of

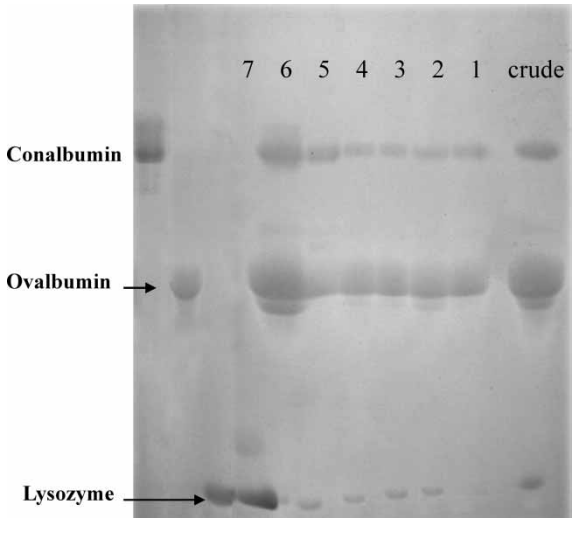

Figure 5. SDS-PAGE profile of egg-white proteins collected before and during scale-up separation. Crude: crude egg-white; 1-7 corresponding to the fractions in Figure 4.

lysozyme in the water cores formed by AOT solubilized in organic phase, since the amount of all samples loaded in scale-up separation were lower than that in the test separation, where 0.9 g/50 mL sample derived from traditional RME of 15 g crude egg-white was loaded and a relatively high recovery (82.4%) was obtained.

Therefore, the reason of lower activity recovery of lysozyme encountered in the separation of crude egg-white should be mainly due to the interference of other proteins, especially ovalbumin, which occupies over 50% of the total egg-white proteins. To overcome this problem, one way may be to remove the main interference proteins before the purification of lysozyme through HSCCC, another way may be to lower the flow rate of mobile phase during forward extraction to increase the retention of stationary phase and leave enough settling time for two phases during HSCCC run. This problem is open to discussion and further approaches are under investigation.

CONCLUSIONS

The above results indicated that the separation of lysozyme by HSCCC using the RM system is practical and promising to be further scaled up if the activity recovery was improved. This method has advantages over traditional RME for its simple continuous elution mode and high separation efficiency.

REFERENCES

1. Mecitoflu, Ç.; Yemenicioflu, A.; Arslanoflu, A.; ElmacÂ, Z.S.; Korel, F.; Çetin, A.E. Food Res. Intl. **2006**, *39*, 12.
2. Bergquist, E. In *Kirk Othmer Encyclopedia of Chemical Technology*, 4th Edn; Grant, M.H., Ed.; Wiley-Interscience: New York, 1993; Vol. 8, 887.
3. Owen, R.O.; Chase, H.A. J. Chromatogr. A **1997**, *757*, 41.
4. Ming, F.; Howell, J.A.; Acosta, F.; Hubble, J. Biotechnol. Bioeng. **1993**, *42*, 1086.
5. Awade, A.C.; Moreau, S.; Molle, D.; Brule, G.; Moubois, J.-L. J. Chromatogr. A **1994**, *677*, 279.
6. McCreath, G.E.; Owen, C.; Nash, R.O.; Chase, H.A. J. Chromatogr. A **1997**, *773*, 73.
7. Junowicz, E.; Charm, S.E. Febs Letters **1975**, *57*, 219.
8. Ghosh, R. Biochem. Engin. J. **2003**, *14*, 109.
9. Ghosh, R.; Silva1, S.S.; Cui, Z.F. Biochem. Engin. J. **2000**, *6*, 19.
10. Wan, Y.H.; Lu, J.R. Separation and Purification Technology **2006**, *48*, 133.
11. Chou, S.T.; Chiang, B.H. Chemistry/Biochemistry **1998**, *63*, 399.
12. Jarudilokkul, S.; Paulsen, E.; Stuckey, D.C. Bioseparation **2000**, *9*, 81.
13. Sun, Y.; Bai, S.; Gu, L.; Furusaki, S. Bioproc. Engin. **2000**, *22*, 19.
14. Shin, Y.O.; Weber, M.E.; Vera, J.H. Fluid Phase Equil. **2003**, *207*, 155.
15. Nishii, Y.; Kishi, Y.; Ito, M.; Morita, Y.; Kanoha, A.; Shintani, M.; Kinugasa, T.; Nii, S.; Takahashi, K. Sepn. Purif. Technol. **2004**, *37*, 161.

16. Noh, K.H.; Imm, J.Y. Food Chem. **2005**, *93*, 95.
17. Bradford, M.M. Anal. Biochem. **1976**, *72*, 248.
18. Laemmli, U.K. Nature **1970**, *227*, 680.

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