Reversed Micellar Extraction of Hen Egg Lysozyme

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ABSTRACT

Eggwhite was diluted to 10 times its original volume with 50 mM phosphate buffer (pH 9.2) containing 0.1M potassium chloride. The aqueous solution was mixed with an equal volume of isooctane containing 50 mM bis-(2-ethylhexyl) sodium sulfosuccinate at 10°C for 50 min. After extraction, the organic phase containing lysozyme was separated from the aqueous phase and mixed with an equal volume of 50 mM phosphate buffer (pH 11.8) containing 1M potassium chloride. Backward extraction was then performed at 30°C for 45 min. The procedures recovered 90% lysozyme from the eggwhite. The specific activity of the extract was near 73,000 units/mg.

Key Words: hen, eggwhite, lysozyme, reversed micelles

INTRODUCTION

REVERSED MICELLES ARE AGGREGATES OF SURFACTANT molecules containing an inner core of water molecules, dispersed in an organic solvent providing an effective separation technology. Many biological products can be solubilized into the core water without loss in activity (Luisi, 1985; Kinugasa et al., 1992). The major application of reversed micelles is the extraction of proteins such as enzymes. Many enzymes can be solubilized in reversed micelle aggregates, including α-chymotrypsin (Jolivalt et al.1990; Marcozzi et al., 1991); lipases (Aires-Barros and Cabral, 1991; Taipa et al., 1992); proteases (Rahaman et al., 1988); α-amylase (Chang and Chen, 1995a; Krei et al., 1995; Brandani et al., 1996); trypsin (Chang and Chen, 1995b) and lysozymes (Kinugasa et al., 1991, 1992; Chang et al., 1994; Lye et al., 1995; Naoe et al., 1995).

Among enzymes studied to assess feasibility of separation and purification by reversed micelles, lysozyme is an important enzyme substrate because of its broad potential for food and clinical applications. Several factors affecting the separation efficiency of lysozyme by reversed micelles have been studied, including pH and ionic strength of the aqueous phase containing lysozyme (Goklen and Hatton, 1987), concentration of surfactant in the organic phase (Dekker et al., 1989; Kelley et al., 1993), extraction temperature (Kunieda and Shinoda, 1980) and time (Dekker et al., 1986). However, previous studies either focused on the partitioning of lysozyme into the reversed micellar solution or the stability of lysozyme during extraction. They were also based on a clean model system prepared by dissolving pure lysozyme in a phosphate buffer, which may not be indicative of separation from biological materials.

When employing reversed micelles to separate lysozyme from a biological material, the existence of other proteins would inevitably interfere with the separation. Our objective was to apply reversed micellar extraction to extract lysozyme from hen eggwhite. Parameters which might affect the effectiveness of the extraction were investigated. The major criteria were the recovery rate and activity of lysozyme after extraction.

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MATERIALS & METHODS

Materials

Hen eggs were obtained from a supermarket in Taipei. Lysozyme (E. C. 3.2.1.17. Mucopeptide N-acetylmuramyl hydrolase) and *Micrococcus lysodeikticus* were purchased from Sigma Chemical Co. (St. Louis, MO). Acetic acid and methanol were from Alps Chemical Co. (Taipei, Taiwan). Tetrabutylammonium bromide and polyacrylamide were from E. Merck Co. (Darmstadt, Germany). The protein assay dye reagent was obtained from Bio-Rad Co. (Hercules, CA). Other chemicals included bis-(2-ethylhexyl) sulfosuccinate sodium salt (also called Aerosol OT, or AOT) (Fluka AG Chemische Fabrik, Buchs, Switzerland), potassium chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan), sodium dodecyl sulfate (BDH Laboratory Supplies, Poole, England), and isooctane (Fisher Scientific Co., Pittsburgh, PA).

A 5 mg/mL aqueous lysozyme solution was prepared by dissolving lysozyme crystals in 50 mM potassium phosphate buffer (pH 9.8). Other potassium phosphate buffer solutions (pH 6 to 11) containing various concentrations of potassium chloride (0.05 to 0.4M) were then used to dilute the lysozyme solution 5-fold to 10-fold. The hen eggwhite was also diluted with the 50 mM buffer solutions of pH 6 to 11 with potassium chloride concentrations from 0.05 M to 0.4 M. The organic solution was prepared by dissolving AOT in isooctane to make a 50 mM solution.

Extraction procedure

The reversed micellar extraction consisted of two steps, forward extraction and backward extraction. The forward extraction was carried out in a stoppered 50-mL centrifuge tube. Equal volumes (ca. 8 mL) of the organic solution and aqueous solution were mixed in the tube and shaken in a constant temperature water bath (5 to 30°C) for selected times (20 to 60 min). The resulting mixtures were then centrifuged at 3000 rpm ($1100 \times g$) for 5 min. The upper layer (5 mL) (reversed micellar solution) was taken for a subsequent backward extraction.

To the reversed micellar solution from the forward extraction, an equal volume (5 mL) of the 50 mM phosphate buffer (pH 11.8) was added. This mixture was shaken in a water bath at 30°C for 45 min. After centrifugation, the lower aqueous layer was collected and analyzed for lysozyme content.

Analytical

The protein concentration was analyzed by a procedure based on the Bradford dye-binding method (Bradford, 1976). Bio-Rad protein assay dye reagent (5 mL) was mixed with 0.1 mL of the protein solution. The optical density of the mixture at 595 nm was observed spectrometrically to determine the protein concentration using a standard curve obtained by recording the $OD_{595\ nm}$ of pure lysozyme solutions. A Micrococcus lysodeikticus turbidity method suggested by Sigma Chemical Co. was used for determining lysozyme activity. The recovery of lysozyme was estimated by:

Lysozyme recovery (%) =
[(Total activity of lysozyme in the aqueous phase after extraction)/
(Total activity of lysozyme in the aqueous phase before extraction)] × 100.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (1970) using 15% polyacrylamide separating gel and 4% stacking gel. Samples containing 1 to 2 mg protein/ mL were dissolved in equal volumes of buffer solution (0.1M Tris-HCl, pH 6.8, 4 mM EDTA, 20% glycerol, 0.05% bromophenol blue, 4% SDS, and 10% β-mercaptoethanol), and heated at 100°C for 5 min. Gels were stained with Coomassie Brilliant Blue R250 for detection of protein, and destained by diffusion in a solution containing 10% acetic acid, 20% methanol, and 70% water.

The residual AOT content in the extract was analyzed with an HPLC system (ICI Instrument Co., Victoria, Australia) and RI detector. The extract was separated on a 5μm, 220 mm × 4.0 mm LiChrospher RP-18 column (Merck Co., Darmstadt, Germany) at 30°C, and eluted at 1 mL/min with ethanol/distilled water (V:V=78:22) containing 2 mM tetrabutylammonium bromide. The Duncan multiple range test was used for analyzing the data statistically (Duncan, 1955). Significance of differences was defined at $p<0.05$.

RESULTS & DISCUSSION

Dilution of eggwhite

Extraction of lysozyme by reversed micelles was first performed on the hen eggwhite without dilution. However, the lysozyme either remained in the aqueous phase or formed insoluble aggregates at the organic solution-aqueous solutions interface. It was suspected that interactions between lysozyme and other eggwhite proteins (Nakai and Kason, 1974; Kato et al., 1975) might have interfered.

Dilution of eggwhite with 50 mM potassium phosphate buffer (pH 9.8) improved the extraction effectiveness (Table 1). Increasing the dilution factor increased lysozyme recovery. However, the recovery of lysozyme from eggwhite was much lower than recovery from the standard lysozyme solution. The reversed micelles recovered nearly 99% of lysozyme from the standard solution. However, when diluting the eggwhite to one-tenth of its initial concentration, the recovery of lysozyme from eggwhite was only about 50%.

The specific activity of the lysozyme extracted from eggwhite remained at ≃60,000 units/mg, suggesting that the AOT/isooctane system selectively extracted lysozyme from eggwhite. In addition, the organic phase (AOT/isooctane) did not exert any adverse effect on the lysozyme molecules.

Effect of pH on extraction of lysozyme

The pH of the aqueous phase is one of the most important parameters affecting reversed micellar extraction (Goklen and Hatton, 1987; Leodidis, 1990; Leser and Luisi, 1990; Luisi, 1985). In general, the recovery of lysozyme increased with increasing pH value (Fig. 1), and reached a plateau at pH 9.2. The isoelectric point of lysozyme is around 11. At pH 9.2, the lysozyme retains mainly positive charges on its surface. The reversed micelles formed by the anionic surfactant Aerosol OT (AOT) display a surface of negative charge surrounding a polar core. Because of the electrostatic interactions, the lysozyme was transferred from the aqueous phase to the micellar solution (Goklen and Hatton, 1987; Leodidis, 1990; Leser and Luisi, 1990; Luisi, 1985; Kinugasa et al., 1991). When the pH of the aqueous phase approached the isoelectric point of lysozyme, the positively charged groups of lysozyme molecules were gradually neutralized, and the electrostatic interaction between lysozyme and AOT were diminished. As a result, solubilization of lysozme within the polar core of the reversed micelles decreased sharply (Fig. 1).

The optimum pH of the aqueous phase for extracting lysozyme from eggwhite was ≃9.2. However, the recovery only amounted to 60%. The electrostatic interaction between lysozyme and AOT at pH 9.2 were not effective in displacing the interactions between lysozyme and other eggwhite proteins.

The extraction efficiency for eggwhite lysozyme was not altered when the KCl concentration was increased from 0.05M to 0.3M (Fig. 2). Since the interaction between lysozyme and other major eggwhite proteins, such as ovalbumin, is electrostatic in nature (Nakai and Kason, 1974), increasing the KCl concentration of the aqueous phase may decrease the interactions between lysozyme and other eggwhite proteins, and increase the extraction efficiency for lysozyme. However, an increase of salt concentration may lead to an electrostatic screening effect (Leodidis, 1990; Nishiki et al., 1993) which reduces the electrostatic interaction between lysozyme and AOT and decreases the size of the micelles, thus decreasing extraction efficiency. Our data suggested that the beneficial effect of decreasing eggwhite-protein interactions was counteracted by the electrostatic screening effect in the salt concentration range 0.10M to 0.30M. When the salt concentration was increased to 0.30M, the electric shielding effect became dominant, and the lysozyme recovery decreased significantly.

Table 1—Effect of dilution on extraction of lysozyme

Dilution factor	Standard lysozyme solution		Hen eggwhite	
	Lysozyme recovery (%)	Specific activity (units/mg)	Lysozyme recovery (%)	Specific activity (units/mg)
5	68.0^{d*}	60287^{a}	28.0^{i}	59132^{a}
6	79.0^{c}	59360^{a}	31.0^{i}	59943^{a}
7	86.5^{cb}	59605^{a}	35.0^{ih}	59066^{a}
8	91.5^{ab}	60083^{a}	39.5^{gh}	59942^{a}
9	95.5^{ab}	59763^{a}	47.0^{gf}	60352^{a}
10	98.4^{a}	59828^{a}	51.6^{ef}	58932^{a}

$^{a-i}$Means within the same column with different superscripts are significantly different ($p<0.05$).

Extraction temperature, time and lysozyme recovery

Since the changes of pH and salt concentration in the aqueous solution did not increase the extraction efficiency, other operating parameters, including extraction temperature and time, were investigated. The recovery rate increased with the a decrease of extraction temperature (Fig. 3). At 10°C, the recovery rate was ≃65%. Dekker et al. (1991) also studied the effect of temperature on reversed mi-

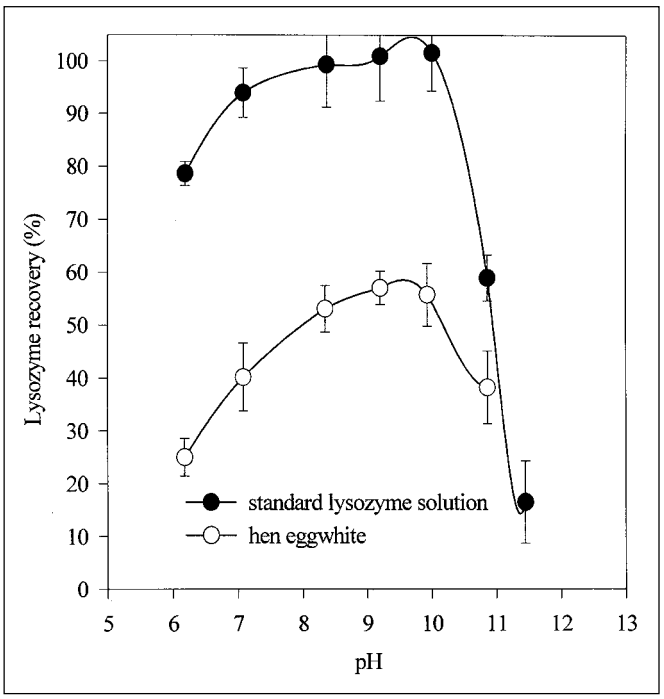

Fig. 1—pH relation to recovery of lysozyme during reversed micellar extraction.

cellar extraction of enzymes and found that the maximum amount of aqueous phase which could be solubilized in the reversed micellar phase was a function of temperature. As the temperature increased the amount of solubilized aqueous solution decreased. Lowering extraction temperature, therefore, facilitated the extraction of lysozyme into the micellar phase.

Decreasing the extraction temperature increased the specific activity of lysozyme in the extract (Table 2). Although the mass transfer rates of the protein molecules in eggwhite were generally decreased with decreasing temperatures, the electrostatic interactions between lysozyme and AOT were not altered significantly. Consequently, the extraction mass transfer rate of lysozyme became relatively higher than that of other eggwhite proteins at lower temperatures, thus increasing the specific activity of lysozyme in the extract.

Because of the decrease in extraction temperature, the extraction time was increased to compensate for the reduced mass transfer rate. When the extraction time was extended to 50 min, the recovery rate of lysozyme was ≃90% (Fig. 4), almost the recovery rate of lysozyme from the standard solution.

The specific activity of the extracted lysozyme could be maintained at a high level within 50 min of extraction time (Table 3). Further increase of extraction time decreased the specific activity of lysozyme, possibly due to increased contact of lysozyme with the organic solvent isooctane, which could inactivate the enzyme.

Electrophoretic patterns of the hen eggwhite, the standard lysozyme solution, and the extracts of these protein solutions by reversed micelles were compared (Fig. 5). The reversed micellar extraction removed the nonlysozyme proteins and yielded a highly purified lysozyme product.

Residual AOT in the extract

Although the LD_{50} of AOT is 1900 mg/kg, the residual AOT in the extract may still cause concern if the purified lysozyme were used for food applications. The HPLC we employed could detect AOT as low as 667 mg/kg, but it still did not detect any AOT in the extract. There-

Table 2—Forward extraction temperature and specific activity of extracted lysozyme[d]

Temp (°C)	Specific activity (units/mg)	
	Standard lysozyme solution	Hen eggwhite
5	60618[c]	63131[b]
10	71228[a]	70763[a]
15	68851[ab]	68267[a]
20	65617[b]	64099[b]
25	65873[b]	64129[b]
30	61225[c]	59794[c]

[a-c]Means within the same column with different superscripts are significantly different ($p<0.05$).
[d]The forward extraction time was 30 min.

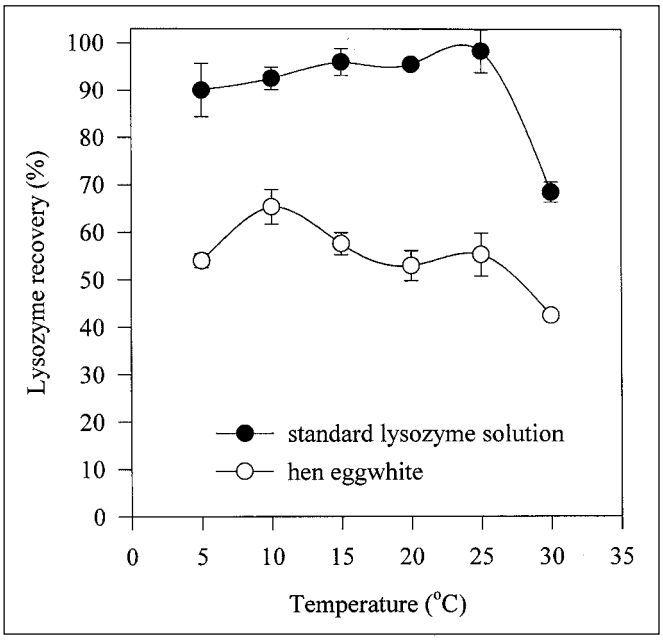

Fig. 3—Temperature effects on the recovery of lysozyme during reversed micellar extraction.

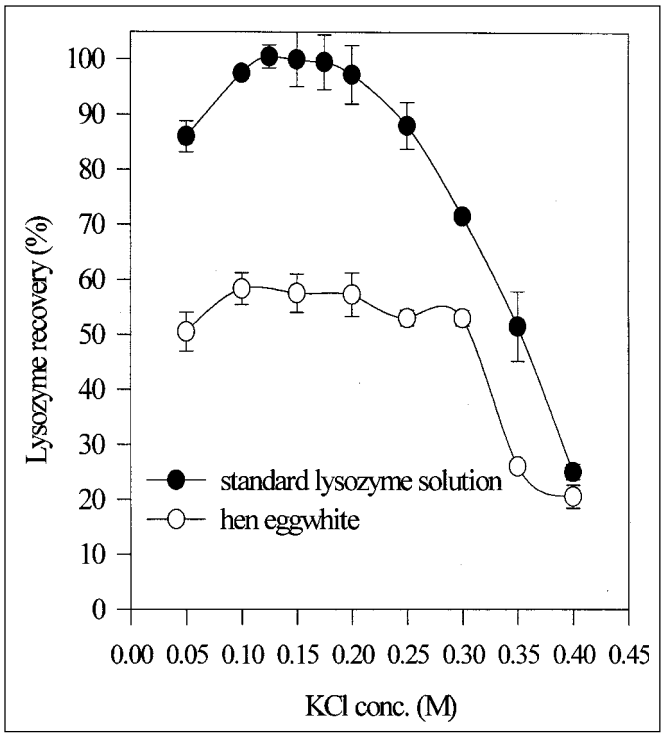

Fig. 2—Concentration of KCl as related to recovery of lysozyme during reversed micellar extraction.

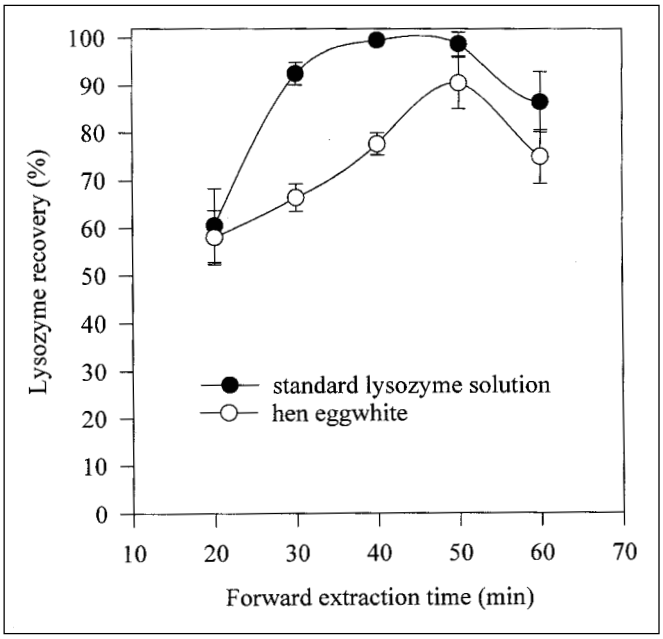

Fig. 4—Forward extraction time effects on the recovery of lysozyme during reversed micellar extraction.

Table 3—Forward extraction time and specific activity of extracted lysozyme[c]

Extraction time (min)	Specific activity (units/mg)	
	Standard lysozyme solution	Hen eggwhite
20	70241[a]	69743[a]
30	71299[a]	72450[a]
40	71142[a]	72558[a]
50	71566[a]	73066[a]
60	60733[b]	62831[b]

* The forward extraction was carried out at 10 ºC.
** Means within the same column with different superscripts are significantly different ($p<0.05$).

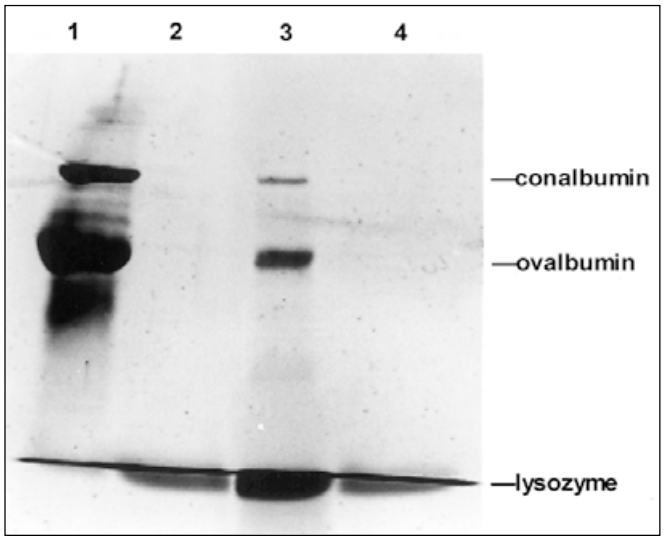

Fig. 5—SDS-PAGE of hen eggwhite proteins during reversed micellar extraction. (1) hen eggwhite; (2) extract from (1); (3) standard lysozyme solution; (4) extract from (3).

fore, we may conclude that AOT was lower than that level, in the lysozyme solution obtained by reversed micellar extraction.

CONCLUSIONS

LYSOZYME IN HEN EGGWHITE COULD BE EXTRACTED BY REVERSED micelles using an AOT/isooctane system. Eggwhite must be diluted 10-fold with phosphate buffer (pH 9.2) containing 0.1M potassium chloride. The extraction was best conducted at 10°C for 50 min and almost 90% of lysozyme could be recovered. The specific activity of the extracted lysozyme was almost 73,000 units/mg, higher than that of most commercially available lysozyme products.

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Ms received 4/15/97; revised 11/29/97; accepted 12/20/97.