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One-step separation of lysozyme by reverse micelles formed by the cationic surfactant, cetyldimethylammonium bromide

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Abstract

Lysozyme was selectively extracted from reconstituted freeze-dried egg-white, using reverse micelles formed by the cationic surfactant, cetyldimethylammonium bromide (CDAB). The major egg-white proteins, including ovalbumin and ovotansferrin, were solubilized into the organic phase while lysozyme was recovered in the aqueous phase. The solubilization behaviours of proteins were manipulated by processing parameters, including pH and salt concentration in the aqueous phase and concentration of surfactant in the organic phase. The optimum extraction was achieved with sodium borate buffer (50 mM, pH 9, no added KCl) and organic phase containing 50 mM CDAB. After the forward extraction, 96% of total lysozyme activity was recovered. Lysozyme was efficiently purified, more than 30-fold with only a single forward extraction. The suggested extraction procedure has advantages in terms of time and cost compared to traditional reverse micellar extraction which requires both forward and backward extraction steps.

Keywords: Lysozyme; Reverse micelle; Extraction; Cationic surfactant

1. Introduction

Lysozyme is an enzyme which hydrolyzes the glycosidic bond between the C-1 of *N*-acetylmuramic acid and the C-4 of *N*-acetylglucosamine in bacterial peptidoglycan (Durance, 1994). Due to its antimicrobial activity, lysozyme has been considered as a natural food preservative and a valuable functional constituent in chicken egg-white. Traditionally, the separation of lysozyme from chicken egg-white has been done either by direct crystallization (Alderton & fevold, 1946) or by chromatographic procedures (Li-Chan, Nakai, Sim, Bragg, & Lo, 1986). However, these methodologies are time-consuming and not well suited for scale-up. Therefore, simple and cost-effective separation methodology is needed for mass preparation of lysozyme.

As an alternative for conventional separation and purification procedures, reversed micellar extraction has a great potential for continuous separation and concentration of bioactive substances, including proteins. Reverse micelles are aggregates of surfactant molecules containing polar cores of solubilized water, dispersed in a continuous solvent medium (Luisi, Giomini, Pileni, & Robinson, 1988). This system is derived principally from the ability of the water droplets to dissolve proteins and the solubilized proteins are shielded by surfactant molecules from the organic medium without losing biological activity (Dekker, Hilhorst, & Laane, 1989).

In the liquid–liquid reverse micellar extraction process, a target protein is selectively solubilized into organic phase (forward extraction) and subsequently is stripped into the aqueous phase (backward extraction) by the

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addition of fresh aqueous buffer (Matzke, Creagh, Haynes, Parausnitz, & Blanch, 1992). The forward extraction is governed primarily by electrostatic interactions between the charged protein and polar head of surfactant (Luisi & Magid, 1986) and is also affected by hydrophobic interactions between the non-polar region of the proteins and surfactant tail (Pires & Cabral, 1993). For backward extraction, the working pH and ionic strength of aqueous buffer should be controlled to provide the same charges for proteins and surfactant. This backward extraction often causes problems, such as low yield and coextraction of surfactant (Goto, Ishikawa, Ono, Nakashio, & Hatton, 1998; Rahaman, Chee, Haynes, Cabral, & Hatton, 1988).

Up to now, several enzymes and proteins, including chymotrypsin (Shin, Rodil, & Vera, 2004), lysozyme (Chou & Chiang, 1998) and lipase (Aires-Barros & Cabral, 1991) have been extracted by reverse micelles but most previous works were done using the well known anionic surfactant, sodium di-(2-ethylhexyl) sulfosuccinate (AOT) and by typical forward and backward extraction procedures.

In the current study, one-step separation method was devised and drove solubilization of unwanted proteins into reverse micelles formed by the cationic surfactant, cetyldimethylammonium bromide (CDAB) while target protein remained in the aqueous phase. Therefore, target protein (lysozyme) was efficiently recovered after only forward extraction without performing the troublesome backward extraction step. The aim of this study was to examine parameters affecting one-step lysozyme separation from egg-white and to find optimal separation procedures.

2. Materials and methods

2.1. Materials

Fresh hen eggs were supplied by Join poultry farm (Kyunggi, Korea). Egg-white was carefully collected from fresh eggs after removal of chalazae and freeze-dried egg-white was prepared. Lysozyme, *Micrococcus lysodeikticus* and cetyldimethylammonium bromide (CDAB) were obtained from Sigma Chemical Co. (St. Louis, USA). Isooctane and hexanol were obtained from J.T. Baker (Phillipsburg, USA) and Acros (Fairlawn, USA), respectively. All other chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, USA).

2.2. Extraction procedure

Freeze-dried egg-white (FDEW, 0.25%, w/v) was solubilized in the buffers (pH 6–12) at various ionic strengths. The desired pH was adjusted using sodium citrate buffer (50 mM, pH 6), sodium phosphate buffer (50 mM, pH 7–8), sodium borate buffer (50 mM, pH 9–10) and Na_2HPO_4/NaOH buffer (50 mM, pH 11–12), while ionic strength was adjusted by the addition of KCl (0–200 mM). The aqueous egg-white solution (10 ml) was mixed with an equal volume of organic phase (isooctane: hexanol = 1:1, v/v) containing a given concentration of cetyldimethylethyl ammonium bromide (CDAB, 50–200 mM). This forward extraction was carried out in a tightly-stopped glass flask (50 ml) using a shaking incubator (Vision Scientific, Korea) with agitation speed of 300 rpm and was continued for 20 min at 25 °C. After extraction, the resulting mixture was separated by centrifugation at 2800*g* for 5 min and the aqueous phase was collected for analysis.

2.3. Protein content and profiles in the aqueous phase after forward extraction

Total protein concentration and profiles of aqueous proteins in the aqueous phases, before and after forward extraction, were determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) and SDS-PAGE (Laemmli, 1970). SDS-PAGE was carried out on 11% separating and 4% stacking gel using a mini-PROTEAN II cell (Bio-Rad Laboratories, Hercules, USA). The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R 250 in 45% (v/v) methanol containing 10% glacial acetic acid and destained with 10% glacial acetic acid. The intensity of each protein band was analyzed by scanning gels with an image analysis system (Kodak 1D Analysis, Eastman Kodak Company, Rochester, USA). The profile of each protein (ovalbumin, ovotransferrin and lysozyme) remaining in the aqueous phase was expressed as a percentage based on total band intensity.

2.4. Lysozyme activity determination

The lysozyme activity of the aqueous phase after extraction was determined by turbidimetric assay (Li-Chan et al., 1986). *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. 2.98 ml of cell suspension were mixed with 20 μl of sample and changes of absorbance at 450 nm were determined by spectrophotometer (Ultrospec 2100 pro, Amersham pharmacia biotech, England) up to 5 min with 30-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 expressed as units/ml using the following equations:

$$\text{Activity (U/min)} = (\Delta A_{450}/\text{min})/(0.001/\text{min} \times 0.02\ \text{ml})$$

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

$$\text{Lysozyme recovery (\%)} = [(\text{Total activity of lysozyme in the aqueous phase after extraction})/(\text{Total activity of lysozyme in the aqueous phase before extraction})] \times 100$$

2.5. Measure of water content

The changes of water content in the micellar organic phase at designated extraction conditions were determined by a Karl–Fischer moisture titrator (Karl Fischer Titrino Model 701, Metrohm Ltd., Swiss). The water content in the organic phase was calculated as the molar ratio of water to initial CDAB in the organic phase, $Wo = [H_2O]/[CDAB]$.

2.6. Statistical analysis

All analytical measurements were done in triplicate and the data were analyzed using Minitab (Ver. 13.1, Minitab Inc., USA). Analysis of variance (ANOVA) was used to determine the effect of processing parameters on lysozyme recovery. When ANOVA revealed significant effects at $p < 0.05$, the data were further analyzed using Tukey's multiple comparison test.

3. Results and discussion

3.1. Effect of pH

The effect of aqueous pH on protein solubilization was determined by mixing the buffered protein solution (pH 6–12) with the organic phase containing 50 mM CDAB. To exclude the effect of ionic strength, KCl was not added to the buffered protein solution. As shown in Table 1, total lysozyme activities in the aqueous phases did not show significant differences in the pH range of 6–11 after the extraction. This result indicates that the lysozyme content initially present in the aqueous phase was not critically changed in the tested pH range. However, only about 35% of initial activity was recovered in the aqueous phase when the extraction was conducted at pH 12. The reduced lysozyme activity was probably due to solubilization of lysozyme into the organic phase.

Solubilization of protein molecules into reversed micelles is largely governed by aqueous pH since electrostatic interaction between charged protein molecules and polar head of surfactant is a major driving force causing protein transfer. The cationic surfactant, used in this study, for solubilization of proteins into the organic phase, probably facilitated aqueous pHs above their isoelectric point (p*I*) by providing net negative charges. As the aqueous pH was increased from pI of major egg-white proteins (ovalbumin: 4.5, ovotransferrin; 6.0), the uptake of ovalbumin and ovotransferrin into the micellar phase was greatly increased. These proteins were no longer detected in the aqueous phase after the extraction was performed above pH 9 (Fig. 1).

The relative proportion of the lysozyme band was significantly increased at pH 9 and 10 but levelled off at pH 11. The reduced lysozyme proportion at pH 11 was mainly due to increased unidentified protein bands (<35,000 Da) rather than lysozyme solubilization. Thus, total activity remained unchanged under this condition. At pH 12, marginal lysozyme activity was observed but the lysozyme band was not detected by SDS-PAGE because of the very low protein content in the recovered aqueous phase. Considering the pI of lysozyme (pI 11), significant uptake of lysozyme into the interface or organic phase could have occurred at pH 12. This result was consistent with the conclusion of Matzke et al. (1992) that electrostatic interaction is one of the predominating driving forces for protein solubilization in reverse micelles. Kawakami and Dungan (1996) reported that solubilization of proteins was the result of the balance between electrostatic interactions and hydrophobic interactions between non-polar protein

Table 1
Effect of pH on recovered lysozyme activity after forward extraction

Condition of extraction			Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
pH	KCl (mM)	CDAB (mM)				
Egg-white control			8850[a]	380[a]	1.0	100[a]
6	0	50	8045[ab]	1241[a]	3.3	90.9[a]
7	0	50	8185[ab]	1276[a]	3.4	92.5[a]
8	0	50	8524[ab]	1368[a]	3.6	96.3[a]
9	0	50	8486[ab]	12,284[b]	32.3	95.9[a]
10	0	50	7820[bc]	11,022[b]	29.0	88.3[ab]
11	0	50	7480[c]	4415[c]	11.6	84.5[b]
12	0	50	255[d]	3000[c]	7.9	2.9[b]

[a–d] Means with different superscripts within the same column are significantly different ($p < 0.05$).

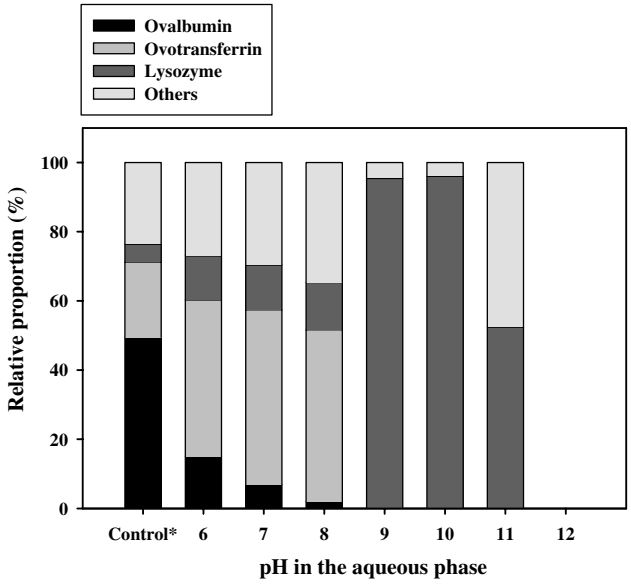

Fig. 1. Proportions of proteins remaining in the aqueous phase after forward extraction of reconstituted freeze-dried egg-white at various pHs. Control*: reconstituted freeze-dried egg-white The extraction was performed with the aqueous phase of designated pHs and the organic phase containing 50 mM CDAB. The extraction time was 20 min.

residues and the non-polar tail region of surfactant in the micellar interface. During the all-extraction processes, a white precipitate was observed at the interface. This indicates the possibility of protein aggregation rather than protein solubilization into the organic phase. However, the final destination of various proteins during extraction was not further characterized since most lysozyme activity remained in the aqueous phase.

The selective protein uptake, either to the organic phase or interface, led to marked differences in terms of specific activity. After the extraction, specific activity of lysozyme remaining in the aqueous phase was about 30 times greater for the extraction at pH 9 and 10 than for the egg-white control. Under these extraction conditions, recovery, based on total activity, was 88–96%. Although specific activity and recovery did not show significant differences between extractions at pH 9 and 10, pH 9 was chosen as an optimal extraction pH since it was a relatively mild processing condition with slightly higher purification efficiency.

3.2. Effect of ionic strength

The ionic strength of the aqueous phase is closely related to the degree of shielding of electrostatic potential imposed by a charged surface and subsequently affects the protein separation process (Pires, Aires-Barros, & Cabral, 1996). The effect of salt (KCl) concentration on the purification efficiency was examined at the predetermined optimal pH (pH 9). As shown in Table 2, total activity and recovery were not changed, regardless of salt concentrations in the aqueous phase, while specific activity was decreased about 14–25 times by the addition of KCl in the aqueous phase. The result clearly indicated that solubilization of other proteins, along with lysozyme, was significantly inhibited in the presence of salt. The protein content in the recovered aqueous phase increased from 0.07 to 2.08 mg/ml as KCl concentration increased from 0 to 200 mM (data not shown). This interpretation was confirmed by SDS-PAGE, indicating the relative proportion of protein band in the recovered aqueous phase (Fig. 2).

The decreased protein solubilization in the presence of salt might be due to two possible factors. First, electrostatic screening reduces the electrostatic interaction between polar part of the surfactant and charged groups. The reduced protein solubilization at higher ionic strength has been reported by other studies (Kamihira, Yanagisawa, Takahashi, & Takeuchi, 1994; Lye, Asenjo, & Pyle, 1995). Second, on the inverse relationship between salt concentration and micelle size was reported in reverse micelles formed by anionic surfactants such as AOT (Nishiki, Sato, Kataoka, & Kato, 1993), and the reduced micelle size possibly results in decreased protein solubilization.

However, salt concentration did not affect micelle size at the tested concentration (0–200 mM) and the size of reverse micelles (Wo) was hardly changed as a function KCl concentration (Fig. 3). This result can be explained by the effect of the cosurfactant, hexanol, used in the organic phase. As depicted by Lu, Chen, Li, and Shi (1998), hexanol is located between the surfactant head groups and acts as a buffer for repulsive electrostatic interaction between surfactant head groups, allowing close packing of the inner core of reverse micelles. Thus,

Table 2
Effect of KCl concentration on recovered lysozyme activity after forward extraction

Condition of extraction			Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
pH	KCl (mM)	CDAB (mM)				
Egg-white control			8850[a]	380[a]	1.0	100[a]
9	0	50	8486[a]	12,284[b]	32.3	95.9[a]
9	50	50	8373[a]	557[a]	1.5	94.6[a]
9	100	50	8755[a]	495[a]	1.3	98.9[a]
9	200	50	8840[a]	887[c]	2.3	99.8[a]

[a–c] Means with different superscripts within the same column are significantly different ($p < 0.05$).

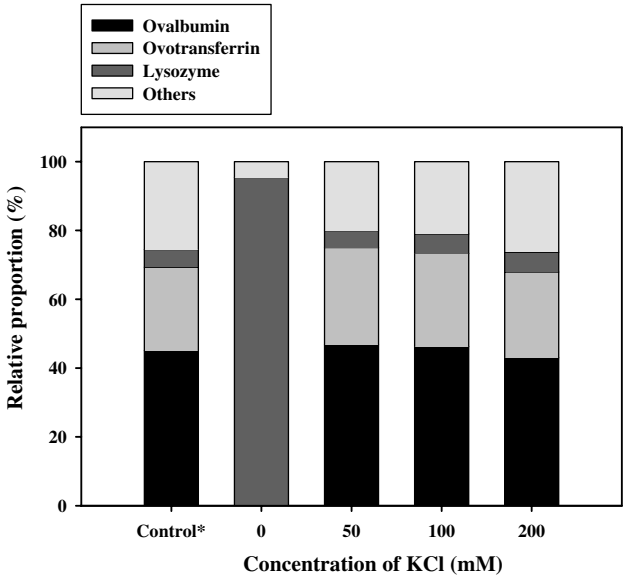

Fig. 2. Proportions of proteins remaining in the aqueous phase after forward extraction of the reconstituted freeze-dried egg-white at various KCl concentrations. Control*: reconstituted freeze-dried egg-white The extraction was performed with sodium borate buffer (50 mM, pH 9) containing designated KCl concentrations and the organic phase containing 50 mM CDAB. The extraction time was 20 min.

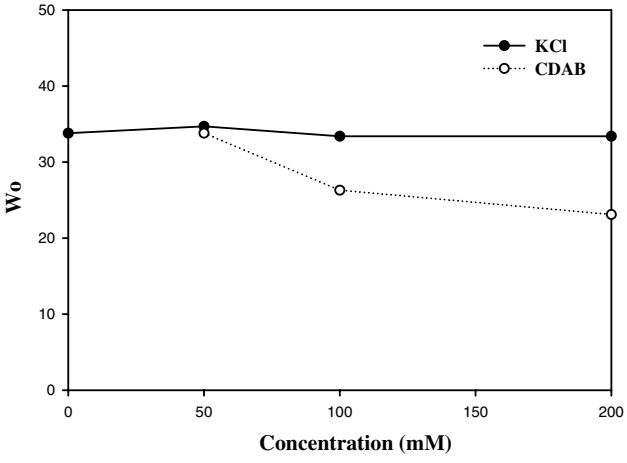

Fig. 3. Effect of KCl and CDAB concentrations on micelle size (Wo).

salt-induced changes in micelle size could be minimized. Additionally, competition between proteins and ionic species (mainly Cl^-) for transfer into reverse micelles might change the overall electrostatic state of the micelle and/or proteins at higher salt concentrations.

Therefore, the decreased protein solubilization in the presence of salt was probably due to an electrostatic screening effect rather than reduced micelle size and resulted in decreased specific activity of lysozyme after the extraction. Although Melo, Aires-Barros, and Cabral (2001) reported that phase separation might not be facilitated below a certain ionic strength of aqueous phase, no such problem was found in phase separation after the extraction. The difficulty of phase separation at low ionic strength was avoided by buffered aqueous phase and inherent salt content of freeze-dried egg powder.

3.3. Effect of CDAB concentration

Protein solubilization is also influenced by parameters related to the organic phase, in addition to the condition of the aqueous phase. Among these, nature of surfactant and its concentration are major contributors affecting protein solubilization. The effect of surfactant concentration on reverse micellar extraction was examined using a predetermined aqueous condition (pH 9, without KCl addition).

Hentsch, Menoud, Steiner, Flaschel, and Renken (1992) reported that protein solubilization was favoured as surfactant concentration increased. However, there was no significant change in specific activity of lysozyme as concentration of CDAB increased from 50 to 100 mM (Table 3). Under these conditions, more than 93% of total recovered protein was lysozyme and major egg-white proteins, including ovalbumin and ovotransferrin, were not detected (Fig. 4). If 200 mM CDAB was used for extraction, no clear separation occured after the extraction. This result might be related to the low ionic strength of the aqueous phase. If high concentration of surfactant (e.g. 200 mM) is present in the organic phase, a certain ionic strength might be required to reduce electrostatic repulsion between the surfactant head groups. Thus, aggregation of surfactant and/or

Table 3
Effect of concentration of CDAB on recovered lysozyme activity after forward extraction

Condition of extraction			Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
pH	KCl (mM)	CDAB (mM)				
Egg-white control			8850[a]	380[a]	1.0	100[a]
9	0	50	8486[a]	12,284[b]	30.4	95.9[a]
9	0	100	8330[a]	12,145[b]	32.0	94.1[a]
9	0	200	–	–	–	–

[a,b] Means with different superscripts within the same column are significantly different ($p < 0.05$).

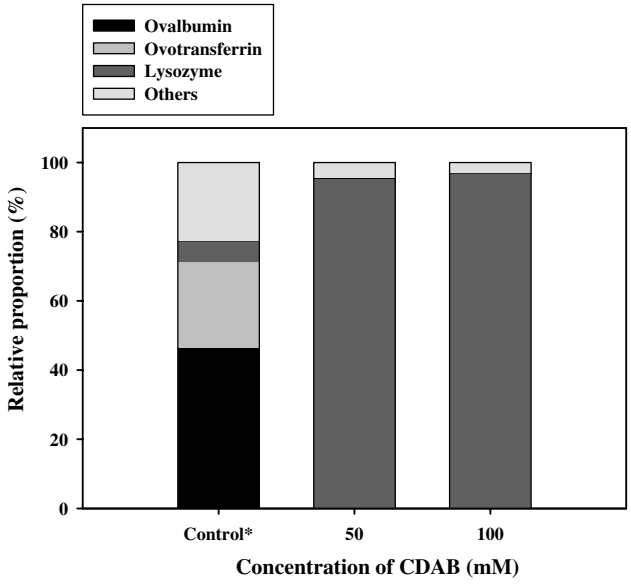

Fig. 4. Proportions of proteins remaining in the aqueous phase after forward extraction of the reconstituted freeze-dried egg-white at various CDAB concentrations. Control*: reconstituted freeze-dried egg-white The extraction was performed with sodium borate buffer (50 mM, pH 9) and the organic phase containing different CDAB concentrations. The extraction time was 20 min.

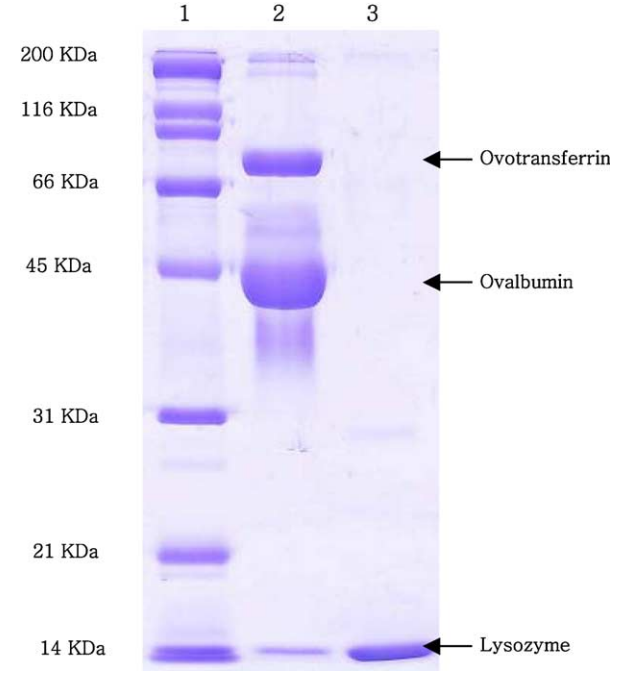

Fig. 5. Electrophoregram of the recovered aqueous phase after forward extraction. The extraction was performed with sodium borate buffer (50 mM, pH 9) and the organic phase containing 50 mM CDAB. The extraction time was 20 min.

the surfactant and protein complex formed a coagulum and induced a phase separation problem.

In addition, the effect of CDAB concentration on micelle size (Wo) was examined. As shown in Fig. 3, micelle size decreased as the concentration of CDAB increased. This result was probably due to the decreased proportion of co-surfactant (hexanol) in the organic phase, since a fixed composition of organic phase (isooctane: hexanol = 1:1, v/v) was used, regardless of CDAB concentration in the organic phase. This reasoning was supported by the report of Wang, Weber, and Vera (1994) that the introduction of a co-surfactant, such as an alcohol, in the cationic surfactant system improved water uptake by reverse micelles, leading to increased micelle size. However, the changes in micelle size might not be large enough to influence protein solubilization.

Based on the above results, 50 mM CDAB was enough for the extraction of lysozyme and 30-fold purified lysozyme was obtained from egg-white solution after reverse micellar extraction. The electrophoregram of recovered aqueous phase under optimum extraction condition (pH 9, no added KCl, 50 mM CDAB) is visualized in Fig. 5. After the forward extraction, most unwanted proteins were successfully moved to the organic phase and 96% of initial lysozyme activity was recovered in the aqueous phase.

4. Conclusion

Lysozyme was conveniently extracted from reconstituted freeze-dried egg-white solution using reverse micelles formed by the cationic surfactant, CDAB. Based on the simplicity, time, cost and yield, the current method has an advantage for the separation of lysozyme compared to classical separation technology or traditional forward and backward extraction procedures.

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