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Separation of Lactoferrin from Model Whey Protein Mixture by Reverse Micelles Formed by Cationic Surfactant

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Abstract The selective extraction behavior of lactoferrin (Lf) from whey protein mixture was examined using reverse micelles formed by the cationic surfactant, cetyldimethylammonium bromide (CDAB). The major whey proteins, including β-lactoglobulin, α-lactalbumin and bovine serum albumin, were solubilized from aqueous phase to organic phase while Lf was recovered in the aqueous phase. The solubilization behaviors of the proteins were manipulated by the process parameters such as the pH and salt concentration of the aqueous phase and the surfactant concentration in the organic phase. Efficient forward extraction was achieved with sodium borate buffer (50 mM, pH 9) containing 50 mM KCl and organic phase containing 100 mM CDAB. Based on SDS-PAGE and densitometry, about 96% of the initial Lf remained in the aqueous phase after forward extraction. The dialyzed Lf fully maintained its bacteriostatic activity against *E. coli* O157:H7.

Keywords: lactoferrin, reverse micelle, extraction, cationic surfactant, whey protein

Introduction

Lactoferrin (Lf) is found in milk or the secretions of some mammals and is present in relatively high concentrations (~20% of total protein) in human milk (1). According to Shimazaki (2), the concentration of Lf ranges from 0.02 to 0.2 mg/mL in bovine milk and human milk contains about 10 times more Lf than cows milk does. Lf is a single chain glycoprotein of about 80,000 Da and bovine and human Lfs have 69% sequence homology (3). Among the various biologically active substances in milk, Lf is of special interest because of its antimicrobial activity against many infectious microorganisms and its immunomodulatory functions in the host defense system (4). Traditionally, proteins have been separated either by ultrafiltration or by chromatographic procedures (5, 6). Chromatographic separations have advantages for the purification of specific proteins but are usually time consuming and frequently require complex preparatory procedures such as ammonium sulfate or ethanol treatments. In this context, chromatographic separations are not well suited for commercial preparation.

As an attractive separation technique, the system of water in oil microemulsion, which is called reverse micelles (RM), has gained attention due to its fast, simple separation procedure and high selectivity. The industrial importance also has been claimed because RM extraction could be operated as a large scale continuous process (7). RM is the spontaneous aggregation of surfactant molecules containing an inner core of water molecules, dispersed in a continuous organic solvent medium. 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 (8).

In liquid-liquid RM extraction process, a target protein is selectively solubilized into organic phase (forward extraction) and subsequently is stripped into aqueous phase (backward extraction) by the addition of fresh aqueous buffer (9). The forward extraction is governed primarily by electrostatic interactions between the charged protein and polar head of surfactant (10) and is also affected by hydrophobic interactions between the non-polar region of the proteins and surfactant tail (11). For backward extraction, the working pH and ionic strength of the aqueous buffer should be controlled to provide the same charges for proteins and surfactant. However, this backward extraction often causes problems such as low yield and coextraction of surfactant (12, 13).

To date, the extensive previous studies regarding RM extraction have been mostly focused on partitioning behaviors of purified proteins using the well-known anionic surfactant, sodium di (2-ethylhexyl) sulfosuccinate, (AOT) by typical forward and backward extraction (14, 15, 16) and only limited studies have been conducted on protein mixtures. In this study, a one-step separation method was devised to drive the solubilization of unwanted proteins into RM formed by cationic surfactant, cetyldimethylammonium bromide (CDAB), while the target protein remained in an aqueous phase. Therefore, the target protein (Lf) was efficiently recovered after only forward extraction was performed, without requiring the troublesome backward extraction step. The aim of this study was to examine the parameters affecting Lf separation from model whey protein mixtures and to suggest optimal separation procedures.

Materials and Methods

Materials Whey protein isolate (WPI) was obtained from Schils Food (Sittard, the Netherlands) and Lf from Tatua (Morrinsville, New Zealand). Isooctane and hexanol were

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obtained from J. T. Baker (Phillipsburg, NJ) and Acros (Fairlawn, NJ), respectively. CDAB and all other chemicals were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

Separation of Lf using reversed micellar extraction The model aqueous phase was prepared by dissolving Lf-fortified WPI in various buffers. The total protein concentration in the aqueous phase was 0.1% (w/v) and Lf-fortified WPI contained 20% Lf on a dry weight basis. The desired pH was adjusted using sodium citrate buffer (50 mM, pH 6), sodium phosphate buffer (50 mM, pH 7-8) and sodium borate buffer (50 mM, pH 9-10), while ionic strength was adjusted by the addition of KCl (0-200 mM). The aqueous phase (10 mL) was mixed with an equal volume of organic phase (isooctane:hexanol=1:1, v/v) containing a given concentration of CDAB. The forward extraction was carried out for 30 min at 25°C in a tightly stopped glass flask (50 mL) using a shaking incubator (Vision Scientific, Korea) with an agitation speed of 300 rpm. After extraction, the resulting mixture was separated by centrifugation at 2800 × g for 5 min and the aqueous phase was collected for analysis.

Protein concentration and profiles in the aqueous phase after forward extraction Total protein concentration and the profiles of proteins in the aqueous phases before and after forward extraction were determined by Lowry method (17) and SDS-PAGE (18). SDS-PAGE was carried out on 12% separating and 4% stacking gel using a mini-PROTEAN II cell (Bio-Rad Laboratories, Hercules, CA). 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 were 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, NY). The extent of each protein transfer was defined as the percentage of individual protein extracted to the micellar phase compared to the protein initially present in the aqueous phase and was determined by the changes of band intensity in the aqueous phase before and after extraction.

Factors affecting Lf recovery The effects of pH, ionic strength and surfactant concentration on Lf recovery were examined. For forward extraction, the pH of the aqueous phase was adjusted with the buffers from pH 6 to 10. The buffer solutions used were sodium citrate buffer (50 mM, pH 6), sodium phosphate buffer (50 mM, pH 7-8) and sodium borate buffer (50 mM, pH 9-10). Based on preliminary experiments, the minimum surfactant concentration (CDAB, 50 mM) and ionic strength (KCl, 50 mM) that allowed protein transfer were used for appropriate pH determination. To determine the effect of ionic strength on Lf recovery, aqueous phases (pH 9, optimal pH region) containing various KCl concentrations (0 - 200 mM) were used for the extraction. The effect of surfactant concentration (50 - 200 mM) was monitored at a predetermined aqueous pH and KCl concentration (pH 9 and 50 mM KCl).

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

Determination of bacteriostatic activity The bacteriostatic activity of Lf was determined by the method of Jo et al. (19) with slight modifications. The iron free Lf was prepared from the recovered aqueous phase (50 mM sodium borate buffer, pH 9, containing 50 mM KCl /100 mM CDAB) and control Lf using EDTA (100 mM) treatment followed by dialysis (12,000 MW cut off, Sigma Chemical Co., St. Louis, MO) against 30 times, double-distilled water. The freeze-dried, iron free Lfs were filtered through membrane filter (0.45 μm, Whatman International Ltd., England) and were added to sterilized basal medium containing 0.65% peptone and 0.0325% $NaHCO_3$. The concentration of Lf was 500 μg/mL in the medium. *E. coli* O157:H7 ATCC 35150 was inoculated to the medium to achieve 3.5×10^7 CFU/mL and the medium was then incubated at 37°C for 11 hr. The bacterial growth was monitored at 660 nm using Ultrospec 2100 spectrophotometer (Amersham Pharmacia Biotech., Sweden). All assays were performed in triplicate.

Results and Discussion

Effect of pH Among the factors affecting forward extraction, the pH of the initial aqueous phase is critical since it dictates the partition between surfactant and protein by controlling the charge distribution of the protein surface. Fig. 1 shows the percentage of individual protein remaining in the aqueous phase after the forward extraction in the pH ranged from 6 to 10. The protein uptake to the organic

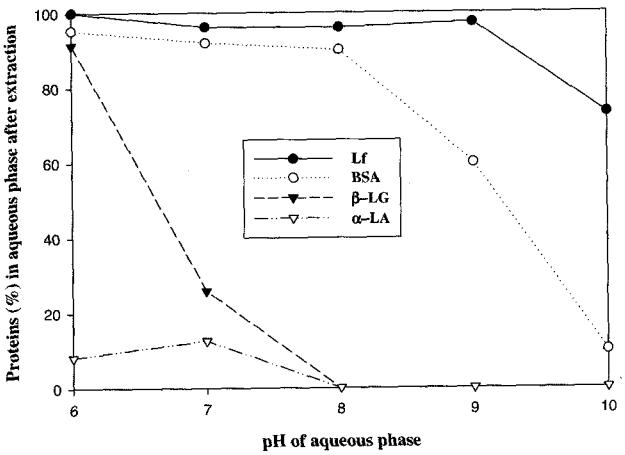

Fig. 1. Proteins recovered in the aqueous phase after forward extraction of the Lf-fortified whey protein isolate at various pHs. Lf: lactoferrin; BSA: bovine serum albumin; β-LG: β-lactoglobulin; α-LA: α-lactalbumin. The forward extraction was performed with various buffers containing 50 mM KCl and the organic phase containing 50 mM CDAB. The buffer solutions used were sodium citrate buffer (50 mM, pH 6), sodium phosphate buffer (50 mM, pH 7-8) and sodium borate buffer (50 mM, pH 9-10).

phase occurred readily when the aqueous pH was above the isoelectric point (pI) of each protein. This result was mainly due to increased electrostatic interaction between the cationic surfactant and negatively charged protein molecules. This simple partitioning behavior was well matched for the proteins with low molecular weight (MW) such as β-lactoglobulin (β-LG, 18,000 dalton) and α-lactalbumin (α-LA, 14,000 dalton). As the aqueous pH was increased from pI of these two proteins (β-LG: 5.2; α-LA: 4.2-4.5) the uptake of proteins to the micellar phase was greatly increased. Thus, only 26% of initial β-LG and 13% of initial α-LA remained after forward extraction at pH 7 and these proteins became undetectable when the forward extraction was carried out at pH above 7.

For high MW proteins such as bovine serum albumin (BSA, 66,000 dalton), the extent of protein transfer to the organic phase was largely inhibited even though its pI (4.7-4.9) is lower than that of β-LG. More than 90% of initial BSA remained in the aqueous phase after extraction at pH 8. As the extraction pH increased, BSA uptake was increased and 60% and 10% of BSA remained in the aqueous phase after extraction at pH 9 and 10, respectively. However, 100% BSA transfer was not feasible in the tested pH conditions. This result was in agreement with the report of Dekker et al. (8) that more extensive electrostatic interactions are required to achieve appreciable solubilization of large proteins.

In the case of Lf, solubilization to organic phase barely occurred in the pH range from 6 to 9 and about 96% of the initial Lf remained in the aqueous phase after forward extraction. Under this pH region, electrostatic interaction between cationic surfactant and Lf was unexpected since pI of Lf is 8.4-9.0 and as Lf has an overall positive charge. When the extraction was made at pH 10, about 27% of the initial Lf had disappeared from the aqueous phase. Considering MW of Lf (80,000 dalton), solubilization of Lf was relatively higher than that of BSA at a given aqueous pH and pI distance. The reason for this phenomenon was not clear but structural characteristics such as strong cation binding potential located in the N-terminal region of Lf might facilitate binding between cationic surfactant and Lf.

One thing that needs to be noted is that the depletion of protein in the aqueous phase does not necessarily mean solubilization of proteins into the organic micellar phase. A large amount of white precipitates were observed at the interface, especially when the extraction was performed at pH 8 and 9. This indicates the possibility of protein aggregation rather than solubilization into the organic phase. However, the final destination of various proteins during extraction was not further characterized since most of the target protein (Lf) remained in the aqueous phase. Goto et al. (20) also reported this interfacial protein complex and they explained that insufficient hydrophobicity was one of the reasons responsible for the formation of the interfacial complex.

Based on the above results, the optimal pH for the separation of Lf was determined to be 9. In order to increase the purity of Lf in the recovered aqueous phase, other parameters, including ionic strength and surfactant concentrations, were adjusted and further experiments were conducted.

Effect of ionic strength The changes in profile and total protein content of the aqueous phase as a function of ionic strength are shown in Figs. 2 and 3. When salt free aqueous buffer (50 mM sodium borate buffer, pH 9.0) was used, only about 10% of the initial Lf was recovered in the aqueous phase after extraction. At this condition, the amount of white aggregate at the interface was greatly increased and a clear separation was not obtained by centrifugation. As the KCl concentration increased, the total protein content in the recovered aqueous phase was increased (Fig. 3). This result suggested that protein solubilization was inhibited by the increased KCl content. Similar behaviors have been reported in other studies (21, 22). The decreased protein solubilization at high salt concentrations can be explained in two ways. Firstly, an

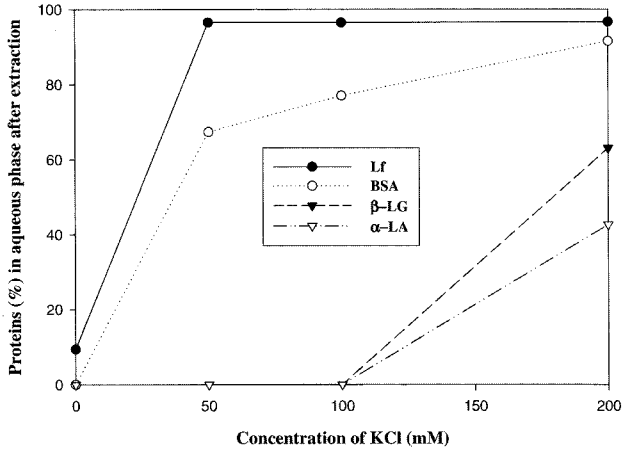

Fig. 2. Proteins recovered in the aqueous phase after forward extraction of the Lf-fortified whey protein isolate at various KCl concentrations. Lf: lactoferrin; BSA: bovine serum albumin; β-LG: β-lactoglobulin; α-LA: α-lactalbumin. The forward extraction was performed with sodium borate buffer (50 mM, pH 9.0) containing designated KCl concentrations and the organic phase containing 50 mM CDAB.

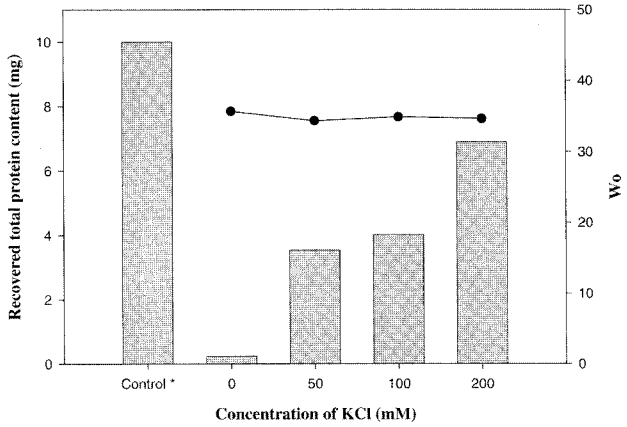

Fig. 3. Changes in total protein content and Wo values after forward extraction of the Lf-fortified whey protein isolate at various KCl concentrations. Control*: protein content in the aqueous phase before forward extraction; solid circles indicate Wo values at the designated extraction conditions. The forward extraction was performed with sodium borate buffer (50 mM, pH 9.0) containing designated KCl concentrations and the organic phase containing 50 mM CDAB.

electrostatic screening effect of the proteins' net negative charge occurs so that proteins can not be solubilized by the positively charged CDAB. Secondly, the high salt concentration decreases the micelle size due to the reduced electrostatic repulsion between the surfactant head groups. The inverse relationship between salt concentra-tion and micelle size was experimentally demonstrated in RM formed by anionic detergents such as AOT (23). However, the Wo values, which indicate the RM size, were almost the same at the tested salt concentrations (Fig. 3). This result was probably due to the effect of the cosurfactant, hexanol, used in the organic phase. As depicted by Lu (24), hexanol is located between the surfactant head groups and seems to act as a buffer for repulsive electrostatic interaction between surfactant head groups, thereby allowing close packing of the RM inner core. Thus, the salt-induced changes in micelle size could be minimized. Based on this result, the electrostatic screening effect is the main contributor to the decreased protein solubilization since the water pool content was barely changed by the salt concentrations.

Lf recovery was greatly increased at 50 mM KCl and about 95% of initial Lf remained in the aqueous phase. As the KCl concentration increased, the BSA content remaining in the aqueous phase was also increased. At 200 mM KCl, solubilization of β-LG and α-LA were significantly reduced and 63% and 43% of the initial β-LG and α-LA, respectively, remained in the aqueous phase after forward extraction. From the above experiment, 50 mM KCl was chosen for Lf separation.

Effect of CDAB concentration and extraction time The optimal aqueous condition, such as pH and salt concentration, for the recovery of Lf was determined by the above experiments. At such condition, major whey proteins, such as β-LG and α-LA, were effectively solubilized into the organic phase whereas about 65% of initial BSA remained in the aqueous phase along with Lf. In order to increase the purity of Lf in the recovered aqueous phase, the effects of other operating parameters, CDAB concentration and extraction time were examined.

The effect of CDAB concentration on the protein partitioning was examined using sodium borate buffer, pH 9.0, containing 50 mM KCl. As shown in Fig. 4, about 65% of BSA remained in the aqueous phase at low CDAB concentrations such as 50 mM. At the extraction with 100 mM CDAB, virtually no BSA was detected in the aqueous phase, having all been transferred to the interface and/or organic phases. The total protein concentration in the aqueous phase was also decreased as the CDAB concentration increased (Fig. 5). It has been known that the concentration of surfactant had little effect on the structure and size of RMs formed by anionic surfactant (8). The increase in surfactant concentration until a certain level improved the solubilization capacity through the increased number of RMs but above a certain limit solubilization potential decreases due to micellar clustering (25).

In the case of cationic surfactant systems the relationship between surfactant concentration and micelle size is rather complicated because of the presence of cosurfactant. Hilhorst et al. (26) reported that Wo of the TOMAC/octane system was varied by the amount and nature of cosurfactant.

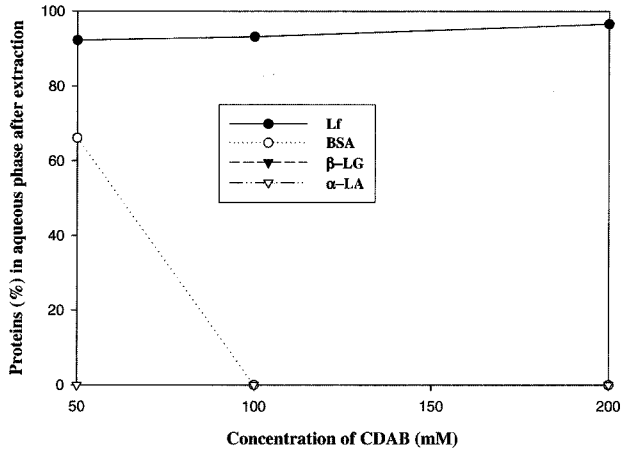

Fig. 4. Proteins recovered in the aqueous phase after forward extraction of the Lf-fortified whey protein isolate at various CDAB concentrations. Lf: lactoferrin; BSA: bovine serum albumin; β-LG: β-lactoglobulin; α-LA: α-lactalbumin. The forward extraction was performed with sodium borate buffer (50 mM, pH 9.0) containing 50 mM KCl and the organic phase containing designated CDAB concentrations.

As shown in Fig. 5, Wo values decreased from 34 to 23 as CDAB concentration increased. This result might have been due to the decreased proportion of cosurfactant (hexanol) 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 et al. (27) that the introduction of cosurfactant such as an alcohol in a cationic surfactant system improved the water uptake by RM, leading to increased micelle size.

Generally, the solubilization of protein within RM depends on the protein size relative to that of RM droplets. However, the decreased Wo at high CDAB concentrations did not critically affect the movement of BSA and it did not remain in the aqueous phase when 200 mM CDAB

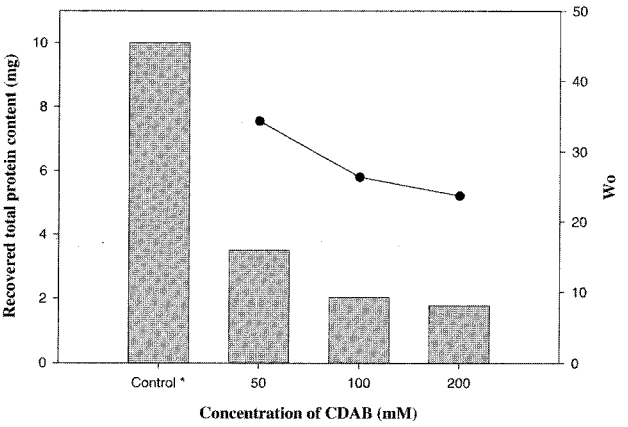

Fig. 5. Changes in total protein content and Wo value after forward extraction of the Lf-fortified whey protein isolate at various CDAB concentrations. Control*: protein content in the aqueous phase before forward extraction. Solid circles indicate Wo values at the designated extraction conditions. The forward extraction was performed with sodium borate buffer (50 mM, pH 9.0) containing 50 mM KCl and the organic phase containing designated CDAB concentrations.

was used for extraction (Fig. 4). This result was consistent with the report of Shiomori et al. (28) that Wo values higher than 15 did not influence BSA solubilization.

The CDAB concentration did not have an effect on the Lf recovery and about 96% of initial Lf remained after forward extraction, regardless of the CDAB concentration. Based on the results, 100 mM CDAB was enough to separate Lf from whey proteins since low surfactant concentration is advantageous in minimizing possible irreversible formation of Lf and surfactant complex.

The effect of extraction time on Lf recovery was examined using the optimized condition (pH 9.0 sodium borate buffer containing 50 mM KCl / 100 mM AOT). As shown in Fig. 6, Lf recovery was not changed for extraction times up to 50 min. This indicates that equilibrium during phase contact was established in a short time and caused the transfer of other proteins into the organic phase. At an extraction time of 60 min, Lf recovery was slightly decreased from 96 to 90%. This extended extraction time might have resulted in partial solubilization of Lf into the organic phase or possibly have formed an aggregate at the interface.

Bacteriostatic activity of recovered lactoferrin The electrophoregram of the recovered aqueous phase at the optimum condition is presented in Fig. 7. After the forward extraction, most unwanted proteins were successfully moved to the organic phase and only the Lf band was detected in the recovered aqueous phase.

To verify the bacteriostatic activity after RM extraction, the growth inhibitory effect of the recovered Lf against *E. coli* O157:H7 was tested at a concentration of 500 μg/mL. As shown in Fig. 8, the bacterial growth was significantly inhibited in the presence of Lf and there was no difference in bacteriostatic activity before and after RM extraction. This result indicated that the bacteriostatic activity of the Lf separated by RM extraction was fully maintained.

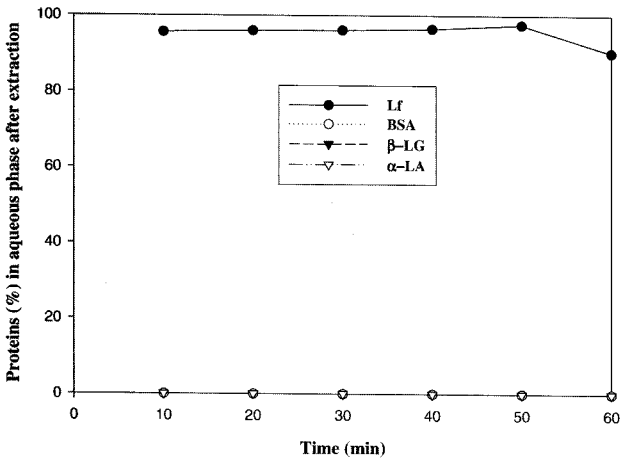

Fig. 6. Proteins recovered in the aqueous phase after forward extraction of the Lf-fortified whey protein isolate at various extraction times. Lf: lactoferrin; BSA: bovine serum albumin; β-LG: β-lactoglobulin; α-LA: α-lactalbumin. The forward extraction was performed with sodium borate buffer (50 mM, pH 9.0) containing 50 mM KCl and the organic phase containing 100 mM CDAB.

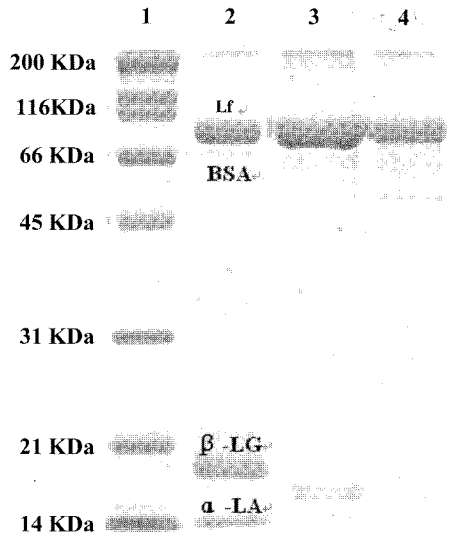

Fig. 7. Electrophoregram of the recovered aqueous phase after forward extraction. Lf: lactoferrin; BSA: bovine serum albumin; β-LG: β-lactoglobulin; α-LA: α-lactalbumin. (1) Molecular weight standard, (2) Lf-fortified whey protein isolate, (3) the recovered aqueous phase after forward extraction, and (4) Lf standard. The forward extraction was performed with sodium borate buffer (50 mM, pH 9.0) containing 50 mM KCl and the organic phase containing 100 mM CDAB.

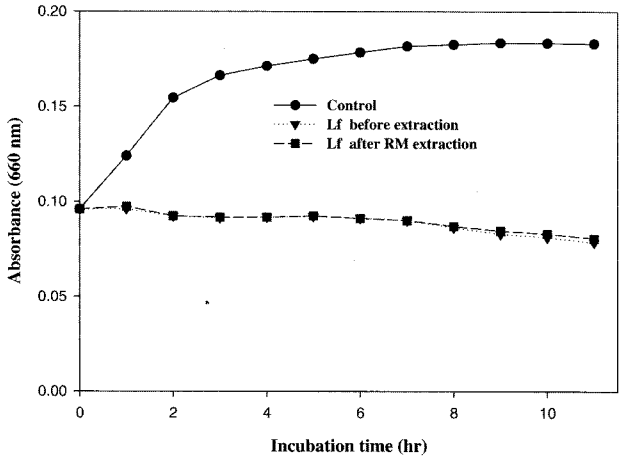

Fig. 8. Bacteriostatic activity of the recovered aqueous phase.

Conclusions

Lf was successfully extracted from the model dairy protein mixture using RM formed by the cationic surfactant, CDAB. The solubilization behaviors of individual proteins could be manipulated with various process parameters including the pH and salt concentration of the aqueous phase and the concentration of surfactant in the organic phase. Considering that surfactants can be coextracted with proteins during backward extraction, the current method has an advantage over traditional forward and backward extraction procedures for the separation of valuable proteins. The suggested separation methodology can be applied to the separation of basic proteins (e.g. Lf and lactoperoxidase) from whey containing a large proportion of acidic proteins (e.g. β-LG, α-LA and BSA).

Acknowledgments

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