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REVERSE MICELLES EXTRACTION OF LACTOFERRIN USING CATIONIC SURFACTANT FROM WHEY

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ABSTRACT

A promising whey protein separation technique, Reverse micelles (RM) extraction could be an important tool for obtaining the valuable food ingredients. RM techniques have the potential to continuously extract specific proteins from an aqueous mixture, achieving simultaneous concentration and purification of specific proteins in an efficient manner. The extraction of lactoferrin (LF) protein, using nano-sized reverse micelles of nonionic surfactant polyoxyethylene p-t-octylphenol (triton-X-100) is attempted here. Suitability of reverse micelles of cationic surfactant cetyl trimethylammonium bromide (CTAB) and triton-X-100/CTAB mixture in organic solvent for LF extraction is also investigated. The raw milk treated with 1M HCl at pH 6.5 and centrifuged to get whey sample. The whey sample was concentrated with 0.1µm membrane enrichment and diluted five times to its original volume with 50 mM phosphate buffer at pH 6.35 containing 100mM sodium chloride. The aqueous solution was then mixed with an equal volume of organic solvents containing the surfactant. The aqueous phase and reverse micelles phase has been separated by centrifugation and the protein is accumulated in the aqueous phase. The extract was fractionated using ammonium sulphate and purity improvements are done by HIC (Hi Trap Phenyl) column and followed by IEX(DEAE cellulose) column chromatography which was previously equilibrated with phosphate buffer with the pH maintained at 7.8 and eluted by increasing ionic strength with sodium chloride. The results show that the LF evolves more in CTAB than in CTAB+ triton-X-100 mode which may be due to reduced hydrophobic interaction**.**

Key words: Reverse micelles extraction, Cationic, CTAB, Nonionic, Triton-X-100, Surfactants.

INTRODUCTION

Lactoferrin is an ion-binding glycoprotein present in milk as well as other exocrine secretions and neutrophil granules in mammals. The lactoferrin concentration in bovine (cows) milk is only 0.5% to 1.0% while human breast milk can contain as much as 15% lactoferrin. The first major application of bovine lactoferrin was the addition to infant formulas to further humanize breast milk replacers. Lactoferrin is considered to be an important host defense molecule. During the past decade, it has become evident that oral administration of lactoferrin exerts several beneficial effects on the health of humans and animals, including anticancer, anti-

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inflammatory effects, antimicrobial/antiviral activities, immune modulatory activity, and antioxidant activity. Generally, chromatographic methods have been used for the separation of LF from mammalian milk but they are time consuming and not well suited for scale up.

The reversed micellar technique on the other hand, is an alternative to chromatographic procedures, since the system can be easily scaled up, and the principle allows for continuous separation processes, similar to the liquid-liquid extraction processes, which are commonly used in the chemical industry. Protein extraction from aqueous solution by reverse micelles is a process that utilizes basic techniques of chemical engineering such as classical liquid–liquid extraction, and thus has the potential for industrial application[3]. RM phase technology is based on charge-charge interaction hydrophobicity, and the size of the protein relative to the droplet. Reverse micelles are able to host the proteins in an aqueous environment, effectively shielding them from a nonmiscible one. The RM solvent contains small droplets of water, stabilized within an organic solvent by a surfactant. Because protein molecules often move from an original water phase into these small, encapsulated water droplets, RM extraction is attractive for separating proteins from an aqueous solution. In RM separation, not only protein molecules remain intact, but also large scale-up of the necessary solutions is relatively uncomplicated. This process can be used to separate biological products such as proteins, which have been solubilized in organic solvents using surfactants, without affecting their functional properties[4]. The process can be performed by a forward extraction of the target protein or contaminants, from an aqueous solution to a reversed micellar organic phase, followed by back-extraction, during which the biomolecules are released from micelles and transferred to a new aqueous phase[5]. In this study, the reversed micellar extraction procedure was investigated to separate Lactoferrin from colostral whey. The objective was to establish a proper procedure and optimal conditions for an efficient separation of Lactoferrin, and obtain a purified product.

EXPERIMENTAL

Materials

Bovine milk

Bovine milk, obtained from a pool of different cows raised in a local dairy farm.

Chemicals used

Cetyltrimethyl ammonium bromide (CTAB), Iso octane (HPLC grade), butanol, Tris HCl, sodium dodecyl sulphate (SDS), β-mercaptoethanol, Coomassie brilliant blue R-250, ethanol**,** Triton X 100.

Methodology

Sample preparation

Skimmed milk was obtained by centrifuging the bovine milk at 10,000 rpm for 10 min at 4^{o}C to remove fats.

Casein removal

Centrifugation of the skim milk in an ultracentrifuge (usually about 50,000 x g or greater) results in pelleting of the casein and in a supernatant called whey (also sometimes called the serum phase of milk) which contains the water, lactose and soluble non-casein proteins. Once casein is removed, then by definition every other protein left in the milk preparation is a whey protein. .

Casein molecules can also be separated from the whey by precipitation of the casein with acid. A classic precipitation method for casein in cow milk which is done in the laboratory is to slowly add HCl (0.1 N) to lower the milk pH to 4.6. The casein will gradually form a precipitate while relatively little of the other milk proteins will precipitate.

Reverse miceller extraction

For the model system study, 5 mL of standard protein solution in phosphate buffers of various pH with ionic strength of 0.05-0.55, were mixed with an equal volume of micellar solution of various concentration of CTAB in iso-octane. For the real system, 5 mL of the aqueous solutions were prepared by adding various amounts of colostral whey (0.2 to 5.0 mL) to 50 mM sodium phosphate buffer at various pH (4.0 to 7.0) and containing various concentrations of NaCl (0 to 200 mM). The aqueous solutions were mixed with 5 ml of the isooctane solutions containing various concentration of CTAB. Forward extraction was carried out by shaking the mixtures at 200 rpm for 10 min at room temperature, and the two phases were separated by centrifugation at 500 g for 30 min. Back extraction was carried out by mixing the organic phase obtained from forward extraction with a fresh aqueous phase, also termed as stripping phase (buffer of known pH containing KBr). The phases were mixed thoroughly for 1 h and centrifuged at 4000 g for 15 min during both forward and back extraction. The recovery of whey protein in the reversed micellar phase (organic phase) or in the aqueous phase after extraction was calculated as the ratio of protein concentration in the reversed micellar phase/aqueous phase after extraction to the initial protein concentration in the aqueous phase before extraction.

To optimize the conditions, a general procedure for determining the effects of pH, concentrations of CTAB as well as NaCl was carried out in which effect of each variable was observed and plotted.

Another system containing the non -ionic surfactant characteristics Triton X-100 combined with the cationic charged CTAB was also employed for the extraction. While carrying out the subsequent separation methods namely liquid chromatography and SDS-electrophoresis for the Triton X-100/CTAB combination,appreciable yield/output was not obtained. Because of this unusual response the output of this system, inferences for this trial has not been include here.

SDS page electrophoresis

SDS-PAGE was performed based on the methods of Laemlli (1970) after modification. The stacking gel was 4.5% polyacrylamide in 0.125 M Tris-HCl buffer at pH 6.8. The separation gel was 12.5% polyacrylamide. Samples were prepared in 0.0625 M Tris buffer at pH 6.8

containing 1% SDS and 2.5% β-mercaptoethanol, and heated at 100°C for 2 min. Coomassie brilliant blue R-250 was used for silver staining and observing bands.

Liquid chromatography (cationic)

IEX (DEAE cellulose) column chromatography were used to adsorb lactoferrin protein from the sample after reverse micellar extraction. Resin was equilibrated before use in 10 mM phosphate buffer (10 mM mono and dibasic sodium phosphate) at pH 6.7. Protein elution was achieved in the same buffer using either gradient (0 to 1.0 M NaCl) or step elutions. Step elutions were carried out by taking 1.0 M NaCl to elute lactoferrin.

RESULTS AND DISCUSSION

Effect of pH on extraction: (Fig. 1)

Protein solubilization in reversed micellar systems mainly depends on electrostatic interactions between biomolecules (proteins) and charged surfactant heads as well as the aggregation properties of surfactant[1]. Effect of pH on lactoferrin extraction was optimized where as salt (30 mM NaCl) and surfactant concentrations (80 mM CTAB/iso-octane) were kept constant. The pH of the first aqueous phase containing our desired protein was varied between 3.0 and 12.0, using different buffer systems according to their pI values. The results showed transfer of protein to the micellar phase is high within the pH range of 8.0-9.0. It suggests that pH 9.0 was more selective, probably due to favorable attractive electrostatic interactions between negatively charged lactoferrin molecules at pH 9.0 (lactoferrin has a pI 7.5 as previously determined in our laboratory) and positively charged surfactant heads[1].

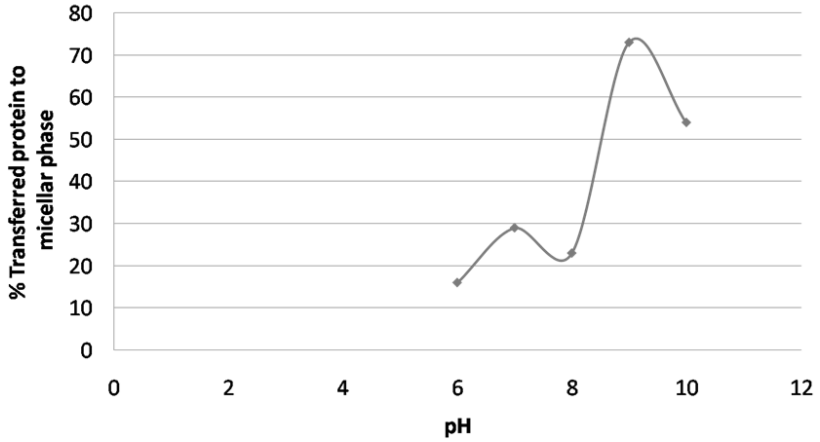

Fig. 1: Effect of aqueous pH

As the pH of aqueous phase increased above 9, the extracted protein decreased ca. of 34% probably due to the degradation of the lactoferrin due to highly basic pH[2]. The protein hydrophobic patches exposed may interact with cationic surfactant hydrophobic tails minimizing

extraction efficiency[2]. Besides the protein charge, the density of surface charge is an important factor on protein solubilization. Therefore, the optimized pH to be used in further steps was 9.0.

Effect of CTAB concentration: (Fig. 2)

Protein solubilization is also strongly dependent on the concentration of surfactant and on the size of the reverse micelle. For a CTAB concentration of 25mM, no phase separation occurred and for CTAB concentrations of 35 mM and 40 mM an increase in phase separation (aqueous phase/micellar phase) was observed, but no protein was transferred to the micellar phase. The protein extraction efficiency was 60% at CTAB concentration of above 50 mM, remaining constant at 70% for CTAB concentrations between 70 and 85 mM and 5% decrease in efficiency for 100 mM CTAB due to critical micelle concentration[3]. Considering these results into account, the 80 mM CTAB concentration was chosen for the subsequent steps since it might be expected that a high surfactant concentration makes it difficult for the backward transfer of proteins into a second aqueous phase[4].

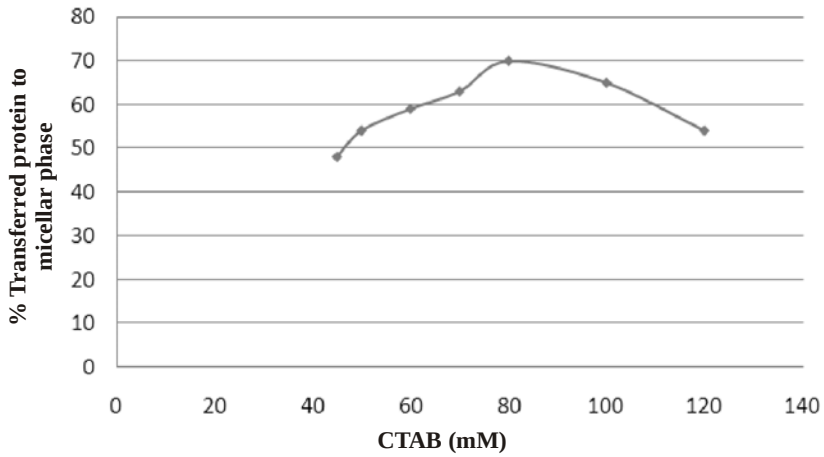

Fig. 2: Effect of CTAB concentration

Effect of ion concentration: (Fig. 3)

The presence of water structure forming salt such as NaCl enhances the stability of the reverse micelles, resulting in higher extent of forward extraction efficiency. The water content decreased as the sodium chloride concentration increased. The positively charged sodium ion could interact with the negatively charged surfactant head and enter the inner core of the reverse micelles. The sodium ion reduced the surfactant head group repulsions and led to the formation of reverse micelles with small sizes. Also, an increase of salt concentration may cause an electrostatic screening effect, which reduces the electrostatic interaction between protein and CTAB and decreases the size of reverse micelle. Hence, in the present study, NaCl concentration in the aqueous phase was increased up to 0.3 M, while maintaining the aqueous pH (8.0) and CTAB concentration 80mM. When NaCl was not added, the phases turned cloudy and the extraction efficiency was very less (17%). The best results were obtained at NaCl concentration

of 0.1 M. When the NaCl concentration was increased above 0.1 M decrease in extraction efficiency was observed. The 'size exclusion' effect caused by the higher salt concentration could be the reason for the above observation. For all further studies, NaCl concentration was maintained at 0.1 M.

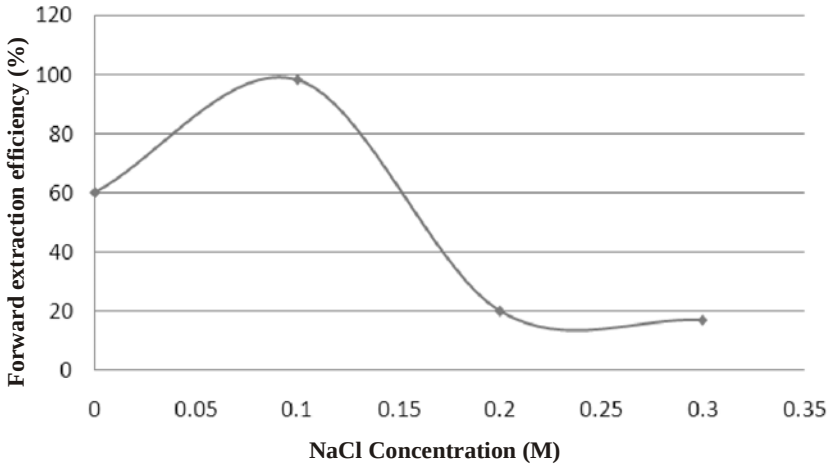

Fig. 3: Effect of ion concentration

Ion exchange chromatography

The chromatograms for the lactoferrin sample is presented in the Fig. 3. The peak obtained at pH 9 in the elution step. From the peak integration the purification and the concentration was found to be 96% and 166 units.

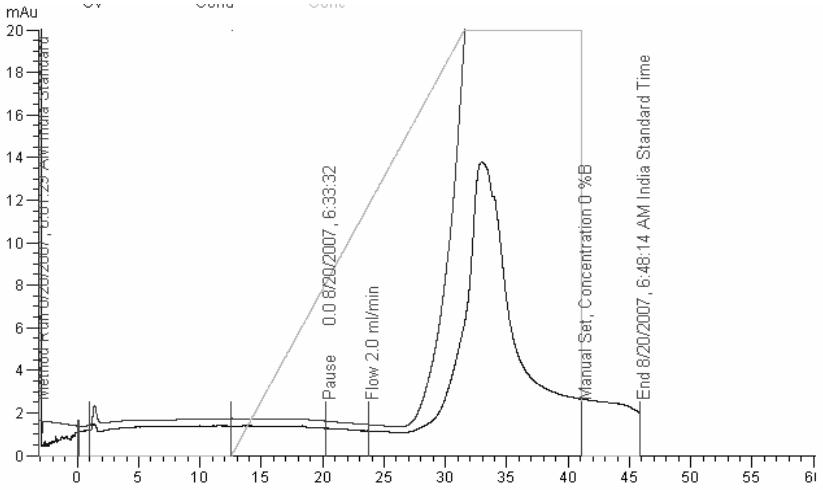

Fig. 4: Ion exchange chromatography: Lactoferrin purification

SDS page electrophoresis

The sample from the LC was used for the SDS page electrophoresis. The sample had been loaded into the gel and electrophoresed for 1 hr at 100 V DC. The staining of gel was done with the help of Coomassie brilliant blue solution and left overnight. Then the gel was washed with distilled water and the band was observed for LF protein at 80kD by comparing with the marker protein as shown in the Fig. 5.

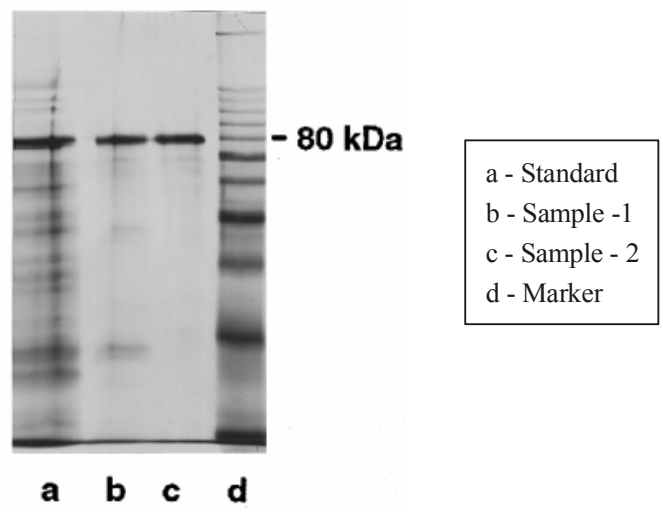

Fig. 5: SDS page electrophoresis

CONCLUSION

Reverese micellar extraction appeared to be a feasible method for separating and purifying Lactoferrin(LF) from other proteins in the colostral whey. The important observation made here is that the best separation was achieved at an aqueous pH 9, CTAB concentration of 80 mM, with an ionic strength of 0.1M NaCl. At this optimal separation condition, more than 96% of Lactoferrin was recovered. Thus, the separation of LF using RM extraction was a simple, highly efficient and an easy to scale up method. Although this study was performed in a model system, the generality of this procedure for other reverse micelle formulations will be explored in the future.

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