Reverse micellar extraction of lactoferrin from its synthetic solution using CTAB/n-heptanol system

Swapnali S. Pawar, Regupathi Iyyaswami & Prasanna D. Belur

Journal of Food Science and Technology

ISSN 0022-1155

J Food Sci Technol
DOI 10.1007/s13197-017-2824-0

Springer

J Food Sci Technol
DOI 10.1007/s13197-017-2824-0

ORIGINAL ARTICLE

Reverse micellar extraction of lactoferrin from its synthetic solution using CTAB/*n*-heptanol system

Swapnali S. Pawar[1] · **Regupathi Iyyaswami**[1] · **Prasanna D. Belur**[1]

Revised: 3 August 2017 / Accepted: 16 August 2017

Abstract The partitioning of Lactoferrin (LF) into the reverse micellar phase formed by a cationic surfactant, cetyltrimethylammonium bromide (CTAB) in *n*-heptanol from the synthetic solution of LF was studied. The solubilization behaviour of LF into the reverse micellar phase and back extraction using a fresh stripping phase were improved by studying the effect of processing parameters, including surfactant concentration, solution pH, electrolyte salt concentration and addition of alcohol as co-solvent. Forward extraction of 100% was achieved at CTAB concentration of 50 mM in *n*-heptanol solvent, pH of 10 and 1 M NaCl. The electrostatic force and hydrophobic interaction have major influence on LF extraction during forward and back extraction respectively. The size of the reverse micelles and their corresponding water content were measured at different operating conditions to assess their role on the LF extraction. The present reverse micellar system has potential to solubilise almost all the LF into the reverse micelles during forward extraction and could able to allow back extraction from the reverse micellar phase with addition of small amount of co-solvent.

Keywords Reverse micellar extraction · Lactoferrin · CTAB · Reverse micelle characterization

Introduction

Lactoferrin (LF), is a multifunctional and an iron-binding protein (Mw ~77–80 kDa) (Adlerova et al. 2008), present in cow milk (>20–200 mg/ml), colostrum breast milk (>7 mg/ml), mature breast milk (>1–2 mg/ml) and biological fluids, such as tears (>2.2 mg/ml), saliva (>7–10 mg/ml) and seminal plasma (>0.4–1.9 mg/ml) (Steijns and Van Hooijdonk 2000). LF plays a key role in maintaining cellular iron level in the body. Along with Fe^{2+} or Fe^{3+} ions, it also binds to metals like Cu^{2+}, Zn^{2+} and Mn^{2+} ions. The antibacterial activity of LF has been proved both in vitro and in vivo against Gram-positive and Gram-negative bacteria and in some acid–alcohol-resistant bacteria (Adlerova et al. 2008). LF shows antiviral activity against RNA and DNA viruses like rotavirus, respiratory syncytial virus, herpes viruses and HIV that infect humans and animals (Gonzalez-Chavez et al. 2009). This protein also provides protection against tumor development and metastasis in several organs including esophagus, tongue, lung, liver, colon and bladder (Ward et al. 2005). LF is incorporated in the milk-based infant formula to mimic the breast milk. For improved oral hygiene, it has been widely used in oral care products also (Steijns and Van Hooijdonk 2000).

Several extraction and purification methods are documented for the extraction of LF individually or with other milk or whey proteins. Ion exchange chromatographic methods are widely employed for the extraction of LF. Carboxy methyl-cellex cation exchange resin was utilized for the separation of LF, lactoperoxidase and lysozyme from camel and bovine milk (Elagamy et al. 1996). Recio and Visser (1999) isolated the antimicrobial peptides (lactoferricin-B) from cheese whey using two different ion-exchange chromatographic methods namely bead-based

Electronic supplementary material The online version of this article (doi:10.1007/s13197-017-2824-0) contains supplementary material, which is available to authorized users.

✉ Regupathi Iyyaswami
regupathi@nitk.ac.in

[1] Department of Chemical Engineering, National Institute of Technology Karnataka, Surathkal, Mangalore 575025, India

Published online: 01 September 2017

cation-exchange chromatography and cation-exchange membrane process. Further, membrane adsorber chromatography method was developed to isolate the bovine LF, lactoperoxidase, lactoferricin from sweet cheese whey (Plate et al. 2006). Isolation of LF from human milk by metal-chelate affinity chromatography was reported by Lonnerdal et al. (1977). Extraction and purification of LF and immunoglobulin G from bovine colostrums and acid whey was carried out by serial cation–anion exchange chromatography (Wu and Xu 2009). A continuous chromatographic technique, Simulated Moving Bed (SMB) technology was utilized by Andersson and Mattiasson (2006) for the separation of lactoperoxidase and LF from whey protein concentrate, and obtained 48% yield with 6.5 times less buffer consumption and 4.8 times higher protein concentration with a better raw material utilization as compared to non-moving bed process. All the reported methods for extraction and purification of LF were conducted in batch process. The high cost of the column, periodic regeneration and maintenance limits the continuous operation of these processes. Moreover, chromatography processes demand the pre-treatment of the sample, which make the separation a multistep purification method. Hence, there is a need for a simple conventional extraction method which can selectively extract the LF and able to operate in the continuous mode. Reverse micellar extraction (RME) can be an alternative, as it is a selective extraction method and all the conventional liquid–liquid extraction equipment may be exploited for the continuous RME.

RME is a commercially potential, purification process to extract the useful proteins (Sadana 1997). Reversed micelles (RM) are water in oil, thermodynamically stable emulsions. It holds protein or enzymes in its inner aqueous core when the organic phase containing selected surfactant is mixed with the feed phase containing the molecule of interest during forward extraction. Further the entrapped proteins in the micellar phase are back extracted to a fresh stripping aqueous phase by mixing vigorously to destabilize the reverse micelle (Chaurasiya et al. 2015). RME of large molecular weight proteins like BSA (Li et al. 2007), haemoglobin (Ono et al. 1996), β-glucosidase (Hemavathi et al. 2010), soy proteins (Zhao et al. 2010) and wide range of enzymes like superoxide dismutase (Wolbert et al. 1989), cutinase (Carvalho et al. 1999), lipase (Gaikaiwari et al. 2012), bromelain (Hebbar et al. 2008; Wan et al. 2016), lysozyme (Shin et al. 2003) and some recombinant enzymes like r-cutinase with commercial importance are reported (Melo et al. 1995). RME process is also used to extract the antibiotics like amoxicillin (Chuo et al. 2014), penicillin G (Mohd-Setapar et al. 2009) with improved extraction efficiency up to 95%. The present work is focused on the RME of bovine LF from the synthetic mixture using CTAB/*n*-heptanol mixture as a potential reverse micellar system and studied the effect of variables like concentration of surfactant, pH of the feed phase, concentration of electrolytes, addition of co-solvents and phase volume ratio during forward and back extraction of LF.

Materials and methods

Materials

CTAB of 99% purity and bovine LF were procured from Sigma Aldrich, India and used without further purifications. Bovine serum albumin (BSA) was purchased from HiMedia, India. Folin–Ciocalteu reagent (FCR) was purchased from Merck, India. Other organic solvents, *n*-heptanol, *n*-butanol, *n*-heptanol, *n*-decanol were obtained from Loba Chemie, India. Inorganic salts like potassium chloride (KCl), Sodium chloride (NaCl) were procured from Spectrum chemicals, India.

Reverse micellar extraction

The CTAB/*n*-heptanol mixture was considered as an organic phase for the formation of RM. The feed aqueous phase was prepared by dissolving the LF at a concentration of 0.1 mg ml^{-1}. 10 ml of reverse micellar system was prepared with a phase volume ratio of 1:1 (organic: aqueous phase) for all the experiments. Forward extraction was carried out by mixing the phases using magnetic stirrer for 20 min at 800 rpm at room temperature. Then the mixture was subjected to centrifugation at 5000 g and 20 min (Remi C-24 plus) for phase separation. The organic phase was carefully separated and used further for back extraction. The effect of surfactant concentration was studied by varying the CTAB concentration in the organic phase between 10 and 100 mM. The effect of pH on the extraction efficiency was studied by adjusting the aqueous phase pH between 2 and 11 using the HCl and NaOH. Influence of ionic strength on protein solubilisation to micellar phase was studied by varying the two salts (KCl and NaCl) concentration from 0.1 to 1.3 M. Further the LF concentration was also varied for a range of 0.04–0.4 mg ml^{-1} to study the effect of protein loading in the system. The effect of co-solvent addition on forward extraction was studied by incorporating the *n*-butanol as co-solvent at 7 and 15% (V/V). The organic phase to aqueous phase volume ratio on the extraction efficiency was also analyzed and reported. In every experiment, the reverse micellar organic phase and the aqueous phases were subjected to the total protein analysis to determine the forward extraction efficiency.

Back extraction was carried out to extract the LF entrapped in the reverse micellar phase. The organic reverse micellar phase (4.5 ml) obtained from forward extraction was mixed with an equal volume of fresh stripping phase using the magnetic stirrer for 60 min at 800 rpm and followed by centrifugation at 5000 g for 30 min. The stripping phase pH was varied to study the effect of pH on the back extraction efficiency. Similar to forward extraction, the ionic strength on protein solubilisation to stripping phase was studied by varying the KCl concentration in the range of 0.3–1.7 M. The destabilization of the micelles was further achieved by adding the alcohols (7–15% volume) like *n*-Propanol, *n*-butanol, *n*-hexanol and *n*-decanol as co-solvents. The effect of phase volume ratio and the contact time (mixing time) were also studied. The separated aqueous and organic phases at each experiment were further subjected to the protein analysis.

LF concentrations in the aqueous and organic phases were measured by Folin–Lowry's assay at wavelength 660 nm using spectrophotometer (UV3000^{+}, Labindia). The BSA was considered as a reference protein. The forward and backward extraction efficiencies were calculated using following equations;

$$\text{Forward Extraction Efficiency}\ (\%) = \left[\frac{\text{LF concentration in organic phase (mg/ml)}}{\text{LF concentration in aqueous feed phase (mg/ml)}}\right] \times 100 \quad (1)$$

$$\text{Back Extraction Efficiency}(\%) = \left[\frac{\text{LF concentration in stripping phase (mg/ml)}}{\text{LF concentration in organic phase (mg/ml)}}\right] \times 100 \quad (2)$$

Reverse micelle characterization

The Karl-Fischer titrator (Metrohm 899 coulometer) was used to determine the amount of water present in the RM phase. The water content (W_0) was represented as a molar ratio of water to surfactant in the RM phase. The W_0 and the size of the RM were measured at the variable combinations at which maximum LF extraction was noticed. The size of the RM was determined using Qudix Scatteroscope, Korea.

HPLC analysis

Qualitative analysis of extracted LF was done by reverse phase HPLC (Shimadzu, LC-20AD, Japan) using C18 (Capcell Pak C18 MG II, Shiseido, Japan) column with size 4.6 ml.D.X 250 mm. Two different mobile phases (A-Trifluoro Acetic Acid (TFA) (0.1%) in water and B-TFA (0.1%) in Acetonitrile) were used. Flow rate was maintained at 0.5 ml/min at column temperature 25 °C to observe elution time of LF using binary gradient mode. Absorbance was recorded at 254 nm using UV detector. Prior to sample injection, column was equilibrated with mobile phase A and B for 15 min.

Results and discussion

The selective extraction of a specific protein in a micellar system is based on the type of surfactant and organic solvent associated with the reverse micellar system. Authors had performed the initial screening of reverse micellar systems formed by different surfactants like, anionic (AOT), cationic (CTAB) and non-ionic (Triton X 100) surfactants with organic solvents for the extraction of LF and the obtained results of the study were discussed elsewhere (Pawar et al. 2017). Based on physicochemical properties like topological surface area (A^{o}), hydrocarbon chain and rotatable bond count of surfactants and organic solvents (Tang et al. 2014), six different RMSs, Triton X 100/Isooctane, AOT/Isooctane, AOT/*n*-decanol, CTAB/Toluene, CTAB/Isooctane, CTAB/*n*-heptanol were screened for the partitioning of LF. CTAB/Isooctane and CTAB/*n*-heptanol were found suitable for favorable LF extraction. Krei and Hustedt (1992) reported that the Hydrophile–Lipophile Balance (HLB) value and W_0 of RM formed by cationic surfactants found to increase as the number of alkyl chain decreases in the cationic surfactants molecule and follow the order of Trioctylmethylammonium Chloride (TOMAC) < dodecyldimethylammonuim bromide (DDAB) < *N*-benzyl-*N*-dodecyl-*N*-bis (2-hydroxymethyl) ammonium chloride (BDBAC) < CTAB = Cetylpyridinium bromide (CPB). The higher W_0 of RM was reported for the RMs formed by CTAB during the partitioning study of α-amylase (Krei and Hustedt 1992) and glucoamylase (Forney and Glatz 1995) due to the presence of single alkyl chain in CTAB. Hence, the CTAB/*n*-heptanol reverse micelle system was employed to extract the LF from the synthetic aqueous solution. The LF was initially extracted from the aqueous LF solution to the reverse micelles during forward extraction. Further, the RM phase which contains the extracted protein was subjected to back extraction with fresh aqueous phase. The effect of different variables on the forward and back extraction of LF was studied for the efficient extraction.

Forward extraction

Surfactant concentration

The CTAB concentration in *n*-heptanol was varied from 10 to 100 mM, which is above the critical micellar

composition of CTAB/*n*-heptanol system (1 mM), to study the effect of surfactant concentration in the micellar system on the forward extraction of LF. The LF transfer from aqueous to organic phase was found to increase till the surfactant concentration of 70 mM. As much as 85% LF was transfer to micellar phase (Fig. 1) at 70 mM CTAB concentration. A gradual increase in the W_0 was also observed till the CTAB concentration of 50 mM and further, it remained constant in spite of increasing surfactant concentration. The increased LF transfer to micellar phase with increasing CTAB concentration was due to the increase in number of reverse micelles and corresponding increase in W_0 (Hebbar et al. 2008; Krishna et al. 2002). Further, the RM size was not varied much by increasing the surfactant concentration which indicates that the enhanced extraction of LF was not because of the change in the size of the reverse micelle, but due to the increase in the number of micelles and interfacial area between the micelles and aqueous phase at higher surfactant concentration. However, the transfer of LF to micellar phase was observed to decrease beyond 70 mM concentration of CTAB due to the inter-micellar collision, micellar clustering and collapse of micellar structure (Nandini and Rastogi 2009). Further, the enhanced surfactant concentration results in decreased protein uptake from aqueous phase due to rupture of reverse micelles (Chuo et al. 2014) and leads to gradual percolation and interfacial deformation along with change in micellar shape as well as clustering (Krishna et al. 2002).

Aqueous phase pH

Aqueous phase pH plays a crucial role in protein transfer to reverse micellar phase as it determines the ionization state of surface-charged groups present on protein. The transfer of protein to RM is regulated by electrostatic interaction between proteins and the surfactant head groups (Ono et al. 1996). The net protein surface charge can be manipulated and modify the interaction between the LF and RM by varying the solution pH. However, the protein transfer to RM phase occurs above the isoelectric point (pI) of the protein in case of cationic surfactants whereas it may happen below the pI for anionic surfactant (Krishna et al. 2002). The CTAB is a cationic surfactant and hence the LF, which has the pI of 9.4 and 9.5 for bovine and human LF, respectively (Steijns and Van Hooijdonk 2000), is expected to interact with the RM around the pI of the protein. The protein charge may be varied by increasing the pH of the aqueous solution higher than the pI of LF (Wolbert et al. 1989). The aqueous phase pH was varied between 2 and 11 to study the effect of pH on the LF extraction (Fig. 2). The extraction efficiency was very low in acidic pH, but it was found to increase at basic pH. Maximum of 96.66% LF entrapment into the RM was observed at a pH 10, which is slightly above the pI of the LF (9.4). Above the pI of LF, the net charge of the LF changes to negative. Thus, negatively charged LF was found to interact with the positively charged head group of CTAB and facilitates the capturing of LF into RM (Li et al. 2007).

Further the size of the RM and protein molecule also has a significant effect on the extraction efficiency. Size of RM can also be increased by increasing the number of charged group on proteins through the manipulation of aqeous phase pH. According to Krishna et al. (2002), proteins with less molecular weight (MW range 12KD-14.5KD) may require lesser pH-pI (i.e. less than 2) as compared to larger molecular weight protein (MW range 33–48 KD), which is around 5, for optimum protein transfer. The size of the RM with LF was found to be much higher than the

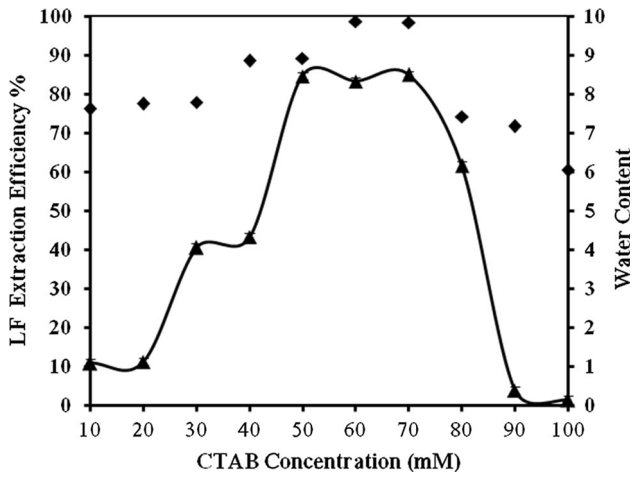

Fig. 1 Effect of CTAB concentrations on LF solubilisation (*triangle*) into CTAB/*n*-heptanol reverse micelles and water content (*filled diamond*) at an initial aqueous phase pH of 7.5

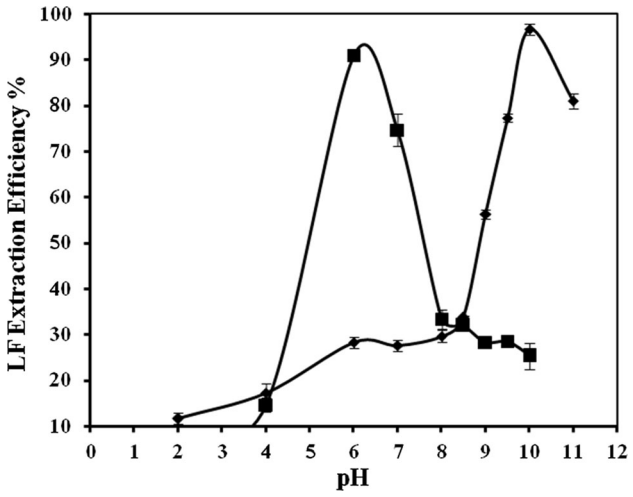

Fig. 2 Effects of aqueous phase pH on the forward (*filled diamond*) and back extraction (*filled square*) efficiency of LF from a solution containing the LF concentration of 0.1 mg/ml

Table 1 Water content and the size of reverse micelles at different process conditions

System parameters	Without LF		With LF		LF extraction efficiency (%)
	W_0 (molar ratio)	Reverse micelle size (μm)	W_0 (molar ratio)	Reverse micelle size	
40 mM CTAB/*n*-heptanol + water	8.532	3.51	8.852	–	43.33
50 mM CTAB/*n*-heptanol + water	8.826	6.15	9.854	6.79 μm	84.66
60 mM CTAB/*n*-heptanol + water	8.854	6.79	9.867	–	83.33
80 mM CTAB/*n*-heptanol + water	9.867	7.67	7.431	–	61.66
50 mM CTAB/*n*-heptanol + water + (0.9 M) KCl	7.085	5.60	9.197	134 μm	97
50 mM CTAB/*n*-heptanol + water + (0.9 M) KCl + *n*-Butanol (7%)	8.234	5.08	8.248	236 μm	46
50 mM CTAB/*n*-heptanol + water + (0.9 M) KCl + *n*-Butanol (15%)	7.931	3.73	7.947	4.89 μm	13

corresponding reverse micelle without LF (Table 1). The higher pH-pI and molecular size of LF (78–80 KD) are favoured for the higher solubilization of LF to RM (Steijns and Van Hooijdonk 2000). Hence the larger RM were formed at the pH of 10. Similar effect was explained for the extraction of bromelain, whose pI is 9.5 (Hebbar et al. 2008).

Effect of ionic strength

The LF extraction efficiency may be further improved by modifying the ionic strength present between the molecules in reverse micellar system. The literature suggests the addition of salts may vary the protein solubilisation in reverse micellar phase, since the size and W_0 of the micelle may differ with respect to the modified electrostatic effect (Hebbar et al. 2008; Ono et al. 1996; Wan et al. 2016). The water forming salts like NaCl and KCl are generally considered to improve the forward extraction (Wan et al. 2016). The effect of ionic strength was studied by adding the KCl and NaCl at different concentration (0.1–1.3 M). The extraction efficiency was found to increase gradually at lower concentrations of both the salts. However, the efficiency was found to decline beyond the salt concentration of 1 M for both the salts (Fig. 3). At lower salt concentration the repulsive force between the surfactant head groups decreased and hence the size of the micelle with protein found to increase (Hebbar et al. 2008; Tonova and Lazarova 2008). The stable emulsion of water was also observed due to less interfacial tension at lower salt concentration. Consequently, the water content of the RM and extraction efficiency was found to increase. As the concentration of salt increases, the stability of the RM tends to increase with the reduction in size. The electrostatic interaction between CTAB head group and LF also decreased due to the interaction between the cationic surfactant head with the chloride ions. Thus the attractive

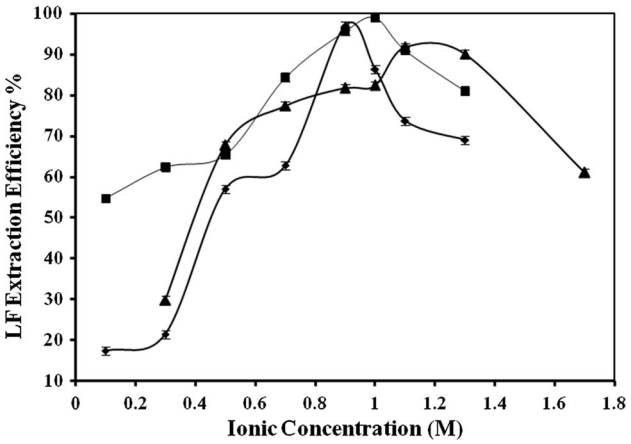

Fig. 3 Effect of inorganic salts KCl (*filled diamond*) and NaCl (*filled square*) on the forward extraction of LF at an initial aqueous phase pH of 10 ± 0.1 and KCl (*triangle*) on the back extraction efficiency at a stripping phase pH of 6

interaction between the LF and CTAB was shielded due to the decreased Debye length and reduction in the thickness of electric double layer (Tonova and Lazarova 2008). Hence, forward extraction efficiency was found to decrease with increased ionic strength due to the smaller RM size (Chuo et al. 2014; Lakshmi and Raghavarao 2010). The presence of different cations, sodium and potassium, pronounce almost similar effect on the LF solubilisation. The maximum capture of protein to RM was observed at KCl concentration 0.9 M (97%) and 1 M (99%) of NaCl concentration (Fig. 3).

Effect of LF concentration and phase volume ratio (V_{org}/V_{aq})

The LF extraction capacity of the reverse micellar system was studied by varying the concentration of LF in the aqueous solution between 0.04 and 0.4 mg ml^{-1} at the optimum surfactant concentration and pH of the system,

since the LF concentration in the whey was 0.03–0.1 mg ml^{-1} (Du et al. 2013). Negligible amount of protein was solubilized into the organic micellar phase till the LF concentration of 0.06 mg ml^{-1} in aqueous phase due to the lesser water content of the micellar system. The lower protein concentration is not sufficient to decrease the interaction between the surfactant heads and hence not able to stretch the micelles. Accordingly, W_0 and size of the RM was found to increase with increasing LF concentration from 0.08 mg ml^{-1} (3.53 μm) to 0.1 mg ml^{-1} (6.79 μm) but RM size and corresponding W_0 was found to be decreased from 6.79 μm to 341.1 nm as LF concentration was increased to 0.4 mg ml^{-1} (Fig. 4). Significant quantity of LF solubility in the reverse micellar phase was noticed at 0.08 mg ml^{-1} and the maximum solubility was observed at a LF concentration of 0.1 mg ml^{-1} (Fig. 4). However, the solubility was not improved beyond the concentration of 0.1 mg ml^{-1}, since all the positively charged head groups of CTAB may be engaged by negatively charged proteins at this concentration. Further, the additional LF solubility was restricted due to almost constant W_0 of the system with increasing concentration (Fig. 4). Similar observations were reported by Mohd-Setapar et al. (2009) in penicillin extraction with anionic surfactant AOT with reasoning that optimum surfactant could be different for increased penicillin concentration in feed phase which ultimately results in low extraction efficiency with same surfactant concentration.

For the effective extraction of the protein, the LF has to be extracted/concentrated in a smaller volume of RM phase. This phenomenon may be examined by calculating the volume ratio (organic/aqueous phase). Generally, this ratio should be lower for forward extraction and higher for back extraction in an effective extraction system (Krishna et al. 2002). Effect of phase volume ratio was studied by varying the ratio from 0.2 to 1.6 by maintaining the other variables at constant value which provided maximum extraction efficiency. As the volume ratio increased, the extraction efficiency was found to increase till the volume ratio of 1. The amount of CTAB increases in total system till the ratio of 1 and hence the extraction efficiency was found to increase. However, the extraction efficiency was found to decrease beyond the ratio of 1 due to the change in organic phase volume which resulted in the change of CTAB concentration in the total mixture (Zhao et al. 2010).

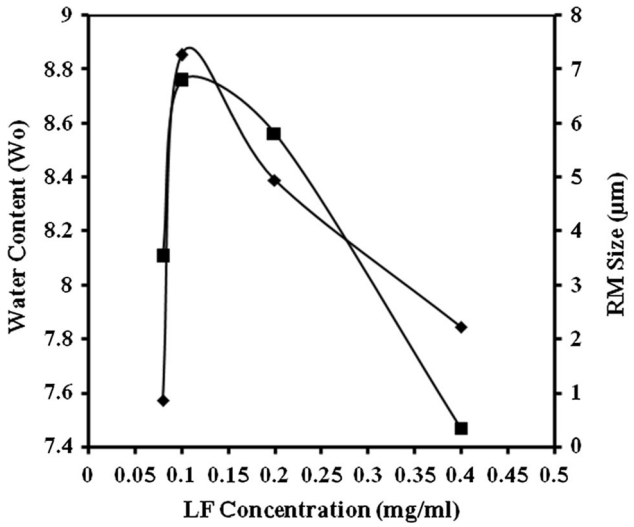

Fig. 4 Effect of various initial concentration of LF on Reverse micelle size (*filled square*) and W_0 (*filled diamond*)

Effect of co-solvent addition

The cationic surfactants tend to form smaller micelle size than any other surfactant type and the size of RM can be modified with the addition of alcohol as co-surfactant/co-solvent (Mathew and Juang 2007) through the interfacial resistance reduction and fusion/fission of the inverse micelles (Mukhopadhyay et al. 1990). The medium chain length alcohols tend to increase the size of the reverse micelles through the reduction of surface tension due to the adsorption of alcohol at the emulsion interface. The presence of alcohol changes the micellar properties due to the micelle–micelle and micelles-protein interactions. The *n*-butanol was considered as a co-solvent during the forward extraction of LF. The effect of alcohol was analyzed in two different LF concentration (0.2 and 0.4 mg ml^{-1}) solutions by adding 7 and 15% (V/V) *n*-butanol with CTAB/*n*-heptanol and (0.9 M) KCl system. The addition of *n*-butanol resulted in increased protein extraction efficiency. Improved extraction may be the result of increased hydrophilic interaction between organic and feed phase (Krishna et al. 2002). The RM size increases (5.81–27.2 μm for 0.2 mg ml^{-1} and 807 μm for 0.4 mg ml^{-1}) due to the increased hydrophilic force and W_0, since the alcohol adsorbed at the interface reduces the hydrophobic interaction between the hydrophobic tails of the surfactant and electrostatic repulsion between the charged head group (Mathew and Juang 2007). However, the higher concentration of alcohol reduces the stability of the RM and lead to denaturation of the protein.

Reverse micelle characterization

The size and number of RM and the resulted W_0 in RM phase are mainly responsible for the solubilisation of LF in the RM phase, apart from the other interactions between the molecules due to hydrophobic and ionic interactions. The experiments were conducted initially to measure the size of RM and corresponding W_0 in the system by changing the surfactant and LF concentration. The

expansion and contraction of the RM size and extraction efficiency of LF are based on the net ionic strength and hydrophobic forces caused due to the addition of alcohol as co-surfactant and electrolyte salts to modify the ionic strength. The characterization of RM was performed at the conditions, which shown a favorable LF solubilisation into RM phase (Table 1).

From the Table (1), it was observed that the size of the RM found to increase from 3.51 to 7.67 μm as the concentration of CTAB increases from 40 to 80 mM without the addition of LF. Accordingly, the W_0 of the RM has been found to increase from 8.532 to 9.867 (Table 1). The size of the RM and W_0 of the RM system found to increase further with the addition of LF at different concentration (Fig. 1). The maximum extraction efficiency of LF and W_0 were observed at 50 mM CTAB, even though the size of the RM was increased beyond this concentration. Size of RM was found to increase from 6.15 to 6.79 μm with a corresponding increase in W_0 of RM from 8.826 to 9.854, when LF was entrapped in RM without altering other process conditions at 50 mM CTAB. The increasing surfactant concentration in organic phase leads to the formation of bigger RM and such larger RM may be useful for enhanced solubilisation of larger biomolecules in the RM phase. The size of the RM with LF was found to be higher than the corresponding RM without LF (Table 1). Increase in RM size with increasing W_0 may be due to the increased number of hydrogen bond at each acceptor due to high water-surfactant molecule ratio. Increased water-surfactant ratio allows more access of hydrogen bonding site to water that help to solubilize LF into RM and resulting in increased RM size compared to empty RM (Jeffrey and Saenger 2012).

The size of RM was found to increase drastically from 5.60 to 134 μm during the addition of KCl at the concentration of 0.9 M with increase in W_0 from 7.1 to 9.2. As Cl^- ions tends to attract towards positively charged CTAB head group, attraction of Cl^- ions to the surfactant head group results in increased thickness of electrical double layer and increase in size of RM (Fathi et al. 2012). Further RM size and W_0 were measured with the addition of co-solvent. When 7% *n*-butanol (V/V) was added at LF concentration of 0.1 mg $ml^{-1,}$ RM size was found to be 236 μm (Table 1). RM size was found to decrease up to 4.89 μm for 0.1 mg ml^{-1} of LF, when 15% *n*-butanol (V/V) was added. Addition of *n*-butanol resulted in decrease in electrical percolation threshold (φt) which tends to reduce the thickness of electrical double layer and ultimately reduce the size of RMs (Mathew and Juang 2007). Hence, addition of co-solvent in forward extraction of LF is not recommended.

Further, RM size and W_0 analysis was carried out for micellar solution containing various LF concentrations (Table 1). Concentration of LF was increased from 0.08 to 0.4 mg ml^{-1}. Size of RM was found to increase from 3.53 to 6.79 μm for LF concentration of 0.08–0.1 mg ml^{-1}, respectively. But further increase in LF concentration of 0.2–0.4 mg ml^{-1}, size of RM was found to decrease up to 341.1 nm even though CTAB concentration of 50 mM was maintained. Perhaps this could be due to weak intermolecular interaction between protein and surfactant molecules as a result of unbalanced charges on protein and surfactant molecules. At low LF concentration, the micellar size was observed to increase due optimal intermolecular interaction between protein and surfactant molecule. As the protein concentration tends to increase, intramolecular forces in protein molecule masks the protein-surfactant interactive forces which results in charge unbalance and ultimately reduction of micelle size (Jeffrey and Saenger 2012). Characterization of RM not only helped to understand the effect of LF concentration on RM size and W_0 but it also confirmed the degree of solubilisation of LF into RM due to variation observed in size and W_0.

Back extraction of LF

The back extraction of LF from reverse micellar phase to fresh stripping phase was carried out by altering the pH of fresh aqueous phase (Mohd-Setapar et al. 2009) and electrostatic interactions using different concentration of electrolytic salt, KCl. The back extraction can be achieved through the electrostatic repulsion between the RM and LF. The effect of pH value of the stripping aqueous solution was studied between the pH values of 2–10 (Fig. 2). Maximum of 91% LF was released from reverse micelles at pH value of 6. In general, the proteins gain the net surface charge based on the isoelectric pH of the protein (pI). Above the pI the net surface charge would be negative and vice versa (Krishna et al. 2002; Steijns and Van Hooijdonk 2000). The pI of the LF was reported as 9.5 (Steijns and Van Hooijdonk 2000) and hence the LF attained the positive charge at the pH of 6 and released from the reverse micelles due to the electrostatic repulsion between positively charged head group of surfactant and protein (Pires et al. 1996).

The ionic strength developed due to pH variation may not be sufficient to overcome the micelle–micelle and micelle-protein interaction exists in the reverse micellar phase due to the strong electrostatic interaction. Further, the protein encapsulated micelles interacted between them and leads to the formation of micelle aggregates and cluster, resulting in decreased back extraction of LF. The ionic strength of the fresh aqueous phase solution reduces this effect to certain extant as a result of Debye screening effect. The electrostatic interaction was further reduced by adding the electrolyte salt KCl with the aqueous phase.

KCl was considered since the larger K^+ ions are capable to cause higher solubilisation as compared to other ions with smaller sizes such as Na^+ (Zhao et al. 2010). Further, K^+ cations are chaotropes in nature and help to destabilize the hydrophobic aggregates and increase the back extraction of proteins (Gaikaiwari et al. 2012). The effect of electrolyte salt KCl on the back extraction of LF was studied by adding the KCl solution at different concentrations of 0.3–1.7 M (Fig. 3) The maximum back extraction of LF (92%) was obtained at 1.3–1.5 M of KCl. Addition of KCl reduces the size of RM due to the stronger interaction between the cationic surfactant head with the chloride ions and thus squeezing out the solutes contained in the RM. However, the K^+ cations represented as a chaotrophic agent which destabilizes the RM by disrupting the hydrogen bonding network between the molecules, resulting in increased extraction of LF into the aqueous phase (Pires et al. 1996). The addition of higher concentration of KCl (>1.7 M) reduces the LF release from RM, since the increased ionic strength resulted in cloudy phase formation (Li et al. 2007) due to the denaturation and precipitation of proteins. Hence the LF back extraction was favorable between the KCl concentrations of 1.3–1.5 M.

Further to reduce the interaction between micelle–micelle and micelle-bio-molecules, different alcohols were studied, since alcohols have amphiphilic property as a co-surfactant. To enhance the back extraction efficiency, many researchers have studied the effect of addition of alcohol as co-solvent (Mathew and Juang 2007; Zhao et al. 2010), since the back extraction was controlled by the interface resistance than the diffusional resistance in the reverse micellar phase and aqueous phase. Hemavathi et al. (2010) reported that addition of alcohol can reduce the interactions by reducing the micellar interface resistance. Alcohol is well known to break and destabilize the RM through coalescence of reduced interface resistance inverse micelles in the solution (Mukhopadhyay et al. 1990). The experiments were conducted to understand the effect of alcohol chain length and their concentration during back extraction. Four different alcohols, namely *n*-propanol, *n*-butanol *n*-hexanol and *n*-decanol were used in the range 3–15% V/V (Fig. 5). Maximum back extraction (98%) was obtained with *n*-propanol and *n*-butanol. Even though higher chain length alcohols are reported as a suitable alcohol for back extraction of solutes than the lower chain length alcohol (Mathew and Juang 2007), the LF was successfully back extracted with *n*-propanol. Back extraction efficiency with higher alcohols (*n*-hexanol and *n*-decanol) were found to be lesser, since the long chain alcohols cannot penetrate into reverse micelles and smaller alcohols can penetrate into interfacial layer of reverse micelles and disrupt the hydrophobic surfactant–protein interaction (Hong and Kuboi 1999). Further the higher chain length alcohols at higher concentration lead to protein degradation (Ono et al. 1996).

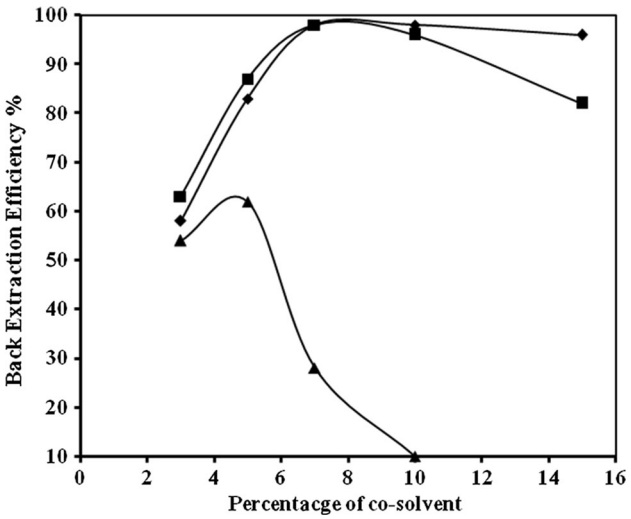

Fig. 5 Effect of co-solvent addition (triangle-*n*-hexanol, *filled square*-*n*-butanol and *filled diamond*-*n*-propanol) during back extraction of LF from CTAB/*n*-Heptanol RM phase using aqueous stripping solution at a pH of 6

Other than the system variables, the effect of operating variables like phase contact time and volume ratio of the phases were studied for the back extraction of LF. The phase volume ratio (V_{aq}/V_{org}) was varied from 0.2 to 1. It was observed that the back extraction increased with increasing phase volume ratio and maximum back extraction was obtained at 1:1 ratio, which indicates that the stripping phase has limited recovery capacity (Li et al. 2007). Similarly, the phase contact duration was also found to have major influence on the recovery of protein as the protein transferred from organic phase to aqueous phase across the interphase by overcoming the interfacial resistance for mass transfer (Chuo et al. 2014). Effect of phase mixing duration on back extraction was studied for range of 15–120 min at a magnetic stirrer speed of 800 rpm. It was observed that less time of phase mixing results in no protein extraction to stripping phase as the external forces fails to destabilize the micellar structure to squeeze out protein. When the mixing time was increased from 30 to 90 min, the recovery of LF increased from 0 to 93%. The optimum mixing time was about 60 min.

Qualitative analysis of extracted protein

Back extraction of LF was confirmed by RP-HPLC. After studying each back extraction parameter HPLC analysis was done to confirm extraction. And final back extracted LF sample was compared with commercially available LF (Fig. S1). With appropriate chromatographic conditions we observed peak elution of pure LF at 5.3 min (a) and back

extracted LF at 5.25 min (b). In case of back extracted LF, slight shift was observed in peak elution due to presence of ions in sample solution. But no structural changes were observed as peak pattern is similar to the pure LF peak.

Conclusion

The studies on the effect of process variables on the RME of LF with CTAB/*n*-heptanol system showed that almost all the LF may be solubilized during the forward extraction at 50 mM CTAB concentration at pH 10 with the addition of 0.9 M KCl. Further, 98% of LF was back extracted at pH 6 with the addition of small amount of co-solvent (7% *n*-propanol or *n*-butanol) and electrolyte (1.3 M KCl). LF was found to be stable after back extraction to the fresh stripping phase. Thus, the selected RM system proved its suitability for LF extraction from synthetic solution. This also implies the potential of RM systems for selectively recovering LF from complex biological sources like whey.

Acknowledgements The authors are thankful for the financial support by Science and Engineering Research Board (SERB), Ministry of Food Processing Industries (MOFPI), Govt. of India to carry out this research (Scheme Number: SERB/MOFPI/0039/2013, dated 16/09/2013).

References

Adlerova L, Bartoskova A, Faldyna M (2008) Lactoferrin: a review. Vet Med Czech 53(9):457–468

Andersson J, Mattiasson B (2006) Simulated moving bed technology with a simplified approach for protein purification: separation of lactoperoxidase and lactoferrin from whey protein concentrate. J Chromatogr A 1107(1):88–95

Carvalho CML, Cabral JMS, Aires-Barros MR (1999) Cutinase stability in AOT reversed micelles: system optimization using the factorial design methodology. Enzym Microb Technol 24(8):569–576

Chaurasiya RS, Sakhare PZ, Bhaskar N, Hebbar HU (2015) Efficacy of reverse micellar extracted fruit bromelain in meat tenderization. J Food Sci Technol 52(6):3870–3880

Chuo SC, Mohd-Setapar SH, Mohamad-Aziz SN, Starov VM (2014) A new method of extraction of amoxicillin using mixed reverse micelles. Colloids Surf A 460:137–144

Du QY, Lin DQ, Xiong ZS, Yao SJ (2013) One-step purification of lactoferrin from crude sweet whey using cation-exchange expanded bed adsorption. Ind Eng Chem Res 52(7):2693–2699

Elagamy EI, Ruppanner R, Ismail A, Champagne CP, Assaf R (1996) Purification and characterization of lactoferrin, lactoperoxidase, lysozyme and immunoglobulins from camel's milk. Int Dairy J 6(2):129–145

Fathi H, Kelly JP, Vasquez VR, Graeve OA (2012) Ionic concentration effects on reverse micelle size and stability: implications for the synthesis of nanoparticles. Langmuir 28(25):9267–9274

Forney CE, Glatz CE (1995) Extraction of charged fusion proteins in reversed micelles: comparison between different surfactant systems. Biotechnol Prog 11(3):260–264

Gaikaiwari RP, Wagh SA, Kulkarni BD (2012) Efficient lipase purification using reverse micellar extraction. Bioresour Technol 108:224–230

Gonzalez-Chavez SA, Arevalo-Gallegos S, Rascon-Cruz Q (2009) Lactoferrin: structure, function and applications. Int J Antimicrob Agents 33(4):301-e1

Hebbar HU, Sumana B, Raghavarao KSMS (2008) Use of reverse micellar systems for the extraction and purification of bromelain from pineapple wastes. Bioresour Technol 99(11):4896–4902

Hemavathi AB, Hebbar HU, Raghavarao KSMS (2010) Mixed reverse micellar systems for extraction and purification of β-glucosidase. Sep Purif Technol 71(2):263–268

Hong DP, Kuboi R (1999) Evaluation of the alcohol-mediated interaction between micelles using percolation processes of reverse micellar systems. Biochem Eng J 4(1):23–29

Jeffrey GA, Saenger W (2012) Hydrogen bonding in biological structures. Springer, Berlin Heidelberg

Krei GA, Hustedt H (1992) Extraction of enzymes by reverse micelles. Chem Eng Sci 47(1):99–111

Krishna SH, Srinivas ND, Raghavarao KSMS, Karanth NG (2002) Reverse micellar extraction for downstream processing of proteins/enzymes. In: History and trends in bioprocessing and biotransformation, Springer, Berlin Heidelberg, pp 119–183

Lakshmi MC, Raghavarao KSMS (2010) Downstream processing of soy hull peroxidase employing reverse micellar extraction. Biotechnol Bioprocess Eng 15(6):937–945

Li X, He G, Lin C, Liu H (2007) Study on the extraction and back extraction of Bovine Serum Albumin using reversed micelles. Sep Sci Technol 42(16):3741–3757

Lonnerdal B, Carlsson J, Porath J (1977) Isolation of lactoferrin from human milk by metal-chelate affinity chromatography. FEBS Lett 75(1–2):89–92

Mathew DS, Juang RS (2007) Role of alcohols in the formation of inverse microemulsions and back extraction of proteins/enzymes in a reverse micellar system. Sep Purif Technol 53(3):199–215

Melo EP, Airesbarros MR, Cabral JMS (1995) Triglyceride hydrolysis and stability of a recombinant cutinase from *Fusarium solani* in AOT-iso-octane reversed micelles. Appl Biochem Biotechnol 50(1):45–56

Mohd-Setapar SH, Wakeman RJ, Tarleton ES (2009) Penicillin G solubilisation into AOT reverse micelles. Chem Eng Res Des 87(6):833–842

Mukhopadhyay L, Bhattacharya PK, Moulik SP (1990) Additive effects on the percolation of water/AOT/decane microemulsion with reference to the mechanism of conduction. Colloids Surf 50:295–308

Nandini KE, Rastogi NK (2009) Reverse micellar extraction for downstream processing of lipase: effect of various parameters on extraction. Process Biochem 44(10):1172–1178

Ono T, Goto M, Nakashio F, Hatton TA (1996) Extraction behaviour of hemoglobin using reversed micelles by dioleyl phosphoric acid. Biotechnol Prog 12(6):793–800

Pawar SS, Regupathi I, Prasanna BD (2017) Screening of reverse micellar system for the extraction of Bovine Lactoferrin. In: 6th international engineering symposium (IES-2017), March 1–3 2017, Kumamoto University, Japan, pp 372–376

Pires MJ, Aires-Barros MR, Cabral JMS (1996) Liquid – liquid extraction of proteins with reversed micelles. Biotechnol Prog 12(3):290–301

Plate K, Beutel S, Buchholz H, Demmer W, Fischer-Fruhholz S, Reif O, Scheper T (2006) Isolation of bovine lactoferrin, lactoperoxidase and enzymatically prepared lactoferricin from proteolytic digestion of bovine lactoferrin using adsorptive membrane chromatography. J Chromatogr A 1117(1):81–86

Recio I, Visser S (1999) Two ion-exchange chromatographic methods for the isolation of antibacterial peptides from lactoferrin: in situ

enzymatic hydrolysis on an ion-exchange membrane. J Chromatogr A 831(2):191–201

Sadana A (1997) Bioseparations of proteins: unfolding/folding and validations, vol 1. Elsevier, Amsterdam

Shin YO, Weber ME, Vera JH (2003) Effect of salt and volume ratio on the reverse micellar extraction of lysozyme using DODMAC. Fluid Phase Equilib 207(1):155–165

Steijns JM, Van Hooijdonk ACM (2000) Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. Br J Nutr 84(S1):11–17

Tang X, Huston KJ, Larson RG (2014) Molecular dynamics simulations of structure property relationships of tween 80 surfactants in water and at interfaces. J Phys Chem B 118(45):12907–12918

Tonova K, Lazarova Z (2008) Reversed micelle solvents as tools of enzyme purification and enzyme-catalyzed conversion. Biotechnol Adv 26(6):516–532

Wan J, Guo J, Miao Z, Guo X (2016) Reverse micellar extraction of bromelain from pineapple peel–effect of surfactant structure. Food Chem 197:450–456

Ward PP, Paz E, Conneely OM (2005) Lactoferrin. Cell Mol Life Sci 62(22):2540–2548

Wolbert RB, Hilhorst R, Voskuilen G, Nachtegaal H, Dekker M, Riet KVT, Bijsterbosch BH (1989) Protein transfer from an aqueous phase into reversed micelles. Eur J Biochem 184(3):627–633

Wu MB, Xu YJ (2009) Isolation and purification of lactoferrin and immunoglobulin G from bovine colostrum with serial cation-anion exchange chromatography. Biotechnol Bioprocess Eng 14(2):155–160

Zhao X, Li Y, He X, Zhong N, Xu Z, Yang L (2010) Study of the factors affecting the extraction of soybean protein by reverse micelles. Mol Biol Rep 37(2):669–675