Screening of Reverse Micellar System for the Extraction of Bovine Lactoferrin

SWAPNALI S PAWAR, IYYASWAMI REGUPATHI AND PRASANNA B D

Department of Chemical Engineering, National Institute of Technology Karnataka, Surathkal, Srinivasnagar Post- 575 025, Mangalore, Karnataka, India

Email: swapnali1706@gmail.com, regupathi@yahoo.com, prsnbhat@gmail.com

Abstract: *Lactoferrin (LF) is a multifunctional and an iron-binding protein (Mw~77–80 kDa) present in animal milk. It is an important additive used in milk based infant formula, cosmetics, health supplements, oral care products due to its unique biological attributes. Present global demand of LF is estimated to be around 2, 62,000 kg/year. Reverse micellar extraction, a selective extraction, can be an alternate for chromatography and simulated moving bed (SMB) technology as it overcomes all the difficulties like high process cost and scale-up. Present work focuses on the selection of the suitable reverse micellar system by considering anionic (Bis (2-ethylhexyl) sulphosuccinate sodium salt), cationic (Cetyltrimethylammonium bromide) and non-ionic (Trition X 100) surfactants with organic solvents for the extraction of LF from the aqueous solution. All the RMSs were characterized (critical micelle concentration, water content, and micelle size) and their extraction efficiency was analysed. Cetyltrimethylammonium bromide with n-heptanol RMS was founded to offer 100% yield. The identified RMS may be further considered for the commercial extraction of LF from complex biological sources.*

Keywords: *Lactoferrin, Reverse Micellar Extraction, Surfactants*

1. Introduction

Lactoferrin is a multifunctional protein majorly available in animal sources [1]. It shows antiviral, antimicrobial, antibacterial activity. LF has high influence on iron distribution on organisms. Apart from these important functions LF also helps to inhibit tumour growth [2]. It is the second most generous whey protein [3]. The demand of LF is observed to be increased yearly and it is expected to reach 2,62,000 kg in 2017. According to market survey huge global shortage of LF has been reported [4]. Inverse proportion of demand and supply of the LF has been observed but countable reports are available for LF extraction from whey [5]. Currently extraction of lactoferrin with ion exchange chromatography [6], Magnetic affinity absorbents [5] at batch scale is reported. However, the Conventional downstream process reported in the literature like chromatography, precipitation techniques used for the separation and purification of LF are difficult to scale up. Hence the present research focused on the development of simplified LF extraction and purification process.

Liquid- liquid extraction with biomolecule compatible solvents has the potential to extract the component like LF with higher purity and opened a new window to scale up the process in an economical way [7]. Reverse micellar based extraction system is an interesting option to extract a specific protein selectively from a complex mixture like whey, since the separation is based on electrostatic interaction and size differences of the target molecules [8]. Reverse micellar extraction of proteins like soy protein [9], BSA [10], lectin[11], bromelain[12] from their respective biological crude mixtures has been reported.

Since the selective extraction of a specific protein is based on the type of surfactant and organic solvent associated with the reverse micellar system, different reverse micellar systems (RMSs) which are formed by anionic (AOT), cationic (CTAB) and non-ionic (Triton X 100) surfactants with organic solvents are examined for the extraction of LF from the aqueous solution. The physicochemical characteristics like Critical Micelle Concentration (CMC), water content (W_0) and size of all the RMSs were measured. The LF extraction efficiency of the RMSs was also studied to ascertain the suitability of the selected systems.

2. Experimentation

2.1. Materials

The surfactants CTAB (cetyltrimethylammonium bromide - cationic), AOT (anionic) and Triton X100 (non-ionic) having 99% purity were procured from Sigma Aldrich, India and used for experiments without further purification. Bovine lactoferrin (LF) were purchased from Sigma Aldrich, India. Bovine serum albumin was purchased from Hi media, India. Organic solvents of analytical grade namely Isooctane, n-Decanol, toluene, n-heptanol were procured from Loba. Folin-Ciocalteu reagent (FCR) was purchased from Merck, India.

2.2 Characterization of RMSs

Based on physicochemical properties of surfactants and organic solvents (Table 1), six different reverse

micellar systems were selected. Triton X 100/ Isooctane, AOT/ Isooctane, AOT/ n-decanol, CTAB/ Toluene, CTAB/ Isooctane, CTAB/ n-heptanol were chosen for the LF partitioning study. CMC of different reverse micellar systems was found by estimating the physical properties like refractive index (Digital Refractometer, RX-500, ATAGO Co. Ltd. Japan) and density (Digital density meter, DDM 2911, Rudolph, USA) of the surfactant/ solvent mixture using properties. Water content (W_0) of all selected micellar system at CMC was measured using Karl Fischer Titrator (899 coulometer, Metrohm, Switzerland). The reverse micelle size (Eq.1) was calculated using the water content and reported in the Table 2.

$$R_m = 0.175W_0 \qquad (1)$$

3. Reverse micellar extraction of Lactoferrin

All the six RMSs are considered for the extraction of LF. Initially equal amount of organic phase (containing surfactant concentration above CMC) were mixed thoroughly with aqueous phase (containing LF concentration 0.1mg/ml) using magnetic stirrer at 800 rpm and room temperature for 20 min. Then the mixture was subjected to centrifugation at 474g and 20 min (Remi C-24 plus, India) for phase separation. The organic phase was carefully separated. Protein concentration after LF concentration in each phase was measured using Folin-Lowry's assay. The absorbance was measured at a wavelength of 660 nm using the spectrophotometer (UV3000+, Labindia). Extraction efficiency was calculated by using following equation,

LF Extraction efficiency (%) = (LF Conc. In organic phase/LF conc. In feed phase) X 100   (2)

The effect of surfactant concentration (5-100 mM), pH (2-9), and addition of electrolyte salts (NaCl and KCl – 0.1 to 1 M) were also studied for the higher extraction efficiency of LF.

4. Results and discussions

4.1. Reverse micellar characterization

The Physical and chemical properties of the surfactants and organic solvents plays important role in reverse micelle formation. Topological surface area, rotatable bond count and number of hydrocarbon chain present in surfactant and solvents mainly responsible for the RM formation and also for the W_0[13]. The physical and chemical properties of all the selected surfactants and solvents are listed in Table 1. The CMC, size of the micelle and W_0 were measured for all the six selected systems and reported in Table 2.

The systems in which the surfactant and solvents are showing topological surface charge (TX100/Isooctane, AOT/Isooctane, AOT/n-decanol), the formation of reverse micelle is best understood

with taking in consideration the sum of the average of total surface area of solvent and surfactant, presence of hydrocarbon chain around central molecule, rotatable carbon atoms in solute and surfactants[14]. Taking all physical and chemical properties in account a relation between CMC value and W_0 of all reverse micellar systems has been found and these parameters are proportional to the sum of averages of Rotatable carbon atom and average charge on participants in group, is lower (28) in TX100/Isooctane system for CMC value 0.3mM and corresponding less W_0 (4.35).

Table 1: Physical and chemical characteristics of phase forming components

System components	Topological surface Area (A°)	Hydro-carbon chain	Rotatable Bond count
TX100	29.5	1	6
AOT	54	2	18
CTAB	0	1	15
Isooctane	18.5	1	2
n-decanol	20.2	1	8
n-Heptanol	20.2	1	5
Toluene	0	0	0

In case of AOT/Isooctane the total rotatable bond count and average charge on the system found to be increased to 46.25, as result CMC and W_0 found to be increased to 1mM and 5.66 respectively. Similar result has been observed in AOT /n-decanol system with a little fall in CMC (0.8mM) which supports the above conclusion. Whereas in systems (CTAB/Isooctane-CMC 2mM, CTAB/ n-heptanol-CMC 1mM and CTAB/Toluene- CMC 2mM) where the solvent and surfactant both are charged or any one of them shows zero topological surface charges, the availability of side chain hydrocarbon in components along with sum of the average of total surface charge of solvent and surfactant plays major role[15].

Table 2: Physical Characteristics of RMSs

RMSs	CMC (mM)	W_0 Without LF (mol ratio)	W_0 With LF (mol ratio)	RM size Without LF (nM)	RM size With LF (nM)
AOT/Isooctane	1	5.66	-	0.99	-
CTAB/ Isooctane	2	7.87	13.4	1.37	2.34
TX100/ isooctane	0.3	4.35	9.88	0.76	1.72
AOT/ n-decanol	0.8	6.48	-	1.13	-
CTAB/ Toluene	2	8.95	-	1.56	-
CTAB/n-Heptanol	1	9.64	15.91	1.68	2.78

The side chain hydrocarbons interact with each other in different symmetry and are responsible for steric repulsion between surfactant and solvent molecules leading to formation of equilibrium for retention of water with in it. As in these cases the CMC value is high if the system doesn't have any net charge them, the RM formation is solely performed by presence of side chain and the steric repulsion between surfactant and solvent molecule.

4.2. Reverse micellar extraction of LF

Out of the six RMSs (Table 2) studied for the extraction of LF, only three RMSs (TX100/isooctane, CTAB/Isooctane and CTAB/n-Heptanol) were found to provide a favourable LF extraction (Fig. 1). There was no LF extraction observed with AOT/Isooctane, AOT/ n-decanol and CTAB/ Toluene due to weak electrostatic interaction between surfactant and solute molecule [11]. LF precipitation at the interphase between the aqueous and organic phase was observed for AOT/Isooctane, AOT/ n-decanol systems. Further the extraction was found less due to the organogel formation in the presence of excess water in organic phase of CTAB/ Toluene system [16]. Hence these three systems were discontinued for the further LF extraction studies.

The surfactants CTAB and TX100 contain only one lipophillic chain, therefore not able to form reverse micelles in organic media without addition of second surface active agent, called as co-surfactant. In case of CTAB/n-heptanol, n-heptanol is used as organic phase which also acts as co-solvent and aid the spontaneous formation of more number of reverse micelle to capture more amount of LF [17]. However, the lesser extraction efficiency was found in case of TX 100/Isooctane and CTAB/Isooctane due to the longer chain alkane (Isooctane) which is more hydrophilic than lipophillic in nature used as organic phase.
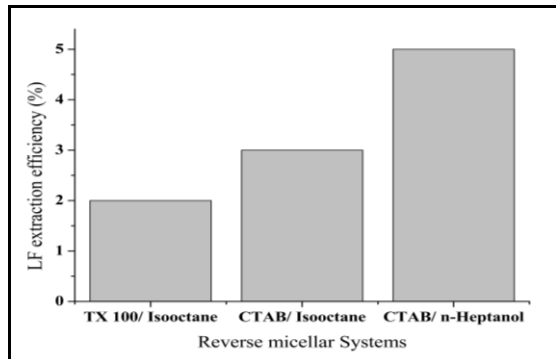
Figure1: *LF extraction efficiency of different RMSs (Without altering variables)*

4.2.1 Effect of pH

Lactoferrin is a basic protein having pI of 9.4. Below pI, LF posses positive charge and above pI it carries negative charge [18]. To improve extraction efficiency pH of the aqueous phase was varied from 2-10 for all three RMSs (Fig.2). Better interaction between positively charged head group of CTAB and negative charge on protein resulted in 5% - CTAB/Isooctane (pH-9.6) and 45% in case of CTAB/n-Heptanol (pH-10) as a result of capture of lactoferrin into reverse micelles [19]. Only 3% of LF transfer has been observed in TX100/Isooctane (pH-7).
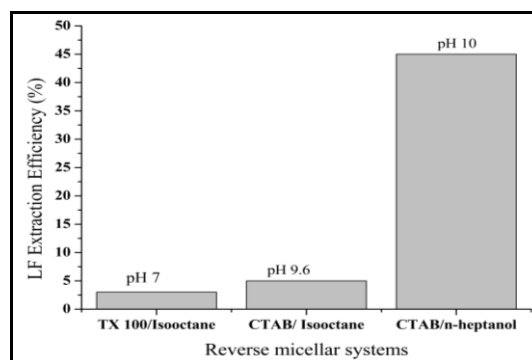
Figure2: *Effect of pH on LF extraction efficiency on TX100/Isooctane, CTAB/Isooctane and CTAB/n-heptanol*

4.2.2 Effect of Ionic strength

Further salt concentration (KCl and NaCl- 0.1to 1.3M) was varied in the aqueous phase to achieve better extraction efficiency. Addition of electrolytes to the aqueous phase of TX100/ Isooctane RMSs could not help to improve LF extraction efficiency. 0.8M NaCl concentration in CTAB / Isooctane found to increase protein transfer efficiency to 7% whereas 1M of NaCl and 0.9M of KCl concentration in case of CTAB / n-heptanol (Table 3) resulted in increased protein transfer efficiency 98% and 100% respectively. Addition of ions to the aqueous phase helps to stabilise the micellar structure and also enhances the electrostatic interaction between polar head group of surfactant molecule and solute and results in better protein partitioning according to Lakshmi et al. (2010)[20].

Table 3: *Effect of Salt concentration on LF extraction efficiency of TX100/Isooctane, CTAB/Isooctane and CTAB/n-heptanol*

Reverse Micellar Systems	NaCl		KCl	
	Conc. (M)	LF extraction Efficiency %	Conc. (M)	LF extraction Efficiency %
TX100/ Isooctane	-	-	-	-
CTAB/Isooctane	0.8	7	-	-
CTAB/n-heptanol	1	98	0.9	100

W_0 of micellar systems after extraction of the lactoferrin has been measured (Table 2) and notable increase in W_0 has been observed after LF transfer to the organic phase in all reverse micellar systems. Comparatively W_0 of non-ionic reverse micellar systems has observed to be low than ionic reverse micellar with isooctane and n-heptanol. Due to the lack of presence of strong interaction forces; partitioning of LF to organic phase is low which also resulted in less W_0 during LF Extraction in case of TX100/Isooctane [21].

5. Conclusions

A suitable reverse micellar system, CTAB/n-heptanol, for the extraction of LF was screened by incorporating

three types of surfactants in six different RMSs. Physicochemical properties of all the components used in the RMSs were studied. Physiochemical properties possible reveres micellar systems were also measured to certain the relation between the properties and LF extraction efficiency. CTAB/n-heptanol RMSs was found to be a favourable system at a pH of 7 and adding 1M of NaCl or 0.9M of KCl as electrolytes for LF extraction. Hence, it can be further used to extract LF from complex biological materials.

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