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Research paper

Reverse micellar partitioning of Bovine Serum Albumin with novel system

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ABSTRACT

To overcome the difficulties associated with the conventional extraction process like poor selective extraction of biomolecule and scale up of the process, the reverse micellar system consist of AOT/n-heptanol was considered to extract Bovine Serum Albumin (BSA) as a model biomolecule. The maximum forward extraction of BSA from aqueous phase to micelle phase was observed at AOT concentration 160 mM, aqueous phase pH value of 4, NaCl concentration 0.8 M and 95% back extraction of BSA from micelle phase to stripping phase was obtained at 1 M NaCl concentration with the pH of 7.5. HPLC analysis confirmed the stability of BSA during extraction. The size and water content of the reverse micelle was also reported. The obtained results emphasize the application of the AOT/n-heptanol reverse micellar system for the extraction of BSA and may be utilized for the selective extraction of similar hydrophilic proteins from the complex sources.

1. Introduction

Serum albumins are the most generous proteins in the circulatory system of a wide variety of organisms, being the major complex molecules contributing to the osmotic blood pressure [1]. Albumin plays a crucial role in the design of media for the culture of mammalian cells, in both the research and commercial areas due to its antioxidant potential [2]. The high demand for proteins from animal sources around the world has raised the search for new sources of proteins [3]. Therefore need for simplified purification techniques for proteins come into focus. Water in oil emulsion has the ability to solubilize hydrophilic proteins and nucleic acids in its hydrophilic core. This property of inverse emulsion, reverse micelles, makes it promising continuous extraction method for bioseparation [4]. The reverse micelles are formed by mixing the surfactant at specific concentrations with the aqueous solution. The hydrophilic head of surfactant protects the proteins from denaturation by the organic phase and due to which little or no damage to their catalytic activity is reported [4–6]. This selective extraction of a target biomolecules from mixture in to reverse micelles can be achieved by varying parameters both in the organic and the feed phases [7].

A new reversed micelle system is prepared for extraction of BSA from the solution contains 0.5 mg/ml. The effect of different factors like pH value, ionic strength in the aqueous phase, surfactant concentration and phase volume ratio which affects the mechanism of protein transfer in forward and backward extraction of protein was studied and optimum conditions were reported. Whereas, the effect of co-solvent addition was examined for better back extraction of the protein. Reveres micelles water content and radius were also calculated to characterize the reverse micelles during forward extraction.

2. Materials and methodology

2.1. Materials

Bovine serum albumin was obtained from High media, India. Sodium bis-2-ethyl hexyl sulphosuccinate (AOT) of 99% purity and other organic solvents n-heptanol, n-butanol, n-octanol, n-decanol were purchased from Loba Chemie, India. AOT used in all experiments without further purifications. Acetonitrile and Trifluoroacetic acid (TFA) of HPLC grade were procured from Merck.

2.2. Forward extraction

Forward extraction was carried out by mixing equal volumes of aqueous and organic phases (n-heptanol with AOT) using mag-

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Nomenclature

BSA	Bovine Serum Albumin
CMC	Critical Micellar Concentration
HPLC	High Performance Liquid Chromatography
L	Liter
M	Molar
mg	milligrams
min	Minutes
ml	milliliter
mMol	millimolar
nM	Nanometer
pI	Isoelectric point
R_m	Reverse Micellar Core Radius
rpm	Revolutions Per Minute
TFA	Trifluoroacetic acid
W_0	Water Content

netic stirrer for 15 min at 500 rpm at room temperature. For all experiments phase volume and phase volume ratio were maintained as 10 ml and 1:1 respectively (except phase volume ratio study). The organic phase had a known amount of surfactant dissolved in it. The aqueous phase was prepared by maintaining BSA concentration at 0.5 mg/ml. Phase separation was carried out using Remi cooling centrifuge at 3000 rpm for 10 min. The organic phase separated from the mixture and further used for back extraction.

Water content (W_0) of reverse micelles was measured using Metrohm 899 coulometer. Further, these W_0 values were used to calculate reverse micellar core radius (R_m) using Eq. (1) [8];

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

2.3. Back extraction

Back extraction was carried out by mixing the organic phase obtained from forward extraction with an equal volume of stripping phase in a magnetic stirrer for 30 min at 500 rpm followed by centrifugation at 3000 rpm for 10 min and the resulted two phases were separated for further analysis.

2.4. Protein content measurement

BSA concentration was measured at 280 nm using Lab India Analytical UV spectrophotometer before and after forward and back extraction. Extraction efficiency was calculated by using the Eqs. (2) and (3):

$$\textit{Forward Extraction Efficiency}\ (\%) = [\textit{Protein concentration in organic phase}\ (\mathrm{mg/ml}) / \textit{protein concentration in aqueous feed phase}\ (\mathrm{mg/ml})] * 100 \quad (2)$$

$$\textit{Back Extraction Efficiency}\ (\%) = [\textit{Protein concentration in stripping phase}\ (\mathrm{mg/ml}) / \textit{protein concentration in organic phase after forward extraction}\ (\mathrm{mg/ml})] * 100 \quad (3)$$

2.5. HPLC analysis

Reverse phase HPLC was performed to confirm the stability of BSA after the back extraction process. Reverse phase C18 column was used with mobile phase Water/Trifluoroacetic acid (0.1%) and Acetonitrile/ Trifluoroacetic acid (0.1%). The flow rate was maintained at 0.4 ml/min at column temperature 25 ± 0.2 °C.

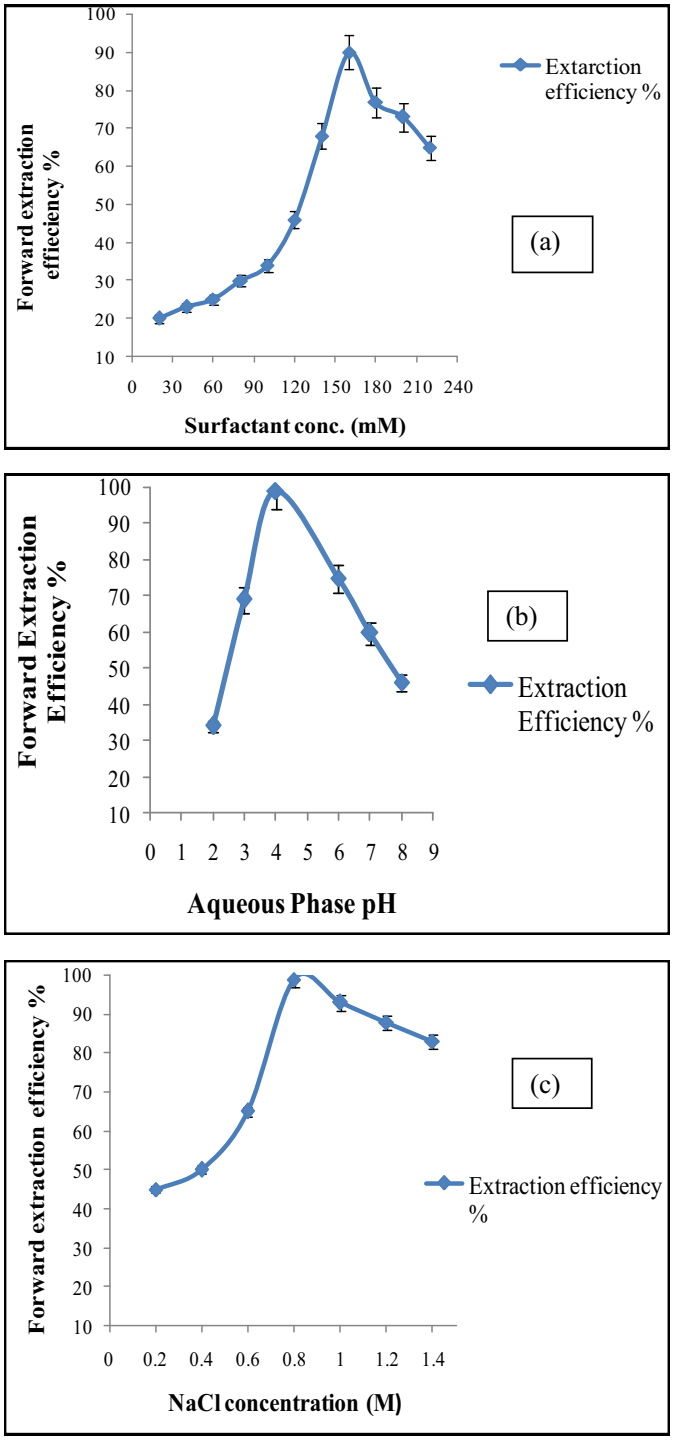

Fig. 1. Effect of (a) surfactant concentration (b) aqueous phase pH and (c) salt concentration on forward extraction of BSA.

3. Results and discussion

3.1. Forward extraction

3.1.1. Effect of AOT concentration

The Critical Micellar Concentration (CMC) of the n-heptanol/AOT system was found to be 6.6 mMol L^{-1} of AOT. The AOT concentration was varied from 20 mMol L^{-1} to 220 mMol L^{-1} (Fig. 1a), which was 3 to 33 times than the CMC concentration of surfactant. The extraction efficiency of the system was ob-

Table 1
Effect of phase volume ratio on forward and back extraction of BSA.

Phase volume ratio for forward extraction (V_{org}/V_{aq})	Forward Extraction Efficiency %	Phase volume ratio for back extraction (V_{aq}/V_{org})	Back Extraction Efficiency %
0.2:1	12.9 ± 0.01	0.2:1	33 ± 0.08
0.4:1	21.6 ± 0.16	0.4:1	46 ± 0.57
0.6:1	40 ± 0.06	0.6:1	62.8 ± 0.12
0.8:1	65 ± 0.11	0.8:1	74.6 ± 0.21
1:1	99.2 ± 0.08	1:1	95 ± 0.30

served to be increased with increased surfactant concentration. At 160 mMol L^{-1} concentration of AOT, 100% capture of protein to reverse micelle is observed. But beyond this concentration, there is a fall in protein transfer to the organic phase. This infers that very less concentration of AOT fails to form sufficient number of reverse micelles in organic phase and higher concentration leads to disruption of reverse micelles which lowers the transfer of protein to organic phase [9].

3.1.2. Effect of aqueous phase pH

Feed pH or aqueous phase pH is one of the crucial parameters during protein extraction as pH decides the net charge on the proteins and also influences the electro-static interaction between the surfactant and any bio molecule [10]. Hence the effect of pH was studied in the range of 2–8 (Fig. 1b). BSA carries positive charge below pH value of 4.7 and negative charge above the pH value of 4.7 [11]. Maximum extraction of BSA was observed at pH 4, i.e. below the isoelectric point (pI-4.7) of the protein. This may be due to the attraction of proteins to the micelle phase since they exhibit opposite charges (protein – positive charge and surfactant – negative charge), whereas the extraction of protein was found to be decreased to 75% above the pI due to the repulsion of protein from reverse micelles [12].

3.1.3. Effect of salt concentration

It is reported that the protein extraction efficiency would decrease as ionic strength increases [13]. The ionic strength of the feed phase makes a notable impact on the degree of sheltering of electrostatic potential urged by a charged surface and consequently affects the protein transfer [14]. Salt concentration effect was studied using NaCl at different concentrations (0.2 M to 1.4 M). Increased BSA extraction is observed with increasing NaCl concentration (Fig. 1c). NaCl concentration 0.8 M gave maximum protein extraction (99%) due to the existence of electrostatic interaction between solute and surfactant, but further increased salt concentration led to decrease in protein extraction due to repulsion [15].

3.1.4. Effect of phase volume ratio

Phase volume ratio V_{org}/V_{aq} makes a remarkable effect on reverse micellar extraction as the extraction capacity of phases depends on the volume of the phases. The effect of volume ratio was studied between the phase volume ratios (V_{org}/V_{aq}) of 0.2 to 1. The better extraction was observed at phase volume ratios of 1:1 (Table 1). As the volume of organic phase varies, the amount of surfactant present in the whole reaction mixture also alters. At low phase volume ratio, sufficient surfactant concentration was not available in the reaction mixture to form the required amount of reverse micelles which ultimately results in less protein extraction [16].

3.1.5. Reverse micelle characterization

The water content and the radius of the reverse micelles were analyzed at a surfactant concentration of 160 mM L^{-1} with additive (NaCl) and protein (Table 2). The addition of NaCl resulted in the reduction of micelle size and correspondingly the water content also found to decrease due to the electrostatic interaction between the surfactant and NaCl molecules. However, the micelle size was found to increase when the protein molecules extracted by the reverse micelles in the presence of NaCl. The increased interaction between the protein and the micelle resulted in the larger reverse micelle size and water content. Reverse micelle radius core raised from 9.7 nm to 11.2 nm after capture of protein confirms the transfer of BSA to organic phase during forward extraction.

Table 2
Reverse micelles characterization.

Process parameters	W_0	R_m
AOT+ n-heptanol	55,642 ppm	9.7 nm
AOT+ n-heptanol+NaCl	53,879 ppm	9.4 nm
AOT+ n-heptanol+NaCl+ BSA	64,568 ppm	11.2 nm

3.2. Back extraction

3.2.1. Effect of ionic strength

Transfer of protein back to aqueous phase is based on squeezing out effect. Hence fresh stripping phase was added to the organic phase with different salt concentrations. NaCl concentration was increased from 0.3 M to 1.5 M (Fig. 2a) to back extract the protein from reverse micelles. It was observed that the protein has transferred to stripping phase at 1 M NaCl, which is comparatively higher concentration than the concentration used in forward extraction. At higher salt concentration, the size of the micelle found to decrease and the proteins present inside the micelles are squeezing out. Further, the electrostatic interaction between the reverse micelles (surfactant polar groups) and the hydrophilic biomolecules found to decrease due to Debye screening effect [17]. The excess unutilized ions present in the stripping aqueous phase also attract the proteins from the reverse micelle phase.

3.2.2. Effect of stripping phase pH

Extraction study was carried out at various pH values from 2–10 (Fig. 2b) to improve back extraction efficiency. It was observed that at the pH value above pI of BSA carries similar (negative) charge to the surfactant polar group which leads to decrease electrostatic interaction between them and ultimately release of protein from reverse micelle to stripping phase [17]. The maximum back extraction of protein as 93% was obtained at stripping phase pH of 7.5

3.2.3. Co-solvent effect

It is reported in many publications that addition of co-solvents helps to enhance the back extraction efficiency. Since the alcohol molecules have the ability to penetrate into the reverse micelle and destabilize the structure. Also, it is reported that smaller alcohols have better destabilizing ability compared to large chain alcohols due to the perforation degree of the alcohols [18]. In the present study, it was observed that addition of 7% of n-butanol tends to increase the back extraction from 95% to 96% (Table 3).

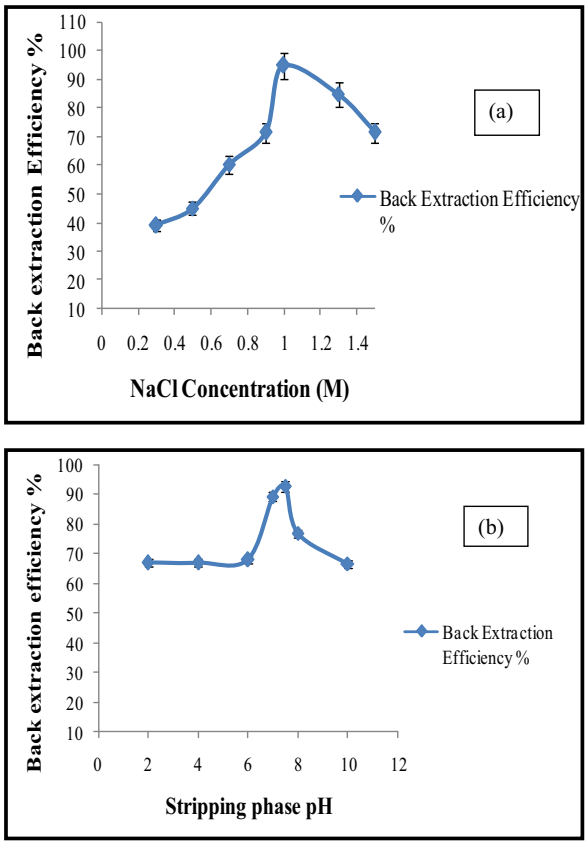

Fig. 2. Effect of (a) salt concentration (b) stripping phase pH on back extraction of BSA.

Table 3
Co-solvent effect on back extraction of BSA.

Co-Solvents	Back Extraction Efficiency %
n-Butanol 7%	96 ± 0.74
n-Butanol 15%	95 ± 0.20
n-Octanol 7%	86 ± 0.28
n-Octanol 15%	86.2 ± 0.20
n-Decanol 7%	79 ± 0.17
n-Decanol 15%	71.6 ± 0.67

3.2.4. Effect of phase volume ratio (V_{aq}/V_{org})

Back extraction was observed to be maximum at phase volume ratio 1:1 (Table 1). As the volume of stripping phase varies, the amount of ions and co-solvents supplied for whole reaction mixture also changes. Lower phase volume ratio resulted in less extraction of BSA since required amount of ions and co-solvents were not available in stripping phase also which were not sufficient to rupture total reverse micelles in organic phase [16,19].

3.3. HPLC analysis

The structural stability of BSA after back extraction was confirmed by HPLC analysis [20]. The chromatogram (S Fig. 1A and 1B) shows the conformation of BSA after back extraction remains same as of pure BSA. Elution time for pure protein and back extracted protein was 4.03 min and 4.04 min respectively. HPLC analysis also proves reverse micellar extraction as a suitable method for proteins or enzymes.

4. Conclusion

The reverse micellar system AOT/ n-heptanol was analyzed for the extraction of BSA from the aqueous phase. The system was optimized for the maximum forward extraction of BSA and the conditions were found as 160 mM L^{-1} AOT concentration, aqueous phase pH value of 4, NaCl concentration 0.8 M. 95% back extraction of protein from micelle phase to stripping phase was obtained at NaCl concentration 1 M, and stripping phase pH 7.5, phase volume ratio 1:1. The obtained results indicate that the AOT/ n-heptanol system is a suitable system for the extraction of the hydrophilic proteins like BSA and may be used to extract the specific protein through selective extraction from the mixtures.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.reffit.2017.06.004.

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