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Reverse Micelle Liquid-Liquid Extraction of Bovine Serum Albumin and Lysozyme

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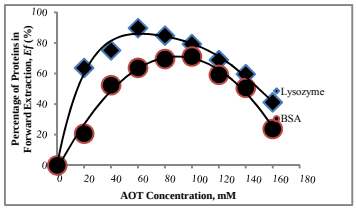
Graphical abstract



Abstract

Reverse micelle extraction by using Sodium bis (2-ethylhexyl) Suffoccinate (AOT) of protein bovine serum albumin (BSA) and lysozyme was investigated in this research. Study of factors affecting the surfactant concentration and pH of aqueous for both forward and backward extraction process was performed in the research. The BSA concentrations were characterized by using the UV-spectrophotometer at wavelength, λ = 280 nm. The result indicated that the extraction percentage of lysozyme was higher than BSA in forward transfer for both parameters; however BSA demonstrated a better extraction performance in backward extraction process. The maximum lysozyme extracted in the forward extraction process was at 60 mM of surfactant concentration while for BSA was 100 mM since BSA is a bulky molecule and the size is larger than of lysozyme.

Keywords: Reverse micelle extraction; AOT; BSA; lysozyme, UV-Spectrophotometer

Abstrak

Pengekstrakan misel terbalik dengan menggunakan Sodium bis (2-ethylhexyl) Suffoccinate (AOT) untuk protein bovine serum albumin (BSA) dan lysozyme disiasat dalam penyelidikan ini. Kajian tentang faktor-faktor yang mempengaruhi kepekatan surfaktan dan pH akueus untuk proses pengekstrakan hadapan dan ke belakang telah dijalankan. Kepekatan BSA telah dicirikan dengan menggunakan spektrofotometer UV pada panjang gelombang, λ = 280 nm. Hasil kajian telah menunjukkan bahawa peratusan pengekstrakan lysozyme adalah lebih tinggi daripada BSA bagi pemindahan ke hadapan untuk kedua-dua parameter, namun begitu BSA menunjukkan prestasi pengekstrakan yang lebih baik di dalam proses pengekstrakan kebelakang. Jumlah maksimum lysozyme yang diekstrak di dalam proses pengekstrakan ke hadapan adalah 60 mM kepekatan surfaktan manakala bagi BSA adalah 100 mM disebabkan BSA adalah molekul yang sangat besar dan ia mempunyai saiz yang lebih besar berbanding daripada lysozyme.

Kata kunci: Pengekstrakan misel terbalik; AOT; BSA; lysozyme, UV-Spectrophotometer

1.0 INTRODUCTION

Reverse micelle technique is extensively used due to its numerous benefits such as cost effective, easier to scale up, environmentally compatible and low potential loss of native activity. Reverse micelle is known as liquid-liquid separation, which has greatly contributed in separating bio-molecules in various medium including proteins, antibiotics, enzymes, amino acids and also organic acids (Ono and Goto, 1997). Moreover, reverse micelle has been used as a treatment technique to remove hazardous dyes from waste water as well (Noritomi *et al*., 2009). Conventionally, separation techniques such as solvent extraction, chromatography, liquid membrane extraction (LME) and aqueous two-phase system (ATPS) were used to separate the desired bio molecules from the impurities; however the methods have several drawbacks. This technique has a high potential in purifying BSA from fermentation medium (Tonova and Lazarova, 2008). Liu *et al*. (2006) studied about the purification of nattokinase by using reverse micelles extraction from fermentation broth for thrombosis therapy and it showed a greater than 80% of activity recovery of nattokinase was purified by using the AOT reverse micelle.

Conventionally, AOT is used as an anionic surfactant (Mohd-Setapar *et al*., 2009; Bera *et al*., 2007) and Triton-X-100 as a nonionic surfactant (Hebbar and Raghavarao, 2007). Reverse micelle was applied since they are able to hold the biomolecule in the core of reverse micelle and therefore minimizing the enzymatic degradation and protecting the active site of bio-molecules (Dasgupta *et al*., 2009). In this research, the application of reverse micelle was investigated to extract

commercialize BSA and lysozyme protein. The extraction process of BSA is effective in reverse micelle phase at high concentrations of AOT (Go`mez *et al*., 1998).

The combination of BSA with V3 peptides also enhanced significantly the capacity to determine the natural antibodies in HIV-1 + sera (Hasegawa *et al*., 1996). Lysozyme (N-acetylmuramidase or muramidase) exists in human body and can be detected in urine, tears, blood, in cell plasma and milk. The isoelectric point (pI) of Lysozyme is 11.35 and has a molecular weight of 14.3 kDa. It is extensively used since it exhibits antimicrobial activity against microorganisms and in food preservation and safety (Marino *et al*., 2003). In adition, lysozyme has the potential in *in vitro* shoot cultures treatment compared to the use of antibiotic (Shiomori *et al*., 1998). AOT is a type of conventional surfactants and commonly used in reverse micelles as an anionic surfactant and consist of hydrophilic head group and hydrophobic tail group. Frequently, in nonpolar organic region, the molarities of AOT are normally used above 10-3 M (Rahimnejad *et al*., 2006). Factors such as temperature, types of salt, types of solvent, surfactant concentration, pH aqueous and size of biomolecules have a great influence on the formation of reverse micelle. A relevant study done by Cason *et al*. examined on the factors of copper particle growth's rate by using two different solvents. It was found that when isooctane solvent was used, the copper particle growth's rate was higher than by using cyclohexane solvent due to the different log K value which refers to the distribution coefficient between water and the organic solvent (Mohd-Setapar, 2008).

■2.0 MATERIALS AND METHODS

2.1 Materials and Chemicals

Sodium di-2-ethylhexyl sulfosuccinate (AOT) was used as an anionic surfactant and isooctane (2, 2, 4-trymethylpentane) as an organic solvent. The salt used for the experiment in aqueous phase is potassium chloride (KCl). Bovine Serum Albumin (BSA) and lysozyme were used as a main bio-molecule. In addition, potassium Hydroxide (KOH) and hydrochloric acid (HCl) were used to adjust the pH of the solution. De-ionized water was utilized to remove the impurities such as microorganisms and the remaining of residues of biological. All chemicals were supplied by Sigma Aldrich Co.

2.2 Extraction Procedures

Experiment was carried out by dissolving the protein in 0.1 M salt solution. Potassium hydroxide (KOH) was then added to the protein solution to adjust the pH aqueous. The forward extraction procedure was conducted by mixing 5 ml of protein solution and the organic phase (5 ml of AOT and isooctane solution) in a 20 ml beaker by using a magnetic stirrer for 10 minutes at the speed of 400 rpm. The solution was completed and kept for 24 hours or above to allow phase separation. Syringe was used to extract the organic phase to remove the sample. The initial and final of pH were recorded. The protein concentrations were measured using the UV-spectrophotometer (Biochrom, Libra S6) at wavelength, $\lambda = 280$ nm. The backward extraction procedure was carried out by mixing the sample of organic phase (forward extraction) with a new aqueous solution for 5 minutes at the speed of 400 rpm and kept for 24 hours or above. The pH of the aqueous solution was determined by using the pH-tester 1 digital pH meter. The experiment was conducted at the temperature of 23±1°C. The extraction percentages for both proteins were computed using the Equation 1 and Equation 2.

2.3 Analysis

The concentration of protein in feed and aqueous phases for both forward and back extractions were determined by using the UV-spectrophotometer. The concentration of the protein in organic phase was identified through mass balance. The efficiencies of forward and back extraction were determined by using the equations below:

$$Ef\,(\%) = (C_i / C_f) \times 100$$

C_i = Protein concentration in organic phase after forward extraction abs

C_f = Protein concentration in feed abs

$$Eb\,(\%) = (C_b /) \times 100$$

C_b = Protein concentration in back extracted aqueous phase abs

C_e = Protein concentration in forward extracted organic phase abs

■3.0 RESULTS AND DISCUSSION

3.1 Forward Extraction

3.1.1 Effect of Sufactant Concentration

Figure 1 shows the effect of surfactant concentration on the extraction percentage of BSA and lysozyme during forward extractions process. The parameters such as aqueous pH, KCl, BSA and lysozyme concentrations were maintained at 7.1, 0.1 M, 7.092 µM and 49.75 mM respectively. The experiment was carried out at room temperature (23±1°C). In the experiment, extraction percentage of lysozyme and BSA extraction is increasing in varios AOT concentration from 20 mM to 60 mM and 20 mM to 100 mM respectively. The increasing extraction percentage can be described as the surfactant concentration increased the number of reverse micelle formed in the organic phase also increase due to higher solute extraction (Hebbar and Raghavarao, 2007).

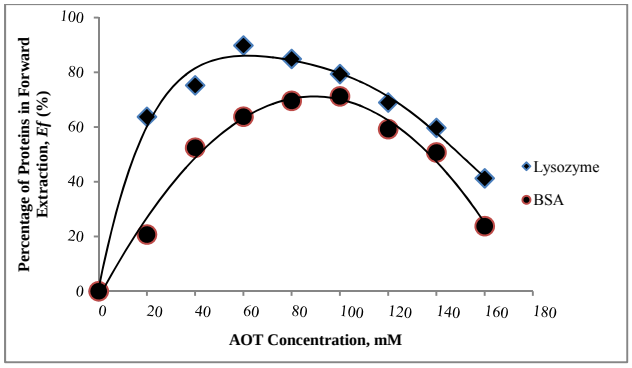


Figure 1 Effect of surfactant concentration on the extraction percentage of BSA and lysozyme, at room temperature (23±1°C), pH 7.1, KCl concentration (0.1 M), BSA (7.092 µM) and lysozyme (49.75 mM)

However, a decreasing line was obtained when AOT concentration above than 60 mM was obtained for lysozyme extraction. From the observation during the process, the

turbidity of the phases was clearly formed, thus making the separation between aqueous phase and lysozyme become difficult. According to the previous researches, when the concentration of surfactant is increased, the number of reverse micelles molecules in the organic phase will also be increasing and therefore preferring the higher solute extraction between two phase (Mohd-Setapar *et al*., 2008). Harikrishna, *et al*. (2002) reported that at high concentration, the surfactant molecule tend to intermicellar collide with each other since the number of molecule is increasing and thus hindering the diffusion of solute. Therefore, the extraction efficiency becomes lower.

Mohd-Setapar *et al*. (2009) also stated that the surfactant concentration affects the surface tension where further addition of the surfactant concentration will increase the reverse micelle formation because of the decreasing surface tension. While for BSA molecules, the higher efficiency was obtained when the surfactant concentration is at 100 mM which is higher than Lysozyme extraction since BSA is a bulky and molecular size of BSA (MW: 65 kDa) and it is larger than that of lysozyme (MW: 14.6 kDa). Thus, more surfactant molecule is needed for BSA partitioning in the organic phase. By increasing the surfactant concentration, the capacity of reverse micelle for the proteins extraction could be enhanced. This is due to the increasing of the micelle population, the size of reverse micelles and water uptake in reverse micelle, W_0 as the electrostatic repulsion inside the micelles are increased. As stated by Mohd-Setapar *et al*., (2008) when the size of reverse micelles is increased, it would lead to the decrease of stearic hindrance of reverse micelles and hence, increasing transfer efficiency of large protein molecules like BSA.

3.1.2 Effect of Aqueous Phase pH

Figure 2 shows the effect of pH on the extraction percentage of BSA and lysozyme during forward extractions process. The parameters such as AOT, KCl, BSA and lysozyme concentrations were maintained at 10 mM, 0.1 M, 7.20 mM and 49.75 M respectively. The experiment was carried at room temperature (23±1°C). The pH value of the aqueous phase was adjusted with 1 M HCl or NaOH. The isoelectric points of BSA and lysozyme are 4.7 and 9.2 respectively. Therefore, the pH region for investigation was selected in the range of 2–11. As the pH of fresh aqueous phase is increased from 2 to 4, for BSA and 2 to 8 for lysozyme which are slightly below the isoelectric point, the protein recovery will decrease significantly. This suggested that the transfer of protein to the organic reverse micellar phase was primarily driven by the electrostatic interactions between positive charges of the protein and opposite charges of AOT hydrophilic molecules (Hemavathi, *et al*., 2010). For lysozyme protein, supposedly at pH 2, the extraction efficiency could be highest and this could be attributed to the increase in the net charge on lysozyme. Conversely, the extraction was obtained in low efficiency at 35.7 %. When pH of fresh aqueous phase was far below pI of lysozyme, net opposite charge will be more, which also resulted in lower activity recovery and denaturation of lysozyme.

Shiomori *et al*. (1998) explained the relationship between salt effect and the BSA extraction, whereby the KCl has a weak electrostatic interaction with AOT compared to $MgCl_2$ and $CaCl_2$. A clear phase was obtained for all experiments in BSA except at pH 3.7 and 10.7. When the pH equals to pI, the electrostatic repulsion will occur between BSA and the surfactant head groups which cause the solubility of BSA into reverse micelle to be lowered. The result shows a similar observation by Shiomori *et al*. (1998).

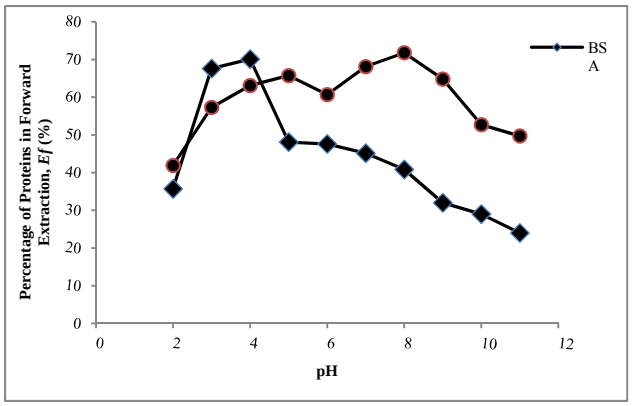


Figure 2 Effect of pH on the extraction percentage of BSA and lysozyme, at room temperature (23±1°C), AOT concentration (10 mM), KCl concentration (7.20 mM), BSA concentration (0.1 M) and lysozyme concentrations (49.75 M)

The graph of lysozyme increased with the addition of pH. The extraction percentage was low at pH range 2.7 to 7.7 due to the large amount of precipitates was observed on lysozyme forward extraction. In addition, the concentration of lysozyme was difficult to examine in some experiments because of the high turbidity in aqueous phase. When pI > pH, the lysozyme molecule behave as positive charge and this cause the attraction with the anionic surfactant to be increased and it could be the reason of the extraction escalation. Conversely, if pI < pH, it will contribute to the descending graph of the lysozyme extraction, limiting for the study the optimum pH value used was 8.

3.2 Backward Extraction

3.2.1 Effect of Surfactant Concentration

Many researchers asserted that high extraction efficiency was difficult to accomplish (Ono and Goto, 1997; Zhang *et al*., 1999; Sun *et al*., 2009). The graph in Figure 3 represents the relationship of AOT on backward extraction for BSA and lysozyme. About 81.92 % of back extracted gained form lysozyme was at the concentration of 20 mM and suddenly drop dramatically, but different results were obtained by Nishiki *et al*. (1995). It was observed that high turbidity and a large amount of white precipitates was obtained during the backward extraction of lysozyme. This implied that the substantial decreased on the extraction percentage with the increasing of AOT concentration. Solution in reverse micelle (AOT/isooctane/water) lysozyme was able to change its conformation and easily denature in consequence of strong specific interaction between lysozyme and AOT molecules in the system (Gochmn-Hecht and Bianco-Peled, 2006). The denature process was affected by the surrounding temperature. According to Nishiki *et al*., (1995) the backward extraction of lysozyme can be enriched for 23 times when the reverse micelle concentration was 1 mg/ml. They studied the back extraction of lysozyme and myoglobin in the reverse micelle. They obtained the highest lysozyme extraction (95% in their case) at 1.5 kmol/m^3 KCl solution while the AOT concentration and pH were maintained at 50 mM and 11.5 respectively.

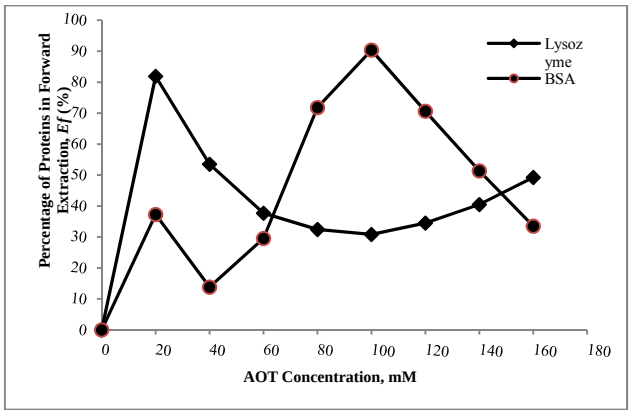


Figure 3 Effect of AOT concentrations on backward extraction for BSA and lysozyme, at room temperature (23±1°C), pH 7.1, KCl concentration (0.1 M), BSA (7.092 µM) and lysozyme (49.75 mM)

The graph of BSA shows ascending and declining line. It was found that, at 100 mM BSA (90.38%) achieved the highest extraction percentage but in vice versa to lysozyme (30.84%). The increasing line can be described as the increasing of the hydrophobicity between the surfactant and BSA molecules. Higher hydrophobicity enhanced the extraction percentage (Hebbar and Reghavarao, 2007). According to Gochman-Hecht and Bianco-Peled (2006), BSA does not easily denature and BSA structures are able to survive to a large extent compared to lysozyme. This behaviour might enhance the extraction of BSA. The head groups of AOT and the surface of BSA played major role on the backward transfer.

3.2.2 Effect of Aqueous Phase pH

Figure 4 displays the effect of pH on backward extraction of proteins. The data obtained from the experiment shows that improvements graph of BSA extraction percentage with 91% the highest extraction percentage achieved. Extraction of BSA at pH higher than the pI might due to the hydrophobic interaction. Hebbar and Roghavarao (Hebbar and Reghavarao, 2007) explained that the existence of terminal groups (side chains on amino acids) for pH higher than pI of BSA leads to a high hydrophobicity (represents the ability of water to exclude non-polar molecules). This can also be explained with the interaction between BSA and the micelle molecule was considered reversible with the help of the less denaturation phenomenon of BSA (Go`mez *et al*., 1998) which lead to the high extraction and the graph of lysozyme shows a descending line.

Based on the experiment observation, at a range of pH between 2 and 11, a clear solution was obtained between the organic and aqueous phases. According to Mohd-Setapar *et. al*., (2008), the clear phase between organic and aqueous phase indicated a successful partitioning of protein in reverse micelle phase. In this case it can be considered that the higher precipitates affect the extraction percentage of lysozyme. In previous literature by Tonova and Lazarova (2008), they mentioned that it was difficult to extract lysozyme in native form (especially backward extraction) due to the strong and extensive electrostatic occurring between its molecules and AOT molecules. At this state, lysozyme has high tendency to aggregate and leads to the gel-like complexes formation.

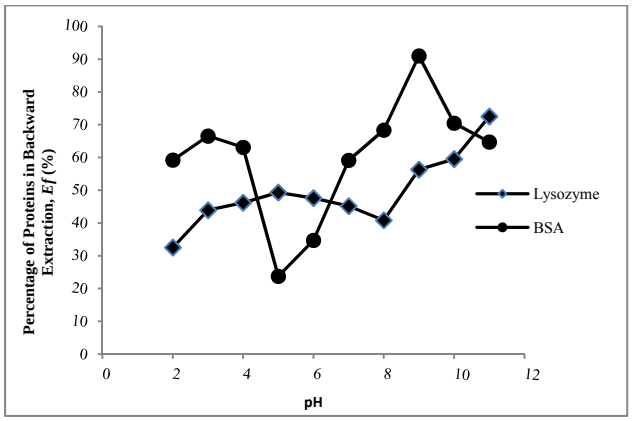


Figure 4 Effect of pH on backward extraction of for BSA and lysozyme, at room temperature (23±1°C), AOT concentration (10 mM), KCl concentration (7.20 mM), BSA concentration (0.1 M), lysozyme concentrations (49.75 M)

■4.0 CONCLUSION

The AOT concentration and the aqueous pH effect on reverse micelle were investigated. The BSA and lysozyme extraction percentages were examined in both forward and backward extraction. The result shows that BSA can be highly extracted during backward extraction for varied pH values and AOT concentrations due to the enrichment of hydrophobic behaviour between BSA and AOT molecules and its structure that does not easily denature or aggregates. Percentage of BSA extraction decreased with the further addition of pH. BSA molecules carry negative charge when pH < pI thus, repulsion forces occur between negative charge molecules of BSA and the anionic surfactant (AOT) and vice versa. The increasing BSA extraction efficiency for further addition of AOT concentration is because of the increasing hydrophobicity between AOT and BSA molecules.

In contrast, lysozyme shows higher extraction percentage during forward extraction in varied pH values and AOT concentrations. A great interaction between the AOT surfactant (negative charge) and the lysozyme molecules (positive charge) occurred when pH > pI. The size molecule of lysozyme is smaller compared to BSA and thus, causing the reverse micelle to extract the lysozyme in an easier way. However, lysozyme structures denature easily especially when the temperature of surrounding is high. High phase turbidity on organic phase also affects the extraction percentage of lysozyme. Therefore, the AOT concentration and pH aqueous influenced the reverse micelle formation.

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