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Application of Aqueous Two-Phase Systems in the Extraction of Bovine Serum Albumin

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Abstract

Protein partitioning in an Aqueous Two-Phase System (ATPS) has been found to be a powerful method for extracting and separating mixtures of biomolecules. In Aqueous Two-Phase Systems, many factors influence the Partition coefficient (K) (which is the ratio of protein concentration in the top phase to that in the bottom phase) and the Recovery percent (Rec%). Two systems of ATPS were used: first, polyethylene glycol (PEG4000)/sodium phosphate (SPH), and second, PEG4000/Dextran. The behavior of Rec% and (K) of pure Bovine Serum Albumin (BSA) in (ATPS) has been investigated throughout the study of the effects of five parameters: (temperature (T), the concentration of polyethylene glycol (PEG4000), the concentration of sodium phosphate or Dextran, pH, and the addition of sodium chloride as a supporting agent). In both systems, pH was the more significant parameter on a Recovery percent (Rec%) and partition coefficient (K). In the first system, the maximum (Rec%) and (K) was 98.08% and 50.99, respectively, at a temperature of 31°C, the concentration of PEG4000 of 1.5 g/10 ml, the concentration of sodium phosphate at 2.4 g/10 ml, pH 10, and the concentration of NaCl at 0.5 M. While in the second system, the parameter that has a more significant effect on (Rec%) and (K) was the temperature. The maximum (Rec%) and (K) were 97.54% and 39.7 respectively at a temperature of 31°C, with a concentration of PEG4000 1.5 g/10 ml, the concentration of Dextran 2.4 g/10 ml, pH 5, and concentration of NaCl at 0.1 M.

Keywords: Aqueous two-phase system (ATPS), Polymer/Polymer, Polymer/Salt, Protein partition, recovery percent, partition coefficient, Bovine serum albumin (BSA)

INTRODUCTION

An aqueous two-phase system is typically produced by mixing two water-soluble substances that are incompatible with each other. The system can be produced by mixing two different water-soluble substances, such as mixing two types of polymers like polyethylene glycol (PEG) and dextran, or a polymer and a salt, like a phosphate or citrate in water over a threshold concentration. This creates two immiscible liquid phases [1–3]. Water makes up the majority of both liquid phases, and each component is saturated inside one of the phases. ATPS provides a number of benefits over conventional downstream processing methods for extraction, recovery, and biomolecule separation, including biocompatibility, technical simplicity, high capacity, and easy scalability [4, 5]. Given these benefits, ATPS has been used as a moderate liquid-liquid extraction and bioseparation process with extensive applications in biotechnology, biochemistry, and cell biology, which would include diverse biological products, such as proteins, enzymes, and nucleic acids, on a massive scale separated, partially purified, or recovered in their parent forms since the mid-20th century [6, 7]. PEG-salt ATPS has been used mainly for bio

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separation due to the inexpensive nature, lower viscous, and rapid separation period of the phase-forming constituents [8–10].

Despite the many benefits of using salt ATPS, there is a lack of understanding of how biomolecules partition in these systems, which makes it difficult to predict how they will behave. The concentration of a particular chemical (e.g. phosphate) and the surface characteristics of the target biomolecules (e.g. proteins) are both important factors when it comes to how the biomolecules behave in a system (e.g. water). In addition, the interactions between the biomolecules and system variables (e.g. pH, temperature, etc.) are also important [11–13].

Bovine serum albumin (BSA) is a protein that is like other proteins-horse serum albumin (ESA), leporine serum albumin (LSA), and human serum albumin (HSA) [14] has been studied extensively and is considered a model protein in a number of fields. Various techniques have already been used to extract and purify it, including using reverse-micelles [15], ultrafiltration [16], and tangential flow filtration [17].

The partitioning of Bovine Serum Albumin between two aqueous phases is a complex process that can be difficult to understand. It is important to know that the partitioning of a biomaterial into separate phases is due to the interactions between it and the surrounding molecules, these interactions create a variety of forces that help to create the desired structure [18, 19]. In order to study the effects of partitioning biomolecules in a liquid mixture, scientists have to conduct many experiments. This can often be expensive, so it is important to make the best choices when doing these experiments. This is because the process of partitioning a substance between different substances is complicated and unpredictable, based on the system variables and the characteristics of the biomolecules involved [20].

In this study, two different systems were used to study the partitioning of bovine serum albumin (BSA): PEG4000/Sodium sulphate and PEG4000/Dextran. The impact of system variables on the behavior of partitioning for our model protein was investigated, such as PEG4000 concentrations (1.5–2.4 g/10 ml), phase-forming salts or polymers, Sodium phosphate (SPH)/Dextran 1100 concentrations (1.5–2.4 g/10 ml), temperature (17–38°C), pH (7–10) and (5–8) for PEG/SPH and PEG/Dextran, respectively, and NaCl (0.1–0.5 M). The protein content for PEG/(salt or polymer) ATPS was (0.1 g/100 ml). We can use the different physical characteristics of model proteins to predict how they might interact with other substances. Additionally, a thorough investigation into the partitioning behavior of a model protein was conducted using Classical Factorial Design (CFD). This was done as a design experiment to improve our understanding of the proteins' behavior.

This research is aimed at finding out which factors (temperature, Concentration of PEG4000, sulphate, and Dexran1100, pH or adding NaCl) have the best effect on the partitioning of BSA, and then designing a manufacturing process that results in the highest recovery rate and partition coefficient.

MATERIALS AND EXPERIMENTAL

Materials

Polyethylene glycol (PEG) was purchased from HIMEDIA Laboratories Pvt. Ltd., India, the molecular weight of the item was 4000 g/mol, disodium phosphate (Na_2HPO_4) were acquired from Central Drug House Ltd., India, Dextran 1100 from AVONCHEM, UK, and Sodium chloride (NaCl) was provided by Alpha Chemika, India. The Coomassie Brilliant Blue G-250 dye and bovine serum albumin (BSA) were bought from HIMEDIA Laboratories Pvt. Ltd., India. A high quality of reagents was used in the analysis. The water utilized in this investigation was double distilled and deionized, which was acquired from a nearby store.

Methods

Aqueous Two-phase Preparation

We need to weigh the amount of different system components of PEG4000 to make it work. This includes Sodium phosphate (Na_2HPO_4) and Dextran1100 to prepare solutions with the desired concentration (1.5–2.4 g/10 ml) each. 0.01 g of BSA is dissolved in the solution of Sodium phosphate or Dextran 1100 to obtain of simulated solution with a concentration of 0.01 g/10 ml of BSA. ATPS of PEG/SPH were created based on the requirement of binodal curves [21–23]. The appropriate pH 9 value was used to create the phosphate stock solution. ATPS of the PEG/Dextran system were prepared based on binodal curves [24, 25]. PH value for the prepared system was 7.

The two ATPS of the PEG/SPH and PEG/Dextran that were investigated to extract the BSA were made with screw tubes. All the two system parts were vortexed and completely blended with a magnetic stirrer for 10 minutes. The samples were spun around in a centrifuge device at 6000 rpm for 20 min so that the different parts of the samples could be separated. To make sure the systems were in equilibrium, they were left to sit at the desired temperature for around 24 hours. A syringe was used to properly separate the phases once phase equilibrium had been reached. The bottom phases' volumes were finally calculated, and samples were collected for protein concentration analysis [15, 24, 26, 27].

BSA Concentration Determination

To measure the concentration of the protein in a sample, a Bradford technique using Coomassie Blue G250 was used [28]. To determine the protein concentration, we diluted samples taken from the bottom phase with a known volume of distilled water. Their ultraviolet absorbance was then analyzed using a single-beam spectrophotometer or photometer equipment [28–30]. Similar solutions that avoided protein interference were used as blanks to make the necessary adjustments to prevent interference from PEG and phosphate or dextran. At 595 nm, the optical density was determined. At a minimum, three duplicates of each experiment or measurement were performed. The degree of protein separation in the polymer phase is determined by the partition coefficient (*K*). To ensure the system is working as intended, we also calculated the percentage of recovery using the partition coefficient [6, 31, 32] The partition coefficient (*K*) is a measure of how much protein is left over after being extracted from a liquid. The higher the partition coefficient, the more protein is left over in the bottom phase, i.e.,

$$K = C_T / C_B \quad (1)$$

Where; C_T is the protein concentration in the top layer.

C_B is the protein concentration in the bottom layer [2, 20, 33].

The recovery percent (Rec%) measures the degree of how well the extraction process is. The following equation was used to calculate the recovery percentage (Rec%):

$$Rec\% = \left(1 - \frac{C_B}{C_{in}}\right) \times 100\% \quad (2)$$

Where; C_{in} is the initial concentration of protein in the bottom phase [20].

RESULTS AND DISCUSSIONS

Temperature Effect

The effect of temperature on the recovery percent of BSA and its partition coefficient has been studied when the concentrations of PEG4000 and Na_2HPO_4 (Or Dextran) are 1.5 g/10 ml each, pH 9 for the first system and pH 7 for the second system, and without adding NaCl. The way that temperature affects an aqueous composition behaves is very complicated because of the way its parts are connected–electrostatic and hydrophobic forces, for example [26]. When the temperature is elevated, proteins can experience changes in their shape, structure, and stability, so the range of temperature is taken from (17–38°C). Figures 1 and 2 show the effect of temperature for systems

PEG4000/Sodium phosphate and PEG4000/Dextran respectively. It can be seen from Figures 1 and 2 that the partition coefficient for protein increases as the temperature rises. This means that the percentage of protein that is recovered after being heated in the range of (17–31°C) is greater at higher temperatures, then they start decreasing with increasing temperature from (31–38°C). Higher temperature and excess heating make proteins vibrate more energetically, breaking hydrogen bonds and disrupting non-polar hydrophobic interactions. This can cause proteins to start to break down and denature [34]. However, the liquids in an ATPS will also change, as the temperature goes up, the PEG molecule becomes more extended, which means it has a less favorable interaction with the protein molecules, which in turn decreases the partition coefficient [35]. Temperature changes cause the molecules in a substance to move around more, which can affect how easily the substance can be divided into smaller pieces. This is because molecules move more easily through substances that are less viscous, denser, and tension-free [36, 37]. This result is in agreement with [20] which allowed the increase in temperature to affect the phase composition of ATPS alongside the protein structure, which increases *K* and Rec% [22]. Had a different opinion when he discovered that the increase in temperature reduces the amount of protein that is recovered in the upper phase. Because of temperature rises, the polymer becomes more hydrophobic; as a result, the contacts between the molecules of the polymer and water tend to weaken, and the amount of water in both phases decreases over time. As scientists know that the temperature of an ATPS experiment may influence the results, they still recommend keeping the temperature constant.

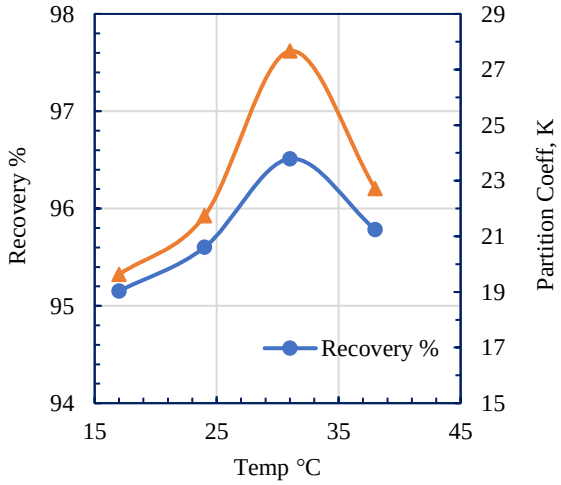

Figure 1. Effect of temp on the recovery % and *K* at conc. of PEG 4000 and SPH of 1.5 g/10 ml each, pH 9, and without adding NaCl.

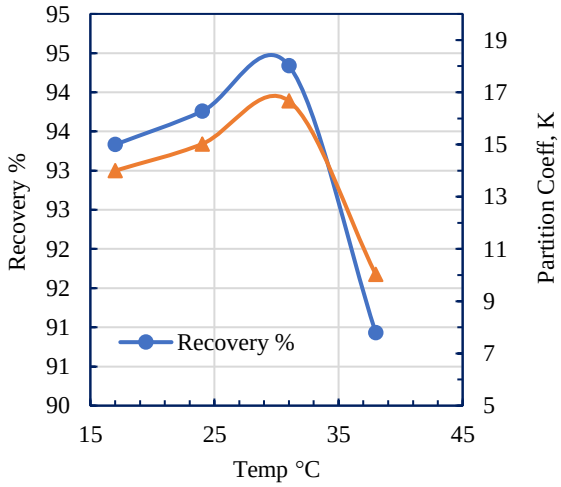

Figure 2. Effect of temp on the recovery% and *K* at conc. of PEG4000 and Dextran of 1.5 g/10 ml each, pH 7, and without adding NaCl.

Concentration Effect of PEG

Protein and PEG interact differently; sometimes the interaction between the two is more hydrophobic, which means that the proteins stay apart more easily, and as a result, more BSA can move to the top phase. Raising the PEG concentration in the system makes it harder for the protein to recover and makes the partition coefficient (*K*) much lower [38]. The concentration of PEG4000 has a significant impact on how quickly and evenly BSA is partitioning, which affects how much of the protein can be recovered. The effect of the concentration of PEG for system PEG4000/Sodium phosphate and system PEG4000/Dextran is shown in Figures 3 and 4 respectively. For 1.5 g/10 ml of PEG4000 with (Na_2HPO_4 or Dextran) at pH 9 and pH 7 respectively without adding NaCl, Rec% and *K* of BSA were found to be (96.62%, 28.59) and (94.44%, 16.97) respectively. It can be seen from Figures 3 and 4 that as the concentration of Polyethylene Glycol (PEG) increases in the range of (1.5–2.4 g/10 ml), the percentage of protein recovered decreases. This result is agreed with [39] who illustrated in their research that when PEG concentration is raised, proteins move from the lower to

the upper phase in ATPS, which leads to rising in the partition coefficient automatically. That is what we call the salting-out effect, when you salt something, the salt molecules move from the bottom up and this happens to the proteins as well. In contrast to what is shown in this work, [40] and [41] had a conflicted opinion when they concluded that increasing PEG concentration levels had a beneficial effect on Rec% and *K* which are increased according to PEG increment. The most hydrophobic anions or cations will tend to spread out in the most hydrophobic phase, while co-ions, which are less hydrophobic, will spread out in the hydrophilic phase. BSA is hydrophilic and is therefore drawn towards the bottom phase. This causes water molecules to be expelled to the upper phase, which translates into more BSAs being partitioned to the salt phase [42].

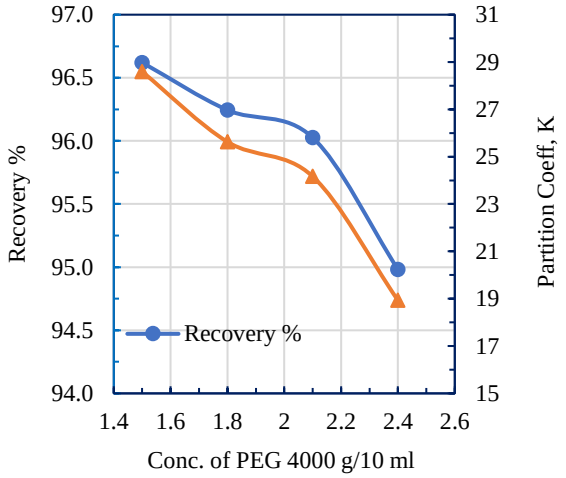

Figure 3. Effect of PEG 4000 on the recovery % and K at T = 31°C, conc. of SPH 1.5 g/10 ml, pH 9, and without adding NaCl

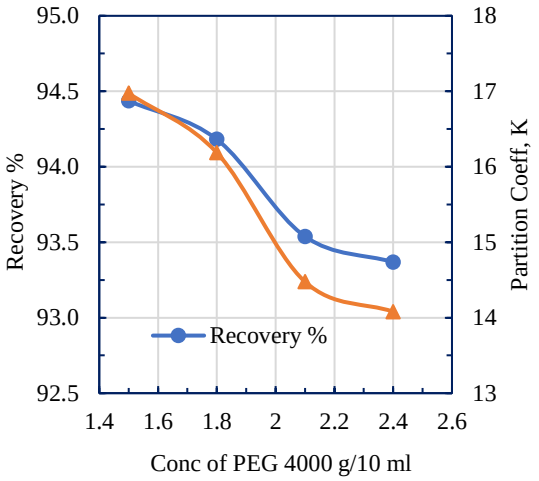

Figure 4. Effect of PEG 4000 on the recovery% and K at T = 31°C, conc of Dextran 1.5 g/10 ml, pH 7, and without adding NaCl

Concentration Effect of Salt/Dextran

By doing experiments, it was found that the amount of Na_2PO_4 salt (or Dextran) in a solution has an effect on how much protein is extracted in ATPS and how well it is partitioned. According to [23, 43], they found in PEG/(salt or Dextran) systems, the recovery percentage and partitioning coefficient increases when the salt or Dextran concentration increases. By checking Figures 5 and 6, it is clear to see that the recovery percentage is increased because when salt and/or Dextran are added, this makes the solution more concentrated and helps to improve the extraction process and thus the partition coefficient rises. It was found by increasing salt concentration from 1.5 g/10 ml to 2.4 g/10 ml for PEG/SPH system the recovery percent increased from 96.56% to 97.71%, and the partition coefficient increased from 28.07 to 42.72, as shown in Figure 5. In PEG/Dextran system, it is shown that the recovery percent increased from 94.38% to 94.99%, and the partition coefficient increased from 16.78 to 18.98, by increasing Dextran concentration from 1.5 g/10 ml to 2.4 g/10 ml as in Figure 6. In this study, it was found that the optimum proportion of PEG to (Dextran/SPH) was 1.5 g/10 ml of PEG to 2.4 g/10 ml of (Dextran/SPH). This result was convenient to [20, 23, 40, 41, 44], and there were no incompatible opinions found.

pH Effect

Biomolecules can be partitioned between two phases according to their different charges and surface properties. This can change depending on the pH of the aqueous solution. Protein molecules with a positive net charge will try to pull electrons away from other proteins, creating an electric force. However, if the pH is higher than the pI (which stands for "pH-indicator" or "Potential isoelectric point"), the electric force is weakened, and the proteins will end up with a net negative charge, and if it is lower, it becomes positive and there will be no net fee if pH and pI values are the same [37]. According to reports, when a system has a higher pH, the negatively charged biomolecules

will partition more easily and the target biomolecule will tend to be found in the top phase. Because of the positive dipole moment, proteins with a high pH value than their (potential isoelectric point) pI, are more attracted to PEG-rich phases [45, 46]. In Figure 7, the effect of pH change shows as the pH of the solution goes up, the partition coefficient increases which means that more protein will be recovered for PEG/SPH system. At pH 10, the Rec% and *K* values are the highest and were 98.08% and 50.99 respectively. In Figure 8, you can see how pH changes can have different effects on the PEG/Dextran system. In the pH range (5–7) the system shows decrease in Rec% and *K* with increasing pH, and higher values of Rec% and *K* were 96.28% and 25.89 respectively at pH 5, then for pH (7–8) the values of Rec% and *K* were increased. After pH 8 the system lost the two-phase formation and specifically in pH 8.3. [22, 25, 31, 40, 44, 47, 48] all had a good agreement with our investigation, while [20, 49] had different results according to their studies for different ATPS systems. They went with an opinion that negatively charged protein molecules partition to the bottom phase at high pH, because of the negative net charge of the protein, which has a pI of 4.7. At pH levels of 5 and 7, the protein is partially negatively charged, whereas at higher pH levels, the protein is fully negatively charged.

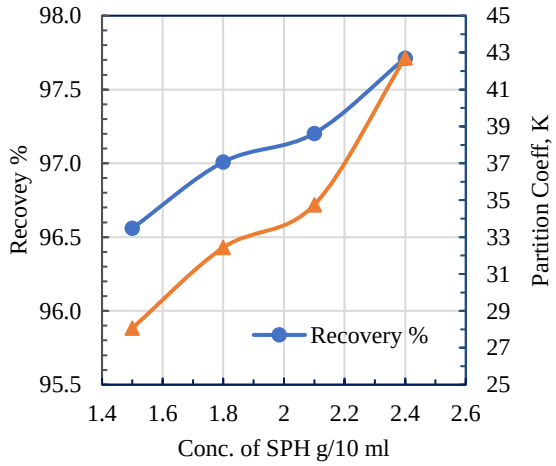

Figure 5. Effect of SPH on the recovery % and K at T = 31°C, conc. of PEG4000 = 1.5 g/10 ml, pH 9, and without adding NaCl.

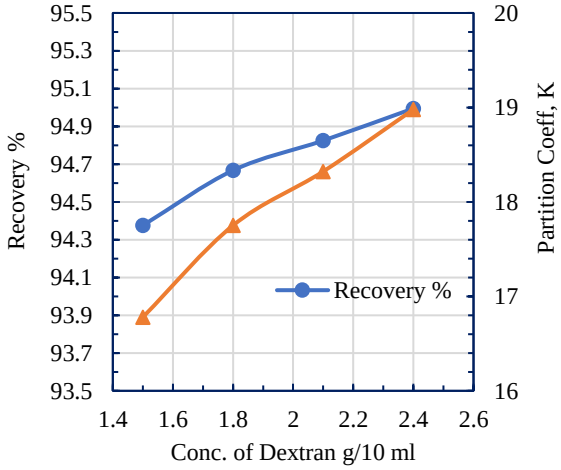

Figure 6. Effect of Dextran on the recovery% and K at T = 31°C, conc. of PEG4000 = 1.5 g/10 ml, pH 7, and without adding NaCl.

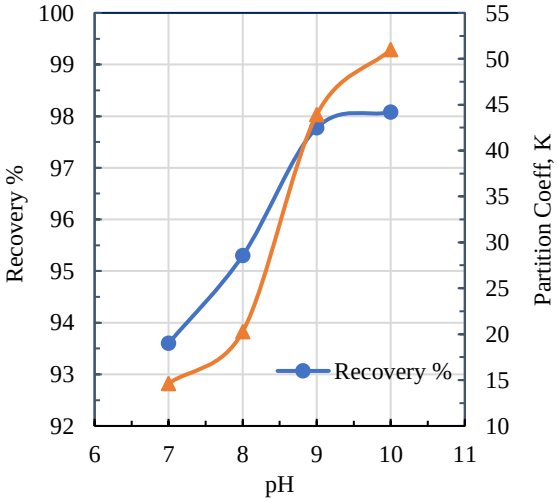

Figure 7. Effect of pH on the recovery% and K at T = 31°C, conc. of PEG4000 and SPH of 1.5 g/10 ml each, and without adding NaCl.

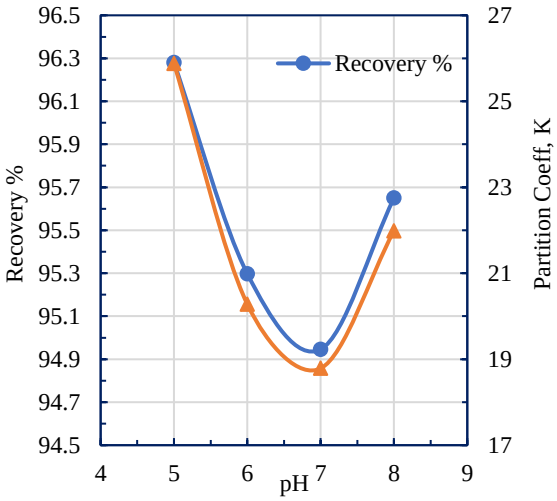

Figure 8. Effect of pH on the recovery% and K at T = 31°C, conc. of PEG4000 and Dextran of 1.5 g/10 ml each, and without adding NaCl.

Through the results obtained and based on scientific facts, we recommend that the pH can be adapted to be 7 in industrial processes because acidic or alkaline media can lead to damage and corrosion in industrial equipment. Furthermore, proteins are comprised of amino acids, which are small molecules, held together by strong chemical bonds. At very high pH (over 10), some of these bonds are broken, which may affect the structural stability of the targeted proteins. Thus, pH may be considered an improvement factor rather than a major factor in these systems.

Effect of NaCl Adding

As a final step in the present work, the effect of NaCl addition has been studied as a way to improve the protein partitioning and the recovery rate of a protein, called BSA. The partition coefficient in ATPSs is influenced by the addition of salts such as NaCl, and KCl [50]. These salts work as partitioning accelerators, ions in these salts have varying degrees of hydrophobicity, and the hydrophobic ions drive the partitioning of the opposite ions to a phase that has ions of higher hydrophobicity and vice versa [37, 38]. The biomolecule transitions from a phase that is salt-rich to one that is polymer-rich due to the salting-out effect [2]. In Figure 9 the addition of NaCl at a concentration range (0.1–0.5 M) to the PEG/SPH system shows an increment in the recovery percent and partition coefficient, higher values recorded at (NaCl 0.5 M) were 98.97% of recovery, and 105.34 for partition coefficient. [20, 21, 23, 25, 31, 33, 42, 51] all went with similar findings and there is no any incompatible opinion. However, a different behavior was recorded in the PEG/Dextran system, as shown in Figure 10. Adding NaCl concentrations of (0.1–0.5 M) results in a change in how much of the protein is recovered and how well it partitioned at a concentration of NaCl = 0.1 M, where Rec% and *K* reached 97.54% and 39.70 respectively. Over 0.1 M of NaCl concentration, a noticeable drop in the Rec% and *K* with increasing NaCl concentration as they dropped from 97.54% and 39.70 at (NaCl 0.1 M) to 96.16% and 25.04 respectively at (NaCl 0.5 M). When the system included neutral salts, partition coefficients dropped linearly as the electrostatic potential between the phases differed [49]. The results of these experiments disagreed with the findings of [36, 52], as they found that adding salt (NaCl) to the systems made the top phase (PEG-rich phase) more negatively charged. As the concentration of salt in the system increases, the positively charged proteins and other molecules are more likely to end up in the top phase. At higher NaCl concentrations, the PEG/dextran systems have a higher percentage of recovery and partition coefficients. According to the case studies, certain biomolecules can have various impacts depending on how they are investigated, which might lead to different results.

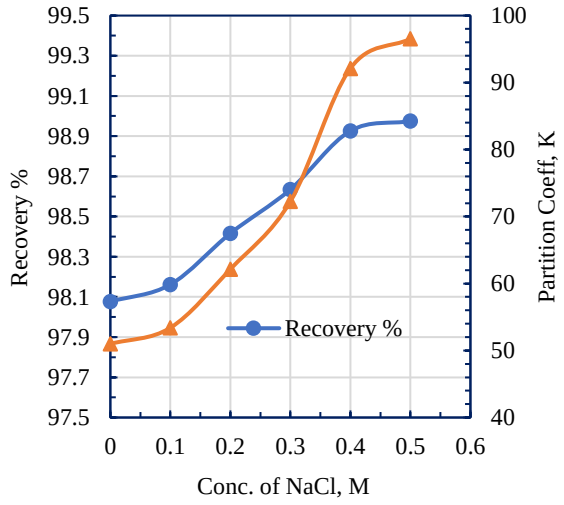

Figure 9. Effect of NaCl conc. on the recovery% and K at T = 31°C, conc. of PEG and SPH are 1.5 and 2.4 g/10 ml respectively, pH 10.

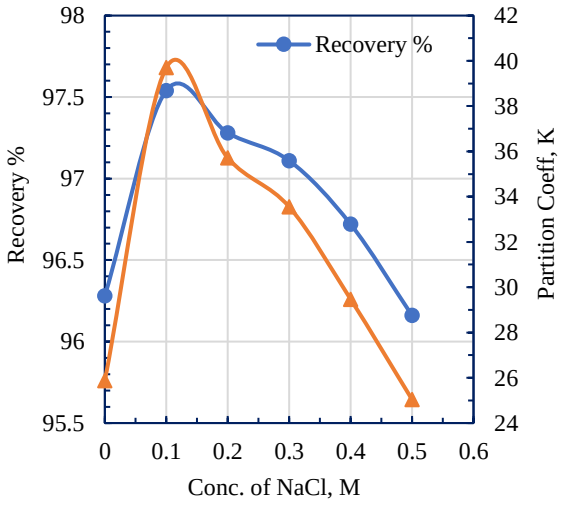

Figure 10. Effect of NaCl conc. on the recovery% and K at T = 31°C, conc. of PEG and dextran are 1.5 and 2.4 g/10 ml respectively, pH 5.

CONCLUSIONS

An aqueous two-phase system (ATPS) is an alternative to traditional methods of extraction, which involve using solvents to extract the proteins. It is a type of method that can be used to recover valuable soluble proteins from aqueous phases. The two systems that have been used in this study for the extraction of BSA were (PEG4000/Sodium phosphate and PEG4000/Dextran); they have a high degree of efficiency. The ATPS is a simple process that can be used to separate bioproducts. This is a cheaper option than using traditional methods, and it is likely to be more effective in purifying products. The results showed that under certain conditions like concentration of PEG, sodium phosphate/Dextran, pH, temperature (T) and NaCl salt addition, it was possible to change the way BSA was partitioned. The study found that the optimal conditions for using a PEG4000/SPH system were, T = 31°C, with concentrations of PEG and SPH of 1.5 and 2.4 g/10 ml, respectively, pH = 10, and NaCl = 0.5 M. The optimal conditions for the PEG4000/Dextran system were as follows: T = 31°C, PEG and dextran concentrations of 1.5 and 2.4 g/10 ml, respectively, pH = 5, and NaCl = 0.1 M. According to these conditions, the BSA recovery percentage reaches 98% and 97% in a single stage for the first and second systems, respectively, implying that no more stages are necessary.

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REFERENCES

1. Philipson L, Albertsson PÅ, Frick G. The purification and concentration of viruses by aqueous polymer phase systems. Virology. 1960; 11 (3): 553–71.
2. Iqbal M, Tao Y, Xie S, Zhu Y, Chen D, Wang X, et al. Aqueous two-phase system (ATPS): an overview and advances in its applications. Biol Proced Online [Internet]. 2016; 18 (1): 1–18. Available from: http://dx.doi.org/10.1186/s12575-016-0048-8
3. Ammar SH, Abdul-nabi WA, Rasheed MK. Extraction of Zn (II) and Cu (II) Ions Using PEG (300)-KCl Salt Aqueous Two-Phase Systems. 2011; 7 (2): 68–74.
4. Phong WN, Show PL, Chow YH, Ling TC. Recovery of biotechnological products using aqueous two phase systems. J Biosci Bioeng [Internet]. 2018;126(3):273–81. Available from: https://doi.org/10.1016/j.jbiosc.2018.03.005
5. Pereira JFB, Outline C. Chapter 5. 2020; 157–82.
6. Khayati G. Aqueous Two-Phase Systems Composed of Different Molecular Weight Phosphate. 2019;(January 2012).
7. Albertsson P-Å. Partition oF Cell Particles and Macrcomolecules in Polymer Two-Phase Systems. in: Advances in protein chemistry. 1970. p. 309–41.
8. Ahuja S. Bioseparations: An overview. Sep Sci Technol. 2000; 2 (C): 1–21.
9. Benavides J, Aguilar O, Lapizco-Encinas BH, Rito-Palomares M. Extraction and purification of bioproducts and nanoparticles using aqueous two-phase systems strategies. Chem Eng Technol. 2008; 31 (6): 838–45.
10. Jaffer ZM, Hameed KW, Imran SG. Extraction of prodigiosin using aqueous two phase system. IOP Conf Ser Mater Sci Eng. 2021; 1076 (1): 012026.
11. Yau YK, Ooi CW, Ng EP, Lan JCW, Ling TC, Show PL. Current applications of different type of aqueous two-phase systems. Bioresour Bioprocess. 2015; 2 (1).
12. Grilo AL, Aires-Barros MR, Azevedo AM, Glyk A, Scheper T, Beutel S. Partitioning in Aqueous Two-Phase Systems: Fundamentals, Applications and Trends. Sep Purif Rev. 2016; 45 (1): 68–80.
13. Pereira JFB, Freire MG, Coutinho JAP. Aqueous two-phase systems: Towards novel and more disruptive applications. Fluid Phase Equilib. 2020 Feb 1; 505.
14. Pereira MM, Pedro SN, Quental M V., Lima ÁS, Coutinho JAP, Freire MG. Enhanced extraction of bovine serum albumin with aqueous biphasic systems of phosphonium- and ammonium-based ionic liquids. J Biotechnol. 2015; 206: 17–25.

15. Lu YM, Yang YZ, Zhao XD, Xia CB. Bovine serum albumin partitioning in polyethylene glycol (PEG)/potassium citrate aqueous two-phase systems. Food Bioprod Process. 2010 Mar; 88 (1): 40–6.
16. Báguena C, Blanco A, Vela V, Cleaning MC. This paper must be cited as : Vol. 125. 2014. 1–10 p.
17. Zydney A, Roush D, Reis R Van, Zydney A. Membrane separations in biotechnology Membrane separations in biotechnology.
18. Du Z, Yu YL, Wang JH. Extraction of proteins from biological fluids by use of an ionic liquid/aqueous two-phase system. Chem-A Eur J. 2007; 13 (7): 2130–7.
19. Dismer F, Alexander Oelmeier S, Hubbuch J. Molecular dynamics simulations of aqueous two-phase systems: Understanding phase formation and protein partitioning. Chem Eng Sci [Internet]. 2013; 96: 142–51. Available from: http://dx.doi.org/10.1016/j.ces.2013.03.020
20. Raja S, Murty VR. Optimization of Aqueous Two-Phase Systems for the Recovery of Soluble Proteins from Tannery Wastewater Using Response Surface Methodology. J Eng (United Kingdom). 2013; 2013.
21. Settu S, Velmurugan P, Jonnalagadda RR, Nair BU. Extraction of bovine serum albumin using aqueous two-phase poly (ethylene glycol)-poly (acrylic acid) system. Vol. 74, Journal of Scientific & Industrial Research. 2015.
22. Pirdashti M, Heidari Z, Fashami NA, Arzideh SM, Khoiroh I. Phase equilibria of aqueous two-phase systems of peg with sulfate salt: Effects of ph, temperature, type of cation, and polymer molecular weight. J Chem Eng Data. 2021 Mar 11; 66 (3): 1425–34.
23. Baskaran D, Chinnappan K, Manivasagan R, Mahadevan DK. Partitioning of crude protein from aqua waste using PEG 600-inorganic salt Aqueous Two-Phase Systems. Chem Data Collect [Internet]. 2018; 15–16: 143–52. Available from: https://doi.org/10.1016/j.cdc.2018.05.004
24. Dumas F, Roger E, Rodriguez J, Benyahia L, Benoit JP. Aqueous Two-Phase Systems: simple one-step process formulation and phase diagram for characterisation. Colloid Polym Sci. 2020 Dec 1; 298 (12): 1629–36.
25. Gündüz U. Partitioning of bovine serum albumin in an aqueous two-phase system: Optimization of partition coefficient. J Chromatogr B Biomed Sci Appl. 2000; 743 (1–2): 259–62.
26. Baskaran D, Chinnappan K, Manivasagan R, Selvaraj R. Liquid-Liquid Equilibrium of Polymer-Inorganic Salt Aqueous Two-Phase Systems: Experimental Determination and Correlation. J Chem Eng Data. 2017 Feb 9; 62 (2): 738–43.
27. Essam Fadel Alwan Al-Jumaily, Muntaha A. Al-Safar ZASA-T. Purification and Characterization β-lactamase produce from local isolate Klebsiella pneumonia. 2009;(March). Available from: https://www.researchgate.net/publication/299624173_Purification_and_Characterization_b_-_lactamase_produce_from_local_isolate_Klebsiella_pneumonia
28. Kielkopf CL, Bauer W, Urbatsch IL. Bradford assay for determining protein concentration. Cold Spring Harb Protoc. 2020 Apr 1; 2020 (4): 136–8.
29. Bradford MM. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Vol. 72, Analytical Biochemistry. 1976.
30. Haweel CK, Ali AJ, Alias SN. Separation of Bovine Serum Albumin Using Chromatographical Column : Parameters and Simulation. 2010; 11 (1): 1–9.
31. Saravanan S, Rao JR, Murugesan T, Nair BU, Ramasami T. Partition of tannery wastewater proteins in aqueous two-phase poly (ethylene glycol)-magnesium sulfate systems: Effects of molecular weights and pH. Chem Eng Sci. 2007 Feb; 62 (4): 969–78.
32. He J, Wan J, Yang T, Cao X, Yang L. Recyclable aqueous two-phase system based on two pH-responsive copolymers and its application to porcine circovirus type 2 Cap protein purification. J Chromatogr A [Internet]. 2018; 1555: 113–23. Available from: https://doi.org/10.1016/j.chroma.2018.04.032
33. Ramesh V, Murty VR. Partitioning of thermostable glucoamylase in polyethyleneglycol/salt aqueous two-phase system. Bioresour Bioprocess [Internet]. 2015; 2 (1): 7–11. Available from: http://dx.doi.org/10.1186/s40643-015-0056-6

34. Kongraksawech T. Characterization by optical methods of the heat denaturation of bovine serum albumin (BSA) as affected by protein concentration, pH, ionic strength and sugar concentration. 2006; 1–109. Available from: http://ir.library.oregonstate.edu/xmlui/bitstream/handle/1957/4100/MS Thesis-Teepakorn Kongraksawech.pdf?sequence=1
35. Dallora NLP, Klemz JGD, Pessôa Filho P de A. Partitioning of model proteins in aqueous two-phase systems containing polyethylene glycol and ammonium carbamate. Biochem Eng J. 2007;34(1):92–7.
36. Ferreira L, Fan X, Mikheeva LM, Madeira PP, Kurgan L, Uversky VN, et al. Structural features important for differences in protein partitioning in aqueous dextran-polyethylene glycol two-phase systems of different ionic compositions. Biochim Biophys Acta-Proteins Proteomics. 2014 Mar; 1844 (3): 694–704.
37. Raja S, Murty VR, Thivaharan V, Rajasekar V, Ramesh V. Aqueous Two Phase Systems for the Recovery of Biomolecules – A Review. Sci Technol. 2012; 1 (1): 7–16.
38. Shad Z, Hussin ASM, Akbari-adergani B. a Primary Approach for Separation and Characterization of A-Amylase From White Pitaya (Hylocereus Undatus) Peels By Polymer/Salt Two Phase System. J Microbiol Biotechnol Food Sci. 2022; 11 (4): 1–6.
39. Shad Z, Mirhosseini H, Hussin ASM, Forghani B, Motshakeri M, Manap MYA. Aqueous two-phase purification of α-Amylase from white pitaya (Hylocereus undatus) peel in polyethylene glycol/citrate system: Optimization by response surface methodology. Biocatal Agric Biotechnol [Internet]. 2018; 14: 305–13. Available from: https://doi.org/10.1016/j.bcab.2018.01.014
40. Faravash RS, Modarress H, Nasernejad B. Structural and partitioning studies of bovine serum albumin in mixture of (poly(ethylene glycol) + K2HPO4 + H2O). J Chem Eng Data. 2007; 52 (1): 71–6.
41. Antelo FS, Costa JAV, Kalil SJ. Purification of C-phycocyanin from Spirulina platensis in aqueous two-phase systems using an experimental design. Brazilian Arch Biol Technol. 2015; 58 (1): 1–11.
42. Regupathi I, Kalaivani S, Sindhu N. Bovine Serum Albumin Partitioning in Aqueous Two-Phase Systems: Effects of Variables and Optimization. Bioprocess J. 2013; 12 (1): 29–41.
43. Johansson G, Joelsson M, Olde B, Shanbhag VP. Affinity partitioning of biopolymers and membranes in ficoll-dextran aqueous two-phase systems. J Chromatogr A. 1985; 331 (C): 11–21.
44. Kee PE, Lan JCW, Yim HS, Chow YH, Chen PT, Ng HS. Efficiency of polymer/salt aqueous two-phase electrophoresis system for recovery of extracellular Kytococcus sedentarius TWHKC01 keratinase. Process Biochem [Internet]. 2021;100(September 2020): 199–206. Available from: https://doi.org/10.1016/j.procbio.2020.10.005
45. Lagomarsino G, Andrews BA, Asenjo JA. Effect of electrostatic energy on partitioning of proteins in aqueous two-phase systems. 2004; 807: 81–6.
46. Andrews BA, Schmidt AS, Asenjo JA. Correlation for the Partition Behavior of Proteins in Aqueous Two-Phase Systems : Effect of Surface Hydrophobicity and Charge. 2005;
47. Raghava Rao J, Nair BU. Novel approach towards recovery of glycosaminoglycans from tannery wastewater. Bioresour Technol [Internet]. 2011; 102 (2): 872–8. Available from: http://dx.doi.org/10.1016/j.biortech.2010.09.013
48. Santhi JV, Tavanandi HA, Sharma R, Prabhakar G, Raghavarao KSMS. Differential partitioning of coconut whey proteins and fat using aqueous two phase extraction. Fluid Phase Equilib [Internet]. 2020; 503: 112314. Available from: https://doi.org/10.1016/j.fluid.2019.112314
49. Hameed RM. The Application of Aqueous Two Phase Systems to the Analysis of Protein Isoforms of Importance in Clinical Biochemistry and Biopharmaceutical Production. 2016; (December).
50. Nuno R, Ferreira LA, Teixeira JA, Uversky VN, Zaslavsky BY. Effects of sodium chloride and sodium perchlorate on properties and partition behavior of solutes in aqueous dextran-polyethylene glycol and polyethylene glycol-sodium sulfate two-phase systems. J Chromatogr A [Internet]. 2018; 12–4. Available from: https://doi.org/10.1016/j.chroma.2018.11.015

51. Perumalsamy M, Murugesan T. Partition behavior of bovine serum albumin in peg2000-sodium citrate-water based aqueous two-phase system. Sep Sci Technol. 2007; 42 (9): 2049–65.
52. Asenjo JA, Andrews BA. Aqueous two-phase systems for protein separation: A perspective. J Chromatogr A [Internet]. 2011; 1218 (49): 8826–35. Available from: http://dx.doi.org/10.1016/j.chroma.2011.06.051

