Aqueous Two-Phase Extraction Advances for Bioseparation

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Abstract

Aqueous two-phase extraction (ATPE), unique liquid-liquid extraction, involves a transfer of solute from one aqueous phase to another. ATPE includes polymer–polymer type and polymer–salt type systems for the recovery of proteins. The protein must be recovered in a highly purified form in order to improve its quality, decrease energy consumption, reduce waste and minimize costs. To acquire the high value and achieve good control over processes, the reliable, multi-component products are required especially those with the ability to investigate complex processing conditions. The current reviewing paper discusses the most recent progresses for the recovery of biomolecules by using the ATPE, covering the mechanism, which controls the phase formation and the behavior of solute partitioning in aqueous two-phase systems (ATPS) processes. The review discusses also the increasing application for the recovery of high-value bioproducts, the recent development of alternative low cost ATPS and disadvantages attributed to ATPS.

Keywords: Aqueous Two-Phase Extraction; Protein; Bioseparation

Introduction

Aqueous two-phase extraction (ATPE) system is composed of either two different immiscible mixing polymers, or one polymer with salt, which are water-soluble in a certain concentration [1,2]. It has been well known as a useful technique for separation and purification of biomolecules, such as proteins [3-14] and antibodies [15-17]. Partitioning of biomolecules in ATPE systems is affected by many factors, including molecular weight/size of polymer and concentration of polymer. Also, the ionic strength of salt and the addition of salts, such as NaCl, improve the hydrophobic resolution of the system. Furthermore, the partitioning of biomolecules can also be influenced by the degree of pH and affinity of the macromolecule for the phase-forming polymer [1,18-21].

The conventional liquid–liquid extraction using organic-aqueous phase systems was previously established. However, due to the poor solubility and possible denaturation of the protein in organic solvents, the technique leads to limit their application in partitioning of many biomolecular products [22]. Now, the application of liquid–liquid extraction based on aqueous two-phase systems (ATPS) has been increased. The recoveries of high-value biomolecules were achieved from various plants using different applications, such as papain from Papaya fruit [23], α- and β- amylases from Zea mays malt [9], glutenin from special wheat [24] and recombinant protein from alfalfa [25]. In addition, the high-value bioproducts also are obtained from different fruits, such as bromelain (EC 3.4.22.33) from pineapple [26], serine protease from Mango [12], invertase from tomato [11] and papain from Papaya fruit [23]. In recent years, the high-quality biomolecules have been recovered from various sources, such as theanine from waste liquid of tea [27] and flavonoids from pigeon pea roots [28]. There are also other bioproducts, such as recombinant human serum albumin from Pichia pastoris broth [29], luciferase from fireflies [30] and immunoglobulin G [17,31].

The ATPE has some advantages in the downstream processing of biomolecules, for instance, the system characterized with high-water content (70–80%, w/w) and low interfacial tension between conjugated phases. This, in fact, provides a secure separation and purification technique for biomolecules [1,32,33]. In addition, it decreases energy consumption, reduces waste and minimizes costs due to a few steps of unit operation, which require low-energy input and easy to scale up. The ATPE also increases biomolecules recovered in a highly purified form. Moreover, it can be used in combination of other separation methods such as liquid chromatography [1,34], packed column [17], magnetic particle adsorption [35].

The ATPE in downstream processing of biomolecules in the bench-scale prototype has been successful with potentially commercial application. However, the scale-up of the ATPS of some biological products were not achieved [15]. Most of those methods have some limitations such as low capacity, several steps and fewer chemicals and proteolytic stability, which may lead to the contamination of the final product [36,16]. Moreover, there are maybe some difficulties during the large scale-up of the ATPS in the industry, especially polyethylene glycol (PEG)/salt system causing corrosion of equipment and precipitation of target product. Thus, the development of alternative methods is very important to achieve the desired biomolecules in high quality and purity. The objective of this paper reviews recent progresses for the recovery of biological products by using the ATPE and the factors affecting partitioning in ATPS processes. The paper also reviews...

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the modern development of the alternative low cost ATPS, and some disadvantages associated with the ATPS.

Separation and Purification of Bioproducts by the Atpe System

The ATPS is considered a powerful and versatile technique, having low-cost and good efficiency downstream process. A high-water content (>70% (w/w) water) generates low interfacial tension, non-flammable, slightly toxic and safe to the environment. This system has high selectivity and recovery yield of biomolecules [37,38]. It has been widely used in the field of biotechnology for separation and purification of various biological products, such as proteins, amino acids, enzymes, cells, antibodies and other bioproducts [1,7,39,40]. Also the ATPS method has received a special application in the area of non-biotechnology, including the recovery of glycosaminoglycans from tannery wastewater [41], crocins from saffron stigmas [42], papaverin from pericarpium papaveris [43].

Protein is one of the most important bio-molecules in the living organism, which is responsible for many reactions and functionalities, such as metabolism, bioprocess, signal transduction, cellular and extracellular structures [44]. A protein in a purified form would be very useful in the applications (e.g. food, chemical, pharmaceuticals). The challenging problem is that the downstream processing mostly accounts for 50 – 80% of the total production costs of proteins [45]. Conventional methods for separation and purification of proteins usually are expensive, time-consuming and difficult to scale up [44,46]. The good news is that the ATPE, an achievable alternative method, has been recognized as an economical and effective technique for recovery and purification of proteins with a variety of advantages, such as simple, fast, low-cost and easily scaled-up. These make it possible strategy for purification of a desired protein in large-scale [35,47]. Partitioning of proteins in the ATPS depends on many factors, namely, hydrophobicity, molecular size, weight and conformation, net electrical charge and environmental conditions [48-50]. A protein purified by this method will be considered to be very important biomolecular product for the bioprocess as it can be used in commercial scale at low cost with relative reliability and accessibility. Optimizing conditions of some selected examples of bioproducts (proteins, enzymes and other products) are summarized in Tables 1-3, respectively.

Mechanism Controlling Phase Formation and the Behavior Solute Partitioning in the Atps Processes

Partitioning of biomolecules in the ATPS is decided by main
electrostatic, hydrophobic and steric hindrance interactions that are very important to the ATPS composition. The ATPS made up by the polymer and the nonionic surfactant results in hydrophobic interaction, [8]. Although electrostatic interactions and salting-out effects during protein extraction in ionic liquid-based aqueous two-phase extraction are important for the transfer of the proteins, the thermodynamics of hydrophobic interactions plays the most important role as a main driving force [44].

Generally, the partitioning of biological products is a result of Van der Waals and ionic interactions of biomolecules with the surrounding phase [37]. However, it is still not well understood the responsible mechanism for the partitioning of biomolecules in the ATPS, which is very important for developing the reliable technique for the industrial application. Therefore, the factors influencing the partitioning of biomolecules are a useful way to study the behavior of solute partitioning in the ATPS process.

Factors Influencing Partitioning of Biomolecules in the Atps

Impact of polyethylene glycol (PEG) characteristics

The PEG characteristics, including weight, size and concentration, are very important factors in the properties of the phase-forming system [51]. The influences of these factors on the partitioning of biomolecules have been reported previously [11,33,41,52].

Molecular weight and size: The partitioning of biomolecules depends on the molecular weight of polymers and the other components constituting the phase. Molecular weight has a strong effect on the partitioning behavior of biomolecules [11,12,53]. Higher molecular weight of PEG has less coefficient factor and then lower polymer concentration needed for high separation [38]. The low molecular weight of PEG has a hydrophilic end group with shorter polymer chains that reduces the hydrophobicity [41], while better partitioning can be achieved due to the low interfacial tension of low molecular weight. An increase in the PEG molecular mass reduces free volume by increasing the chain length of the PEG polymer [11,25,30], resulting in partitioning of the biomolecules to the bottom phase [4]. The increase in polymer weight causes the reduction of free volume of the top phase, so the partition of biomolecules in the salt-rich bottom phase decreases the partitioning coefficient [54].

Another study indicated the influence of different molecular weights of PEG (4,000, 6,000, and 8,000) on partitioning of myoglobin and

| Bioproduct      | Source                  | System and Composition (%w/w) | $K_p$ | Yield (%) | TLL (%w/w) | pH | Reference |
|-----------------|-------------------------|-------------------------------|-------|-----------|------------|----|-----------|
| Protein         | Cheese whey             | PEG6000/polassium phosphate (11.7/10%) | 0.9   | 83.4      | 23.9       | 7.0 | [67]      |
| Glutatin        | Wheat flour             | PEG15000/Li$_2$SO$_4$ (14.0/13.23%) | 5.90  | 64.9      | 35.51      |    | [24]      |
| Recombinant     | Alfalfa                 | PEG8000/phosphate (16.1/10.0%)  | 0.1   | 88        | 35.7       | 7.0 | [25]      |
| (α-la), (β-lg)  | Milk whey               | PEG20000/phosphate (13.0/13.0%) | 0.48, 0.01, 0.92 | 81.1, 97.3, 97.8 | - | 6.7 | [6]       |
| OVA            | Chicken egg             | PEG40000/poly(acrylic acid) (PAA) + 1 M NaCl | 5.5   | 87.4      | 54.7       | 8.0 | [37]      |
| Protein         | Zea mays malt           | PEG 6000/CaCl$_2$             | 4.2   | -         | -          | 7.0 | [39]      |
| Bromelain       | Pineapple Peel          | PEG30000/MgSO$_4$ (15/20%)    | 2.93  | 108.45    | -          | 9.0 | [71]      |
| (α-Al)          | Wheat flour             | PEG20000/(FBP) trisodiumsalt (11.7/19%) | -     | 79        | -          | 7.0 | [69]      |
| Protein         | Corn                    | PEG14500/Na$_2$SO$_4$/8.5% NaCl | -     | 93        | -          | 7.0 | [5]       |

$K_p$ = partition coefficient of protein, TLL= Tie line length, (-) = the value was not given, α-la= α-lactalbumin, β-lg= β-lactoglobulin, Gmp= glycomacropeptide, α-Al= α-amylose inhibitor, FBP= fructose-1,6-biphosphate, OVA= Ovalbumin

Table 1: Optimizing partitioning of proteins in the various ATPS recovered from foods.
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Table 2: Optimizing partitioning of different enzymes recovered in the various ATPS.

| Bioproduct           | Source                   | System and Composition (%w/w)     | Kp  | Yield (%) | TLL (%) | pH  | Reference |
|----------------------|--------------------------|-----------------------------------|-----|-----------|---------|-----|-----------|
| Serine Protease      | Mango Peel               | PEG8000/ phosphate (-/ 4.5%)       | 84.2| 97.3      | 17.2    | 7.5 | [12]      |
| Inverts              | Tomato                   | PEG 3000/ Na₂SO₄ (15/12%) +5% KCl | 1.1 | 90        | -       | 4.5 | [11]      |
| Serine Protease      | Kesanai (Streblus asper) | PEG6000/rich- MgSO₄ (16/ 15%)     | -   | 96.7      | -       | 7.0 | [59]      |
| Papain               | Papaya                   | PEG6000/ (NH₄)₂SO₄ (8/ 15%)       | -   | 89.9      | -       | 5.0 | [23]      |
| Phytase              | Ascogillus niger         | PEG6000+8000/ Citrate (10.5/ 20.5%) | 0.96| 96.0      | -       | 5.6 | [13]      |
| Protease             | Tuna (Thunnus alalunga)  | PEG2000/MgSO₄ (15/15%)            | 0.86| 89.1      | -       | -   | Nalinan et al. |
| Soybean peroxidase   | Soybean (USA)            | PEG4000–IDA–Cu²⁺/Na₂SO₄ (4/ 10%)   | 0.05| 64        | -       | -   | [72]      |
| G6PDH                | Sigma (USA)              | PEG6000/Na₂SO₄ (14/ 13%)          | 2.7 | 98.26     | 92.58   | 6.9 | 6.5       | [53] |
| β-gala; β–gluc.      | Barley                   | PEG1500/ (NH₄)₂SO₄ (14/ 13%)      | 2.8 | 98.05     | 92.58   | 6.9 | 6.5       | [53] |
| Lipase (E.C. 3.1.1.3)| Burkholderia pseudomallei| 2-propanol/phosphate (ATPS) (16/ 4.5%)| 13.5| 99.9      | -       | 9.0 | [40]      |
| PPL                  | Sigma (USA)              | PEG1500/potassium phosphate (17/13%) | 12.7| 94.7      | -       | 7.0 | [14]      |
| PheDH                | Bacillus sphaericus      | PEG-6000/(NH₄)₂SO₄ (8.5/17.5%)    | 0.027| 94.42     | 39.89   | 8.0 | [56]      |
| Invertase            | Baker’s yeast            | PEG3000/MgSO₄ (15/123%)+5%MnCl₂  | -   | 98        | -       | 5.5 | [54]      |
| Plant-esterase       | Wheat flour              | PEG10000/NaH₂PO₄(27.0/13.0%) and PEG1000/ NaH₂PO₄/(NH₄)₂SO₄ (27.0/13.0/6.0%) | -   | 83.16     | -       | 5.0 | [62]      |

Kp = partition coefficient of protein, TLL = Tie line length, IDA = Iminodiacetic acid, G6PDH = Glucose-6-phosphate dehydrogenase, pk = purification factors, β-gala = β-galactosidase, β–glu = β–glucosidase, PheDH = phenylalanine dehydrogenase, PPL = porcine pancreatic lipase, (-) = the value was not given.

Table 3: Optimizing partitioning of some selected products in the various ATPS recovered from different sources.

| Bioproduct          | Source                  | System and Composition (%w/w)       | Kp  | Yield (%) | TLL (%) | pH  | Reference |
|---------------------|-------------------------|-------------------------------------|-----|-----------|---------|-----|-----------|
| IgG                 | Chinese Hamster Ovary   | PEG3350/phosphate-rich phase (cont, ATPE) | -   | 85        | -       | 6.0 | [98]      |
| Lectin              | Caranavila grandiflora Benth | PEG4000/sodium citrate (20/20%)      | 8.67| 104       | -       | 6.0 | Porto et al. |
| Lutein              | Green miroagla (Chlorella protothecoides) | PEG 8000/phosphate (22.9/10.3%) | -   | 81.0      | 49.4    | 7.0 | Cisneros et al. |
| Luciferase          | Fireflies (Photinus pyralis) | PEG1500 rich (NH₄)₂SO₄ (4/20.5%) | -   | 13.69 fold in pk | - | - | [30] |
| Crocins             | Saffron stigmas (Crocus sativus) | Ethanol/ potassium phosphate (19.8/16.5%) +0.1 M NaCl | -   | >75       | 25     | -   | [42]      |
| Glycosaminoglycans  | Tannery wastewater      | PEG4000(PAA)                        | -   | 91.50     | 54.7    | 8.0 | [41]      |

Kp = partition coefficient of protein, TLL = Tie line length, (-) = the value was not given, IgG=Human immunoglobulin G

Ovalbumin, the partitioning of both proteins is higher if the molecular mass of PEG is lower [37]. In PEG (4,000–phenylacetic acid (PA)) system, the percentage yields of extracted myoglobin at 200°C and pH 8.0 in 1 M NaCl increases from 75.2% to 95.2% with the increase of tie line length (TLL). This is due to the increase of hydrophobicity and partitioning coefficient of the ATPS. It also affects the partitioning of proteins present in the phase system, whereas in the case of ovalbumin, the increase is from 67% to 87.4%. Different molecular weights of PEG (1,000, 2,000, 3,000, 4,000, 6,000 and 8,000) have been used to purify invertase enzyme from tomato, while the partitioning of invertase in PEG–Na₂SO₄ system is strongly dependents on the molecular weight of the PEG [11]. Nearly all invertases partitioned into the top phase with PEG-3,000, while most contaminating proteins were partitioned into the lower phase in the ATPS containing the other PEGs (1,000, 2,000, 4,000, 6,000 and 8,000). A wide range of molecular weight PEG (400, 1000, 1,500, 4,000, 6,000 and 8,000) was screened for differential partitioning of α-galactosidase and β-glucosidase from the barley [53]. For low molecular weight PEG, both the enzymes partitioned to top phase whereas for high molecular weight PEG, both the enzymes partitioned to bottom phase. PEG 1,500 had better partitioning of both of enzymes. The highest partitioning (97.22%) of luciferase enzyme from fireflies (Photinus pyralis) was obtained in 1,500PEG/(NH₄)₂SO₄ system [30]. In the smaller molecular mass PEG (300, 400 and 600), there was a tendency for glucose 6-phosphate dehydrogenase (G6PDH) to stay in the top phase (PEG phase) [55]. In the higher value of PEG (1,000 and 1,500), the larger amount of G6PDH stayed in the bottom phase. However, the low molecular mass PEG is also unsuitable for adequate partitioning, due to the decrease of the exclusion effect [56]. It allows the polymer to attract all the proteins to the upper phase. Thus, the choice of the most suitable intermediate molecular mass of PEG is very important for increasing the extraction efficiency of the ATPE system [54,57,58].
**PEG concentration**: Several studies demonstrated the influence of different PEG concentrations (7–21%, w/w) on protein partition coefficient (Kp) and enzyme partition coefficient (Ke) from various sources [53,54,59]. Their results showed the significant effect of the PEG concentration on the partitioning of biomolecules in the ATPS. The highest concentration in PEG lowered Kp and Ke. The intermediate concentration of PEG/salt is more applicable for good separation and purification. When serine protease was extracted from Kesinai (Streblus asper) leaves, the Ks of the enzyme decreased significantly at a low concentration of molecular weight (8% PEG4,000) [59]. On the other hand, the high PEG concentration and high molecular weight gave negative effect on the partition coefficient of the enzyme. In partitioning of a yeast invertase at different PEG-3,000 concentrations (7.5–20%, w/w) together with 20% (w/w) MgSO₄ at pH 5.0, partitioning of invertase in the ATPS is affected significantly by the PEG concentration [54]. The PEG at concentration of 15% (w/w) resulted in the highest value (3.2-fold) of purification factors (PF) with the yield of 134%. At concentration of 20% (w/w), purification factors and yield decreased dramatically to 2.1-fold and 46%, respectively. Similarly, high purification factors and yield were obtained when invertase was partitioned in a 15% (w/w) PEG/12.5% (w/w) Na₂SO₄ at pH 5.0 [11].

**Impact of salt concentration**

The impact of salt concentration has been widely studied. Increases in salt concentration result in an increase in partition coefficients of bioproducts in upper phase or interface due to salting out [11,53,60]. In general, proteins with the negative charge tend to partition to the top phase in PEG/salt systems while those with the positive charge usually go to the bottom phase [54,61,62].

Varied salt concentrations have been used in separation of proteins (cytochrome c, lysozyme, trypsin, bovine serum albumin and myoglobin) by the ATPS system combined with high-performance liquid chromatography (HPLC) [34]. When (NH₄)₂SO₄ concentration increased from 10% to 18%, the top phase volume decreased and the bottom phase volume increased. All proteins retained in the lower phase except lysozyme, which was partitioned in the two phases. Moreover, 23% (w/w) MgSO₄ caused the best partition behavior of a yeast invertase at different concentrations (15–25%, w/w) in the PEG/MgSO₄ aqueous [54]. Besides the salt type, the distribution of invertase is mainly controlled by concentration of the salt. The partition coefficient (Kp) of luciferase increased rapidly as compared to that of total protein (Kt) with an increase in salt concentration from 12% to 16% [30]. The optimum condition for cephalin antibody separation was at a concentration of 20% for both PEG and Na₂SO₄ [60]. Similar finding was reported in the case of penicillin G and PAA extraction [47].

When (NH₄)₂SO₄ concentration increased, and the optimal values could be obtained at pH 8.0 [56]. However, an increase in pH of the ATPS (PEG-4000/K₂HPO₄, 12/13%) from 7.0 to 9.0 reduced the partition coefficient of lipase from 7.94 to 4.45 and activity recovered from 81.1 to 70.6% [70]. Enzyme stability slightly reduced in the acidic area, but it was dramatically lost at pH above 9.0 [71]. Generally, the efficacy of the pH can be either by changing the charge of the solute or by altering the ratio of the charged species presents [37].

**Impact of the type of salts**

The selection of salts for the ATPS depends on their ability to promote hydrophobic interactions between biomolecules [64]. The PEG/phosphate system is widely used for recovery of bioproducts [65]. Other salts having similar properties to phosphate, such as sulphate and citrate, have been also used. The changes of the environmental phase system, due to use of different salts, lead to change the behaviors of partitioned protein [62]. Anions are the most effective in partitioning (SO₄²⁻ > HPO₄²⁻ > acetate) than cations (NH₄⁺ > K⁺ > Na⁺ > Mg²⁺ > Ca²⁺) [66]. Recently, use of salts, like citrate (biodegradable) and ammonium carbonate (volatile) are favored because of their high selectivity, less pollution, biocompatibility and easy to scale-up [38,50]. The partition coefficient values of methionine in systems containing Na₃PO₄ are greater than values in systems containing NaH₂PO₄ or Na₂HPO₄ because of more abilities of Na₃PO₄ to enhance hydrophobic interactions between particles [7]. The PEG 6,000/potassium phosphate system of 23.9% (w/w) TLL gave the best partitioning results with the highest recovery of proteins from cheese whey than the PEG/ammonium sulphate and PEG/potassium dihydrogen phosphate systems [67]. In fact, a significant aggregation tendency of proteins was observed in PEG/ammonium sulphate and PEG/potassium dihydrogen phosphate systems, whose pH was close to isoelectric pH of whey proteins. The PEG/ammonium sulphate could be the suitable purification phase for differential partitioning of β-galactosidase and β-glucosidase rather than other types of salts, such as sodium sulphate, sodium phosphate, potassium phosphate and sodium citrate. This PEG/ammonium sulphate was found to be the best in terms of activity recovery and differential partitioning of both the enzymes [53], while potassium salts have much better effects on partition of lipase than that sodium salts and ammonium salts. This is due to the partition coefficients increased according to the following order: K⁺ > Na⁺ > NH₄⁺ [68].

**Impact of pH**

Partitioning of proteins and enzymes to the phases in the ATPS system depends on their isoelectric points (pl) [63]. The pH of the system, however, affects the charge of target protein and ion composition as well as introduces differential partitioning into the two phases [56]. Accordingly, the initial pH of the system must be above the pl of target bio-molecules [65]. A pH value above 7 is suitable for the PEG/phosphate system and a pH below 6.5 is compatible with the PEG/salt system. Most of the biomolecules, especially proteins and enzymes, are stable at neutral pH that is favorable condition to conduct the ATP partitioning. At pH 7, 79% a-amylase inhibitor was recovered with 3.2 purification factor in 11.7% (w/w) PEG-2000 and 19% (w/w) fructose-1,6-bisphosphate trisodium salt [69]. When the pH rose from 5.8 to 8.0, the Kₘ, yield and recovery of phenylalanine dehydrogenase (pl 5.3) increased, and the optimal values could be obtained at pH 8.0 [56]. However, an increase in pH of the ATPS (PEG-4000/K₂HPO₄, 12/13%) from 7.0 to 9.0 reduced the partition coefficient of lipase from 7.94 to 4.45 and activity recovered from 81.1 to 70.6% [70]. Enzyme stability slightly reduced in the acidic area, but it was dramatically lost at pH above 9.0 [71]. Generally, the efficacy of the pH can be either by changing the charge of the solute or by altering the ratio of the charged species presents [37].

**Tie line length (TLL)**

Tie line length can affect biomolecule partitioning by hydrophobicity and interfacial tension between phases of the ATPS. The ATPS becomes more hydrophobic with increasing TLL due to reduction of water availability [8]. An increase in the TLL causes an increase in the protein partition coefficient that, in turns, increases the yield of proteins in the top phase due to reduction of the bottom phase [12]. Increasing TLL in the PEG-salt system makes salting-out more effectively, leading to shift of proteins to the top PEG-rich phase [72]. If protein solubility in the PEG phase is insufficient, protein will precipitate at the interface. Solubility and salting-out limits depend on the properties of individual proteins. Therefore, a different response will be expected when a mixture of protein is handled [39]. It was reported that glutenin partitions from wheat flour, an increase in TLL caused an increase in protein transfer to the upper phase in systems formed by PEG-1,500/sulphate salts (lithium or sodium) [24]. However, in terms of systems that composed

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of PEG-4,000/sulphate salts (lithium or sodium), an increase in TLL caused an increase in protein transfer to the lower phase.

**Influence of the addition of NaCl**

In general, addition of neutral salts, such as NaCl, to the ATPS results in an increase in the hydrophobic difference due to generation of an electrical potential difference between two phases [5,12,73]. An increase in the hydrophobicity will decrease the amount of bound water, which keeps the final composition of the systems constantly [15]. Furthermore, it increases the ionic strength and enhances the migration of low molecular weight compounds towards the polymer-phase, especially in PEG-4,000/sulphate salts (lithium or sodium), an increase in TLL caused an increase in protein transfer to the lower phase. In 6% (w/v) of NaCl, the purification factor of lipase enzyme increased 15.77 and 2.82 to 5.51, respectively, in the PEG-4,000/PAA system [37].

The addition of different concentrations of salt (0.0 - 1.0 M) increased the concentration of neutral salts may cause denaturation of proteins existing in the system, thus low concentration range from 0.0 to 1.0 M is preferred. High yield (97.3%) of serine protease from mango peel was obtained with addition of 4.5% of NaCl to the PEG/dextran ATPS [12]. The addition of different concentrations of salt (0.0 - 1.0 M) increased the partition coefficient of myoglobin and ovalbumin from 4.20 to 15.77 and 2.82 to 5.51, respectively, in the PEG-4,000/PAA system [37]. Similar results were observed with bovine serum albumin (BSA) in the ATPS [74]. In 6% (w/v) of NaCl, the purification factor of lipase enzyme increased significantly from 59.93 to 141.65 fold. However, further addition of NaCl decreased the K, of lipase [63].

**Recent Process in Atp, Applications Economic Costs**

The ATPS is a powerful method commonly used for separation and purification of biomolecules, such as proteins, enzymes and antibodies. It is composed either polymer (PEG)–polymer (dextran) or polymer (PEG)–salts. However, the polymer-polymer interaction dominates due to the low solubility of amphiphilic proteins in the PEG-salt, which has a high tendency to aggregate in aqueous solution that may damage fragile proteins [37]. The environmental problems were raised from elevated salt concentration in waste disposal [4,73]. Large chemical consumption during the phase-forming [37] will lead to additional cost for the phase recycling in the system. However, the ATPS based on polymer (PEG)–polymer (dextran) is very expensive because of the high cost of some forming phase polymers, such as dextran [37] and ethylene oxide–propylene oxide copolymers [65]. This, in fact, limits the implementation of this system at the large scale.

In recent years, some progress in the ATPE technique has been reported, indicating some modification introduced to the ATPE system to recover biomolecules in high value, good quality and low cost, providing basic materials for bio-product processes and increasing the application of the non-biotechnology. The alternative polymers used as a substitute for dextran, are generally safe, low-cost and compatible with the system, such as PEG/nonionic surfactant polymers (Triton X-100 and Tween 80) [8] and ionic liquid-based ATPE [42,44]. The dextran polymer also substituted by alcohol/salt ATPS system [42,76], microfluidic aqueous PEG/detergent ATPE system [77] and acid polymer ATPS prototype for the recovery of biomolecules [97]. It was used for recovery of protein and α-amylase from soybean, to extract the low-abundant protein from complex mixtures. A continuous ATPE process incorporating three various steps (extraction, back-extraction, and washing) has been introduced with a pump mixer-settler battery. The ATPS process recovered 99% purity of IgG from a CHO cell supernatant and 100% of IgG from a PER.C6 [98]. This new process indicates the ability to successfully recover and purify different antibodies. It could overcome some of the limitations encountered using the typical chromatographic processes, besides inherent advantages of scalability, process integration, capability of continuous operation, and economic feasibility.

Generally, the continuous operation increases partition coefficients with higher recovery efficiencies. The processing time is reduced at least three folds, compared to the batch ATPS. Furthermore, it achieves higher enzyme partitions coefficient (K,>4) and a top phase enzyme recovery (81%) with the purification factor 40-fold than a batch system. It is suggested that the continuous ATPE model can be used in an industry field for the recovery of bioproducts.

**Disadvantages Attributes to the Atps**

The development and application of the ATPS have some drawbacks due to lack of information about the exact mechanism of partitioning and unpredictable [56]. There are two restrictions to limit the wide application of the ATPE [99]. Firstly, it is difficult to predict exactly the behavior of target proteins in the ATPE system. Secondly, monitoring the characteristics of proteins is the basic requirement for assessment of bioprocesses, which are affected frequently by the presence of high concentrations of polymers or salts. Considerable time is needed to build first recovery process of the experiment, while big
budget is needed for installation and limited output of the purification units [69]. Experimental design is needed to determine the optimal ATPS system for partitioning of desired products [65]. Compared with a novel alcohol/salt ATPE, polymer/polymer, polymer/salt and surfactant ATPE systems have some disadvantage such as the high cost and viscosity as well as slow segregation. There are difficulties in isolating the extracted molecules from the polymer phase or micellar phase by re-extraction and environmental pollution resulting from the recycling of phase-forming polymers [68,76].

Conclusion

The ATPE is the suitable technique for separation and purification of bioproducts in biological and biotechnological fields, especially the PEG/salts system. However, the implementation of the PEG/salt in the large scale may cause environmental problems due to the great amount of chemicals (salts and polymers) needed to phase forming and the high cost resulting from effectively recycling. To overcome it, some modifications are introduced into the system to purify good quality of biomolecules with low-cost and safely to the environment. This modification includes synthesized polymers (P \(_{\text{PEG}}, P \_\text{salts}\), reverse micelle solvent ATPS system and interaction of HPLC with the system to increase the extraction rate. Furthermore, the application of a continuous ATPE system is an alternative technique for the recovery of proteins in large scale. This, in fact, provides the ability to recover proteins in higher efficiencies, increases partition coefficients and shortens processing time. Therefore, the continuous separation ATPE system can be used in pharmaceuticals, foods and chemical industries for purification of high-value bioproducts.

Although the ATPE has been extensively used for recovering of biomolecules, the poor understanding of mechanism that governs phase formation and solute partitioning in the ATPE hinders its application in some cases. Therefore, using of the ATPS with some modifications, such as affinity ligands, a substitute of polymers or using copolymers and a combination of the ATPS with other methods (e.g. chromatography), are more effective due to the high efficiency of separation, purification and increasing recovery yields of bioproducts.

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