Efficacy of Alcohol/sugar Aqueous Biphasic System on Partition of Bovine Serum Albumin

Yin Hui Chow
School of Computer Science and Engineering, Faculty of Information and Technology, Taylor's University Lakeside Campus, 47500 Subang Jaya. Selangor, Malaysia

Alagan Sahlini
School of Computer Science and Engineering, Faculty of Information and Technology, Taylor's University Lakeside Campus, 47500 Subang Jaya. Selangor, Malaysia

Hui-Suan Ng
Faculty of Applied Sciences, UCSI University, UCSI Heights, 56000 Cheras, Kuala Lumpur, Malaysia.

Chi-Wei Lan (lanchiwei@saturn.yzu.edu.tw)
Yuan Ze University

Research

Keywords: Aqueous biphasic system, separation, protein partition, alcohol, carbohydrate

Posted Date: November 2nd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-98359/v1

Version of Record: A version of this preprint was published on January 21st, 2021. See the published version at https://doi.org/10.1186/s40643-021-00360-y.

Abstract

A green bio-separation alternative can be performed with a non-toxic and biodegradable aqueous biphasic system (ABS) composed of short-chain aliphatic alcohol-based top phase (1-propanol and 2-propanol) and carbohydrate-based bottom phase (glucose, sucrose, and maltose). A model protein, bovine serum albumin (BSA) was adopted to determine the effects of types and concentration of phase-forming components; protein concentration; and system pH on the protein partition efficiency in the ABS. Results showed that the 1-propanol/maltose ABS gave an overall better partition efficiency of BSA to the alcohol-rich top phase compared to the 1-propanol/sucrose ABS, 1-propanol/glucose ABS, and 2-propanol/sugar ABS attributed to the lower hydrophilicity of 1-propanol and the stronger sugaring-out effect exerted by the maltose. A maximum partition coefficient (*K)* of 20.01 ± 0.05 and recovery yield (*Y)* of 95.42% ± 0.01 of BSA were obtained with the 35% (w/w) 1-propanol/22% (w/w) maltose ABS at pH 5.0 which contained 10% (w/w) BSA. The *K* and *Y* of BSA in 1-propanol/maltose ABS was further enhanced with the addition of 3% (w/w) of ionic liquids, 1-butyl-3-methylimidazolium bromide ([Bmim]Br) as the adjuvants which provides the protein stabilizing effect. The Fourier Transform Infrared Spectrum (FTIR) analysis revealed that the protein structure remained unaltered upon the separation process.

Introduction

Proteins play important roles in many biological processes (Pei et al. 2009) and are widely used for therapeutics and diagnostics applications (Taha et al. 2015). Proteins exhibit poor stability upon removal from their native surroundings. Slight changes in temperature, pH, mechanical stress, and the presence of chemical denaturants can cause the proteins to lose their native structure. Therefore, a biocompatible extraction technique is required. Conventional methods applied in the recovery and purification of proteins, which include precipitation, membrane filtration, electrophoresis, gel and affinity chromatography are expensive, rate-limiting and not economically feasible for large scale applications. Moreover, the recovery of proteins with high purity usually involves multiple purification steps that result in protein loss and large amounts of solvents consumption (Taha et al. 2015).

The aqueous-based aqueous biphasic system (ABS) with high biocompatibility has emerged as an alternative cost-effective approach for protein separation and purification. ABS is formed by mixing two immiscible phase-forming solutions at a critical composition (Ng et al. 2011). The difference in the physiochemical properties and the interactions between the biomolecules and the phase-forming molecules results in differential partitioning behavior of the target biomolecules and thereby result in selective distribution and separation of the target biomolecules from the mixtures. ABS offers several process advantages such as low interfacial tension, short phase separation time, and low toxicity. Polymer/polymer ABS, polymer/salt ABS, and alcohol/salt ABS have been widely applied in protein extraction (Ng et al. 2011). Nevertheless, the industrial-scale applications of polymer-based ABS are limited because of the high viscosity of polymer used and the high cost demanded to recycle the phase-forming components (Ng et al. 2012). Moreover, the use of high charge density salts may cause environmental issues and denaturation of targeted protein due to their high ionic strength or alkalinity.

The substitution of conventional salts with carbohydrates could potentially create a more biocompatible separation environment for the biomolecules (de Brito Cardoso et al. 2013). Carbohydrates are polyhydroxy aldehydes or ketones that encompass a broad range of organic compounds. It can be divided into two large groups: simple sugars (i.e. monosaccharides) and compound sugars (i.e. oligosaccharides and polysaccharides). Sugars are electrically neutral, non-toxic, biodegradable, and highly hydrophilic in nature (Sadeghi et al. 2016). Sugars possess several hydroxyl groups with dual donor/acceptor characteristics that can participate in hydrogen bonding, thereby exerting a sugaring-out effect (also known as soluting-out effect) inherently (de Brito Cardoso et al. 2013). Rising attention has been given to the application of carbohydrates as a sugaring-out agent to form polymer/sugar ABS (Sadeghi et al. 2016), acetonitrile/sugar ABS (de Brito Cardoso et al. 2013), ionic liquid (IL)/sugar ABS (Quental et al. 2018) and alcohol/sugar ABS (Ebrahimi and Sadeghi 2018). The viscosity of alcohol is lower when compared to polymers and ILs, allowing more rapid phase separation and efficient mass transfer of biomolecules from one phase to another (Wang et al. 2010). Therefore, alcohol/sugar ABS can serve as a low-cost and sustainable extraction platform for the recovery and separation of biomolecules. Although the phase composition and properties of alcohol/sugar ABS have been characterized (Ebrahimi and Sadeghi 2018), the separation efficiency of the alcohol/sugar ABS and the stability of the extracted biomolecules remains superficial to-date. This study aims to investigate the protein partition efficiency using the alcohol/sugar ABS formed with alcohols (1-propanol and 2-propanol), and different sugars (glucose, sucrose, and maltose).

Serum albumin is one of the most abundant proteins in the human body fluid with many physiological functions. Bovine serum albumin (BSA) has been extensively applied as the model protein in various extraction and purification studies because of the 76% similarity in the amino acid sequence when compared to human serum albumin (HSA) (Pereira et al. 2015). Hence, BSA was employed as the model protein to evaluate the protein extraction efficiency of the investigated ABS. Carbohydrates such as glucose, sucrose, and maltose are low-cost sugars which can be easily derived from natural sources and are widely used in the food industries. ILs are often known as designer solvents due to their flexibility and tunability in the combination of cations and anions. These greener solvents are generally non-flammable, chemically stable, possess high dissolving capacity and good extractability. Despite the application of ILs as phase-forming components, recent studies have shown that smaller amounts (≤5% (w/w)) of ILs can be added as adjuvants into polymer/salt ABS (Neves et al. 2019; Aziz et al. 2017) and alcohol/salt (Ran et al. 2019) to enhance the partition efficiency of biomolecules to one of the phases. In this study, the effects of the addition of neutral salts and ILs as adjuvants on the partition efficiency of BSA were examined. The stability of BSA upon the ABS separation process was also assessed with Fourier Transform Infrared Spectrum (FTIR) analysis.

Materials And Methods

Materials

Lyophilized BSA (essentially globulin and fatty acid-free, ≥99% pure) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethanol absolute 99.8% (C_2H_5OH), 1-propanol (C_3H_8O) absolute 99.5%, and 2-propanol absolute 99.5% were obtained from VWR Chemicals (Fontenay-Sous-Bois, France). D-(+)-glucose (³ 99.0%), sucrose (³ 98.0%) and D-(+)-maltose monohydrate (³ 99.0%) were obtained from Merck (Darmstadt, Germany). Pierce BCA protein assay kit was sourced from Thermo Scientific (Rockford, United States of America).

Determination of binodal curves

The binodal curves for the ABS which composed of different concentrations of alcohol (i.e. 1-propanol and 2-propanol) and sugar (i.e. glucose, maltose, and sucrose) were constructed using the turbidimetric titration method (Johansson et al. 2011). The mixture containing known concentrations of alcohol and sugar stock solution was weighed and titrated dropwise with an appropriate amount of ultrapure water until a single-phase solution was formed. The resultant mixture was centrifuged at 4000*g* for 10 minutes to ensure that a single-phase system was formed. The final weight of the system was measured to determine the amount of ultrapure water added.

Protein partitioning in sugaring-out assisted ABS

For the protein partitioning experiment, 5 g of ABSs which consisted of various concentrations of phase-forming components were prepared in 15 mL centrifuge tubes by mixing appropriate amounts of 100% (w/w) alcohol (i.e. 1-propanol and 2-propanol), 60% (w/w) stock solution of sugar (i.e. glucose, maltose, and sucrose), 5% (w/w) of 20 mg/mL of BSA protein solution, and ultrapure water (Ng et al. 2018). Subsequently, the mixture was centrifuged at 4000*g* for 10 minutes to ensure total phase separation was attained. The volume of each phase was recorded, and the sample was collected separately from each phase to quantify the BSA concentration. All ABS partitioning experiments were performed in triplicate.

Determination of protein content using bicinchoninic acid (BCA) assay

The concentration of BSA in each phase was determined using the bicinchoninic acid (BCA) assay (Ng et al. 2018). To each of the 25 µL of phase sample solution, 200 µL of the prepared working reagent was added. The resultant mixture was mixed thoroughly for 30 seconds and incubated at 37□C for 30 minutes. Next, the absorbance of the resultant mixture was measured at 562 nm against the blank ABS phase sample solution which was prepared in parallel to eliminate any possible interferences. The calibration curve was constructed using BSA standard solutions diluted to the working range of 25-2000 µg/mL.

Fourier Transform Infrared Spectrum (FTIR) analysis

The Fourier- Transform Infrared Spectrometer (FTIR) analysis was conducted to investigate the natural properties of the protein in the alcohol/sugar ABS phase sample solutions containing BSA (Pereira et al. 2015). The FT-IR spectra (4 cm^{-1} resolution, 16 scans) were recorded using the Perkin Elmer Spectrum 100 FT-IR spectrometer (BioTek, Winooski, VT, USA) in the wavelength ranging from 450 to 4000 cm^{-1} to locate main functional groups.

Determination of protein partitioning efficiency

The volume ratio (V_R) was expressed as the ratio of the volume of the top phase (V_T) to the volume of the bottom phase (V_B).

$$V_R = \frac{V_T}{V_B} \quad (1)$$

The partition coefficient (K) of BSA was calculated as the ratio of the concentration of BSA at the top phase (C_T) to the concentration of BSA at the bottom phase (C_B) (Eq.2) [22].

$$K = \frac{C_T}{C_B} \quad (2)$$

The recovery yield (Y) was used as a parameter to evaluate the percentage of the amount of BSA partitioned between the top phase and the total mixture. The Y was calculated as a function of V_R and K according to (Eq.3) (Ng et al. 2019).

$$Y = \frac{100}{1+\frac{1}{V_R \cdot K}} \quad (3)$$

Results And Discussion

Binodal curves of sugaring-out assisted ABS

Fig. 1(a) depicts the binodal curves of 1-propanol /sucrose, 1-propanol/maltose, and 1-propanol/glucose ABS. Fig. 1(b) depicts the binodal curves of 2-propanol/sucrose, 2-propanol/maltose, and 2-propanol/glucose ABS. The biphasic region of 1-propanol/sugar ABS was larger than that of 2-propanol/sugar ABS, indicating that the 2-propanol/sugar ABS required a higher amount of phase-forming components to form ABS (cf. Supporting Information Data, Figs. A1–A3). According to the octanol/water partition coefficient of 1-propanol and 2-propanol, the hydrophilicity of secondary alcohol

(i.e. 2-propanol) is higher than the hydrophilicity of primary alcohol (i.e. 1-propanol) (Ebrahimi and Sadeghi 2018). In other words, a higher concentration of sugar was required to sugar-out the 2-propanol to form ABS. Thus, the lower phase-forming ability of 2-propanol was observed. Ethanol was also investigated as a potential phase-forming component for the formation of alcohol/sugar ABS. Several systems constituted with various concentrations of ethanol and sugar were examined. However, no phase formation was observed. This could be due to the relatively close hydrophilicity degree between the ethanol and the examined sugars (Ebrahimi and Sadeghi 2018). Moreover, precipitation had occurred in the mixture which contained high concentration of ethanol and low concentration of sugar. Thus, ethanol and sugars are not suitable candidates to form stable sugaring-out assisted ABS.

As shown in Fig. 1(a), the binodal curve of 1-propanol/maltose ABS was closer to the axes compared to the binodal curve of 1-propanol/glucose ABS and 1-propanol/sucrose ABS. A similar trend was observed for 2-propanol/sugar ABS (Fig. 1(b)), indicating that the phase formation of alcohol/sugar ABS was also influenced by the sugaring-out ability of the sugars investigated. The sugaring-out ability and hydrophilicity of the sugars are affected by its stereochemistry and number hydroxyl groups. The molecular structures of sugars used to form the alcohol/sugar ABS are depicted in Fig. 2. Among the disaccharides (i.e. maltose and sucrose) and monosaccharides (i.e. glucose) applied in this study, maltose has the highest number of equatorial hydroxyl group and thus a higher aptitude to be hydrated (Freire et al. 2011). It has been reported that the ability of these sugars in sugaring-out ionic liquids (Ferreira et al. 2016), polymers (Sadeghi et al. 2016), and alcohols (Ebrahimi and Sadeghi 2018) to form ABS decreased in the order of maltose > sucrose > glucose. Fig. 1 showed the amount of maltose required to form 1-propanol/sugar and 2-propanol/sugar ABS was lesser compared to sucrose and glucose (Ebrahimi and Sadeghi 2016).

Effect of types and concentrations of phase-forming components on the partition efficiency of BSA

The partitioning behaviour of BSA in the 1-propanol/glucose, 1-propanol/maltose, 1-propanol/sucrose, 2-propanol/glucose, 2-propanol/sucrose, and 2-propanol/maltose ABS was investigated to evaluate the effect of various types and concentrations of phase-forming components on the partition efficiency of BSA. The systems investigated were chosen based on their relative position in the biphasic region which will give a V_R of about 1.0 at equilibrium.

For 1-propanol/sugar ABS, BSA was preferentially partitioned to the alcohol-rich top phase (i.e. $K > 1$) at lower concentrations of alcohol and sugar (Table 1). The BSA was partitioned to the alcohol-rich top phase which is more hydrophobic because of the favourable hydrophobic interaction between the BSA molecules and the alcohol molecules (Ng et al. 2018). However, increasing the alcohol and sugar concentrations decrease the partition efficiency of BSA in both 1-propanol/sugar ABS and 2-propanol/sugar ABS. This decrease could be attributed to the gradual dehydration of both aqueous phases (Ebrahimi and Sadeghi 2018). When the concentrations of sugar and alcohol used to construct

the 1-propanol/sugar ABS were increased, both aqueous phases with increasing phase-forming components' concentration were gradually dehydrated, which to a stronger extent resulted in the insufficient free water molecules to solubilize the protein in the alcohol-rich top phase, and thereby leading to the decrease in the partition efficiency of the BSA (Ooi et al. 2009). For 2-propanol/sugar ABS, a high K of 2.41 ± 0.39 and Y of 70.51% ± 3.35 were recorded at 32% (w/w) 2-propanol/31% (w/w) maltose ABS. Similarly, increasing the concentrations of 2-propanol and sugars significantly reduced the partition efficiency of BSA in the 2-propanol/sugar ABSs. The K and Y of BSA in 2-propanol/sugar ABSs were mostly lower than unity and 50%, respectively. Moreover, the 1-propanol which is more hydrophobic compared to the 2-propanol has driven more of the protein to partition to the alcohol-rich top phase of the sugaring-out assisted ABS.

Table 1
The effect of types and concentration of phase-forming components on the partition efficiency of BSA with propanol/sugar ABS.

Types of ABS	Concentration of sugar, % (w/w)	Concentration of alcohol, % (w/w)	*K*	Recovery Yield, %
1-Propanol/Maltose	22	35	6.03 ± 0.17	87.84 ± 0.61
	23	37	4.40 ± 0.43	81.70 ± 1.88
	24	38	2.57 ± 0.46	72.58 ± 3.58
	25	39	1.74 ± 0.14	64.39 ± 1.81
	26	39	0.90 ± 0.25	49.40± 4.79
1-Propanol/Sucrose	26	33	1.17 ± 0.07	63.61 ± 1.48
	27	34	1.26 ± 0.11	56.71 ± 2.11
	28	35	1.36± 0.15	62.86 ± 2.64
	29	36	0.93±0.10	48.21 ±2.72
	30	37	0.72±0.08	42.33 ± 3.32
1-Propanol/Glucose	24	36	1.14 ± 0.08	50.42 ± 2.10
	25	38	1.36 ± 0.05	58.11 ± 1.48
	26	39	1.01 ± 0.08	50.76 ± 2.58
	27	40	0.59 ± 0.02	38.04 ± 0.71
	28	41	0.38 ± 0.09	30.75 ± 4.91
2-Propanol/Maltose	31	32	2.41 ± 0.39	70.51 ± 3.35
	32	33	1.19 ± 0.19	54.19 ± 3.88
	33	34	0.85 ±	42.10 ± 0.92

			0.03	
	34	35	0.76 ± 0.14	43.06 ± 4.63
	35	36	0.33 ± 0.03	23.38 ± 1.66
2-Propanol/Sucrose	33	38	1.01 ± 0.03	48.13 ± 0.84
	34	39	0.75 ± 0.02	43.02 ± 0.60
	35	40	0.74 ± 0.04	42.46 ± 1.19
	36	41	0.22 ± 0.05	17.71 ± 3.15
	37	41.5	0.08± 0.03	7.42 ± 2.90
2-Propanol/Glucose	31	39	0.82 ± 0.07	44.95 ± 2.00
	32	40	0.59 ± 0.00	39.84 ± 0.10
	33	41	0.35 ± 0.05	27.38 ± 2.70
	34	42	0.32 ± 0.04	25.82 ± 2.51
	35	43	0.48 ± 0.02	34.33 ± 1.10

The types of sugar applied also exerted a significant effect on protein partition efficiency in the alcohol/sugar ABS. The sugaring-out ability of sugars greatly depends on its hydration extensions (Ebrahimi and Sadeghi 2018). Apart from the number of hydroxyl group, the arrangement of the hydroxyl group on the pyranose ring also affects the intensity of the sugaring-out effect. The equatorial hydroxyl groups possess greater hydration potential than the axial ones as the former could interact with water molecules and form long-lived hydration structures (Ng et al. 2012; Sadeghi et al. 2016). For glucose, its C-2, C-3, and C-4 hydroxyl groups are all on the equatorial positions (Fig. 2). Both maltose and sucrose possess the same number of hydroxyl group. Maltose is composed of two glucose monomers whereas sucrose is composed of a glucose monomer and a fructose monomer. As such, maltose which consists of two six-membered pyranose rings is more easily hydrated than the sucrose (Freire et al. 2011). In other words, maltose has a higher number of equatorial hydroxyl group and could exert a stronger sugaring-out effect than sucrose and glucose, thereby facilitating the transfer of BSA to the alcohol-rich top phase. Results showed that the 1-propanol/maltose ABS gave an overall better protein partition efficiency compared to the 1-propanol/sucrose ABS and the 1-propanol/glucose ABS. A similar phenomenon was

observed in the 2-propanol/sugar ABS. Therefore, 35% (w/w) 1-propanol/22% (w/w) maltose ABS which gave higher K of 6.03 ± 0.17 and Y of 87.84% ± 0.61 compared to other alcohol/sugar ABSs was selected to investigate the effect of amount of protein added on the partition efficiency of BSA in the 1-propanol/maltose ABS.

Effect of BSA amount added to the ABS on the partition efficiency of BSA

The amount of BSA added into the ABS was varied at a range of 5-25% (w/w) to evaluate the partition efficiency of BSA in the 35% (w/w) 1-propanol/22% (w/w) maltose ABS (Fig. 3). When the amount of protein added to the system increased from 5% (w/w) to 10% (w/w), the partition coefficient increased from 6.27 ± 0.16 to 10.21 ± 0.03 and the recovery yield increased from 87.12% ± 1.55 to 91.27% ± 0.02. It was observed that the increase in protein amount from 5% (w/w) to 25% (w/w) resulted in a decrease of V_R by 8.7%. With the decrease in V_R, the free volume available in the top phase to accommodate a higher amount of protein is greatly reduced (Ooi et al. 2009). Hence, the protein partition efficiency decreased to minimum K of 3.38 ± 0.28 and Y of 77.12% ± 1.49 when the protein amount was increased to 25% (w/w). The increase in the amount of BSA beyond 25% (w/w) is not feasible because of the increase in the tendency of protein precipitation at the interface as a result of phase saturation (Ng et al. 2014). Thus, 10% (w/w) of BSA solution which exhibited the highest protein partition efficiency was selected to further investigate the effect of pH on the partitioning behaviour of BSA in the 1-propanol/maltose ABS.

Effect of pH on the partition efficiency of BSA

The pH of the environment will affect the net surface charge and conformation of the protein. Hence, the pH of an ABS can be adjusted to steer and enhance the partition of protein to the targeted phase. In this study, the pH of the 35% (w/w) 1-propanol/22 % (w/w) maltose ABS, which contained 10% (w/w) BSA, was varied between pH 3.0 and pH 8.0 to investigate the effect of pH on the partition efficiency of BSA. The phase system was mixed well while adjusting the pH with the addition of 1.0 M of sulfuric acid or 1.0 M sodium hydroxide and followed by phase separation. The change in the volume of both phases was negligible with the increase in pH.

As shown in Fig. 4, the pH exerted a significant impact on the protein partition efficiency. When the pH was increased from pH 3.0 to pH 5.0, the partition efficiency of BSA increased remarkably from K of 5.71 ± 0.7 and Y of 86.94% ± 1.39 at pH 3.0 to maximum K of 20.01 ± 0.05 and Y of 95.42% ± 0.01 at pH 5.0. The isoelectric point (pI) of BSA is at pH 4.8 and its native form is relatively stable at pH 5.0-8.0 (Chow et al. 2015). When the pH was increased to 5.0, approaching the pI of BSA, the net surface charge of BSA was close to zero. Thus, the hydrophobic interaction between the BSA and 1-propanol molecules was intensified at this pH, facilitating the partition of more BSA to the alcohol-rich top phase (Ng et al. 2018). Thereafter, the protein partition efficiency decreased significantly when the pH was raised to pH 8.0. At pH

8.0, the *K* and *Y* of BSA dropped to a minimum of 2.52 ± 0.27 and 71.50% ± 2.17, respectively. As proteins prone to denature at high pH values, operating the ABS above pH 8.0 would not be favourable for effective protein recovery (Chow et al. 2015).

Effect of types of adjuvants on the partition efficiency of BSA

The effects of the addition of neutral salts (sodium chloride (NaCl) and potassium chloride (KCl)) or ILs, (1-butyl-3-methylimidazolium tetrafluoroborate, [Bmim]BF_4; 1-ethyl-3-methylimidazolium tetrafluoroborate, [Emim]BF_4; 1-butyl-3-methylimidazolium bromide, [Bmim]Br and 1-ethyl-3methylimidazolium bromide, [Emim]Br) on the partitioning behaviour of BSA in the 35% (w/w) 1-propanol/22% (w/w) maltose ABS at pH 5.0 were investigated. These adjuvants were added into the ABS at a fixed concentration of 1% (w/w) and their respective *K* and *Y* were shown in Fig. 5.

According to the Hofmeister series, the salting-out effect is more pronounced for KCl than NaCl (Wan et al. 2018). Thus, higher *K* (13.86 ± 1.03) and *Y* (93.26% ± 0.47) of BSA were obtained in the 35% (w/w) 1-propanol/22% (w/w) maltose ABS which was added with KCl compared to *K* (7.57 ± 0.78) and *Y* (88.48% ± 1.34) obtained with the addition of NaCl. Nevertheless, both *K* and *Y* values attained with the addition of these univalent neutral salts were lower than that without the addition of adjuvants, indicating that the presence of KCl and NaCl has a negative impact on the protein partition efficiency of BSA in the alcohol/sugar ABS.

Among the ILs investigated, only [Bmim]Br exhibited a positive effect in improving the partition efficiency of BSA in the 1-propanol/maltose ABS. When compared to the alcohol/sugar ABS without adjuvants, the addition of [Bmim]BF_4 showed insignificant changes in the protein partition efficiency, while the addition of [Emim]BF_4 and [Emim]Br reduced the *K* and *Y* significantly by more than 60% and 8%, respectively. When the effect of IL's cation on the protein partition efficiency was compared, the *K* and *Y* values of BSA for ILs which share the same type of anion increase in the following orders: [Emim]BF_4 < [Bmim]BF_4 and [Emim]Br < [Bmim]Br. This phenomenon could be attributed to the alkyl chain length of IL's cation. Longer alkyl chain length increases the hydrophobicity of ILs and enhances the molecular interaction between BSA and IL, thereby promoting the partition of the BSA in the top phase containing 1-propanol and IL (Ran et al. 2019).

For the same alkyl chain length, [Bmim]Br exhibited higher *K* and *Y* values compared to [Bmim]BF_4. This variation in the protein partition efficiency is in accordance with the chaotropic order of the IL anions (>), whereby the chaotropic which has a higher tendency in unfolding the protein and destabilizing the hydrophobic aggregates will increase the dissolution of protein in the sugar-rich bottom phase (Ran et al. 2019). Thus, tetrafluoroborate-based IL with high salting-in ability could not act as a suitable adjuvant to enhance the partition efficiency of protein in the alcohol/sugar ABS. Comparing to the ABS without adjuvant (*K* of 20.01 ± 0.05 and *Y* of 95.42% ± 0.01), a slight increase in *K* (27.42 ± 0.02) and *Y* (96.27%

± 0.10) of BSA were observed with the addition of [Bmim]Br. Hence, [Bmim]Br was chosen as the suitable adjuvant for subsequent evaluation.

Effect of concentration of adjuvant [Bmim]Br on the partition efficiency of BSA

The effect of [Bmim]Br's concentration on the partitioning behavior of BSA was evaluated within the range of 1.0-5.0% (w/w) and the results were presented in Fig. 6. When the concentration of [Bmim]Br was increased to 3.0% (w/w), the *K* (34.39 ± 2.26) was increased markedly by 70% compared to the system without adjuvant (*K* of 20.01 ± 0.05). This increase in protein partition efficiency indicates that the addition of IL could serve to enhance the affinity of the 1-propanol-rich phase to the protein (Ran et al. 2019). A maximum *Y* of 97.05% ± 0.35 was achieved at 3% (w/w) [Bmim]Br. The protein partition efficiency decreased thereafter to *K* of 10.08 ± 1.4 and *Y* of 90.88% ± 1.06 at 5% (w/w) [Bmim]Br. This decrease in protein partition efficiency was probably caused by the change in protein stability at a high concentration of IL (Hadzir et al. 2016).

Stability of BSA in 1-propanol/maltose ABS

The protein conformation is highly affected by the change in the pH of the environment and the presence of foreign solute. FTIR analysis was conducted to evaluate the structure of the BSA that partitioned in the top phase of the 35% (w/w) 1-propanol/22% (w/w) maltose ABS at pH 5 with and without the addition of 3% (w/w) [Bmim]Br IL. Proteins are made up of many amino acids which are joined to one another by peptide bonds. The basic unit of the peptide bond is amide. The amide I band, which falls between 1600-17000 cm^{-1}, is associated with the C=O stretching vibration of the amide functional group [1]. As the most sensitive spectral region to the secondary structure of the protein, this absorption band is often used as a structural probe to determine the structural properties of protein. Fig. 7 shows the FTIR spectra of pure BSA and BSA partitioned in the alcohol-rich top phase of the 35% (w/w) 1-propanol/22% (w/w) maltose ABS at pH 5 with and without the addition of adjuvants. As shown in Fig. 7, the amide I absorption band of the pure BSA falls between 1600-17000 cm^{-1}. For the spectra of BSA separated to the top phase of 1-propanol/maltose ABS with and without IL, the amide I absorption band was still identifiable and remained unchanged in the same region. Since there was no disappearance and significant shift of protein absorbance peak, the BSA conformation was unaltered and conserved in the phase solution of the 1-propanol/maltose ABS.

Conclusion

The partitioning behaviour of BSA in ABSs composed various alcohols (i.e., 1-propanol and 2-propanol) and sugars (i.e., glucose, sucrose, and maltose) was investigated to evaluate the protein partition efficiency in the alcohol/sugar ABS. At low concentrations of alcohol and sugar, the BSA partitioned

preferentially to the alcohol-rich top phase of the alcohol/sugar ABS. The effects of other parameters such as protein concentration, pH, types, and concentrations of adjuvants were further investigated to enhance the protein partition efficiency in the 1-propanol/maltose ABS. Maximum protein partition efficiency with *K* of 34.39 ± 2.26 and *Y* of 97.05% ± 0.35 was achieved in the 35% 1-propanol/22% (w/w) maltose ABS at pH 5.0 added with 3% (w/w) [Bmim]Br and 10% (w/w) BSA. The FTIR analysis showed that the BSA structure was conserved in the 1-propanol/maltose ABS. Comparing to the expensive IL/sugar ABS, highly viscous polymer/sugar ABS and the use of inorganic salt in polymer/salt ABS which could lead to corrosion of equipment and environmental pollution, the alcohol/sugar ABS could serve as a green and cost-effective alternative for the separation and purification protein.

Abbreviations

ABS: Aqueous biphasic system; (Bmim)BF_4: 1-butyl-3-methylimidazolium tetrafluoroborate; (Bmim)Br: 1-butyl-3-methylimidazolium bromide; BCA: Bicinchoninic acid; BSA: Bovine serum albumin; C: Carbon; (Emim)BF_4:1-ethyl-3-methylimidazolium tetrafluoroborate; (Emim)Br: 1-ethyl-3methylimidazolium bromide FTIR: Fourier Transform Infrared Spectrum; HSA: Human serum albumin; IL: Ionic liquids; K: Partition coefficient; KCl; Potassium Chloride; NaCl: Sodium Chloride; O: Oxygen; V_R: Volume ratio; Y: Yield

Declarations

Acknowledgment

Special thanks to Taylor's University, Malaysia; UCSI University, Malaysia and Biorefinery and Bioprocess Engineering Laboratory, Yuan Ze University, Taiwan for the lab facilities.

Authors' contributions

Yin Hui Chow: Data curation, Methodology, Validation Writing - original draft. Alagan Sahlini: Data curation, Writing - original draft. Hui Suan Ng: Conceptualization, Funding acquisition, Writing – Reviewing and Editing. John Chi-Wei Lan: Conceptualization, Funding acquisition, Supervision, Writing - Reviewing and Editing.

Availability of data and materials

All the datas obtained in this study are included in this article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Fundings

This work was financially supported by UCSI University Pioneer Scientist Incentive Funds, Malaysia (grant number: PROJ-2019-In-FAS-064), and Ministry of Science and Technology Taiwan (grant number: MOST108-2622-E-155-013-CC2).

References

1. Aziz NFHA, Abbasiliasi S, Ng HS, Phapugrangkul P, Bakar MHA, Tam YJ, Tan JS (2017) Purification of β-mannanase derived from *Bacillus subtilis* ATCC 11774 using ionic liquid as adjuvant in aqueous two-phase system. J Chromatogr B 1055:104-112
2. Chow YH, Yap YJ, Tan CP, Anuar MS, Tejo BA, Show PL, Ariff AB, Ng E-P, Ling TC (2015) Characterization of bovine serum albumin partitioning behaviors in polymer-salt aqueous two-phase systems. J Biosci Bioeng 120 (1):85-90. doi:https://doi.org/10.1016/j.jbiosc.2014.11.021
3. de Brito Cardoso G, Mourão T, Pereira FM, Freire MG, Fricks AT, Soares CMF, Lima ÁS (2013) Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin. Sep Purif Technol 104:106-113. doi:https://doi.org/10.1016/j.seppur.2012.11.001
4. Ebrahimi N, Sadeghi R (2016) Osmotic properties of carbohydrate aqueous solutions. Flu Pha Equi 417:171-180. doi:https://doi.org/10.1016/j.fluid.2016.02.030
5. Ebrahimi N, Sadeghi R (2018) Propanol – Sugar aqueous biphasic systems as a suitable platform for biomolecules extraction. J Chromatogr A 1581-1582:156-167. doi:https://doi.org/10.1016/j.chroma.2018.11.007
6. Ferreira AM, Esteves PDO, Boal-Palheiros I, Pereiro AB, Rebelo LPN, Freire MG (2016) Enhanced tunability afforded by aqueous biphasic systems formed by fluorinated ionic liquids and carbohydrates. Green Chem 18 (4):1070-1079. doi:10.1039/C5GC01610J
7. Freire MG, Louros CLS, Rebelo LPN, Coutinho JAP (2011) Aqueous biphasic systems composed of a water-stable ionic liquid + carbohydrates and their applications. Green Chem 13 (6):1536-1545. doi:10.1039/C1GC15110J

8. Hadzir MH, Abbasiliasi S, Ariff AB, Yusoff SB, Ng HS, Tan JS (2016) Partitioning behavior of recombinant lipase in *Escherichia coli* by ionic liquid-based aqueous two-phase systems. RSC Adv 6 (86):82571-82580. doi:10.1039/C6RA16722E
9. Johansson H-O, Feitosa E, Junior AP (2011) Phase diagrams of the aqueous two-phase systems of poly(ethylene glycol)/sodium polyacrylate/salts. Polymers 3 (1):587-601
10. Neves CMSS, Sousa RdCS, Pereira MM, Freire MG, Coutinho JAP (2019) Understanding the effect of ionic liquids as adjuvants in the partition of biomolecules in aqueous two-phase systems formed by polymers and weak salting-out agents. Biochem Eng J 141:239-246. doi:10.1016/j.bej.2018.10.022
11. Ng H-S, Ng T-C, Kee PE, Tan JS, Yim HS, Lan JC-W (2019) Partition efficiency of cytochrome *c* with alcohol/salt aqueous biphasic flotation system. J Biosci Bioeng. doi:https://doi.org/10.1016/j.jbiosc.2019.08.013
12. Ng HS, Chai CXY, Chow YH, Loh WLC, Yim HS, Tan JS, Lan JC (2018) Direct recovery of *Bacillus subtilis* xylanase from fermentation broth with an alcohol/salt aqueous biphasic system. J Biosci Bioeng 125 (5):585-589. doi:10.1016/j.jbiosc.2017.12.010
13. Ng HS, Ooi CW, Show PL, Tan CP, Ariff A, Moktar MN, Ng E-P, Ling TC (2014) Recovery of *Bacillus cereus* cyclodextrin glycosyltransferase using ionic liquid-based aqueous two-phase system. Sep Purif Technol 138:28-33. doi:https://doi.org/10.1016/j.seppur.2014.09.038
14. Ng HS, Tan CP, Chen SK, Mokhtar MN, Ariff A, Ling TC (2011) Primary capture of cyclodextrin glycosyltransferase derived from *Bacillus cereus* by aqueous two phase system. Sep Purif Technol 81 (3):318-324. doi:https://doi.org/10.1016/j.seppur.2011.07.039
15. Ng HS, Tan CP, Mokhtar MN, Ibrahim S, Ariff A, Ooi CW, Ling TC (2012) Recovery of *Bacillus cereus* cyclodextrin glycosyltransferase and recycling of phase components in an aqueous two-phase system using thermo-separating polymer. Sep Purif Technol 89:9-15. doi:https://doi.org/10.1016/j.seppur.2011.12.028
16. Ooi CW, Tey BT, Hii SL, Kamal SMM, Lan JCW, Ariff A, Ling TC (2009) Purification of lipase derived from *Burkholderia pseudomallei* with alcohol/salt-based aqueous two-phase systems. Process Biochem 44 (10):1083-1087. doi:https://doi.org/10.1016/j.procbio.2009.05.008
17. Pei Y, Wang J, Wu K, Xuan X, Lu X (2009) Ionic liquid-based aqueous two-phase extraction of selected proteins. Sep Purif Technol 64 (3):288-295. doi:https://doi.org/10.1016/j.seppur.2008.10.010
18. Pereira MM, Pedro SN, Quental MV, Lima ÁS, Coutinho JAP, Freire MG (2015) Enhanced extraction of bovine serum albumin with aqueous biphasic systems of phosphonium- and ammonium-based ionic liquids. J Biotechnol 206:17-25. doi:10.1016/j.jbiotec.2015.03.028
19. Quental MV, Pereira MM, Ferreira AM, Pedro SN, Shahriari S, Mohamadou A, Coutinho JAP, Freire MG (2018) Enhanced separation performance of aqueous biphasic systems formed by carbohydrates and tetraalkylphosphonium- or tetraalkylammonium-based ionic liquids. Green Chem 20 (13):2978-2983. doi:10.1039/C8GC00622A

20. Ran L, Yang C, Xu M, Yi Z, Ren D, Yi L (2019) Enhanced aqueous two-phase extraction of proanthocyanidins from grape seeds by using ionic liquids as adjuvants. Sep Purif Technol 226:154-161. doi:https://doi.org/10.1016/j.seppur.2019.05.089
21. Sadeghi R, Ebrahimi N, Tehrani MD (2016) Investigation of carbohydrates as non-charged, non-toxic and renewable soluting-out agent for polymer based aqueous biphasic systems implementation. Polymers 98:365-377. doi:https://doi.org/10.1016/j.polymer.2016.06.050
22. Taha M, Quental MV, Correia I, Freire MG, Coutinho JAP (2015) Extraction and stability of bovine serum albumin (BSA) using cholinium-based good's buffers ionic liquids. Process Biochem 50 (7):1158-1166. doi:10.1016/j.procbio.2015.03.020
23. Wan PK, Lan JC-W, Chen P-W, Tan JS, Ng HS (2018) Recovery of intracellular ectoine from *Halomonas* salina cells with poly(propylene) glycol/salt aqueous biphasic system. J Taiwan Inst Chem Eng 82:28-32. doi:https://doi.org/10.1016/j.jtice.2017.11.003
24. Wang Y, Han J, Xu X, Hu S, Yan Y (2010) Partition behavior and partition mechanism of antibiotics in ethanol/2-propanol–ammonium sulfate aqueous two-phase systems. Sep Purif Technol 75 (3):352-357. doi:https://doi.org/10.1016/j.seppur.2010.09.004

Figures

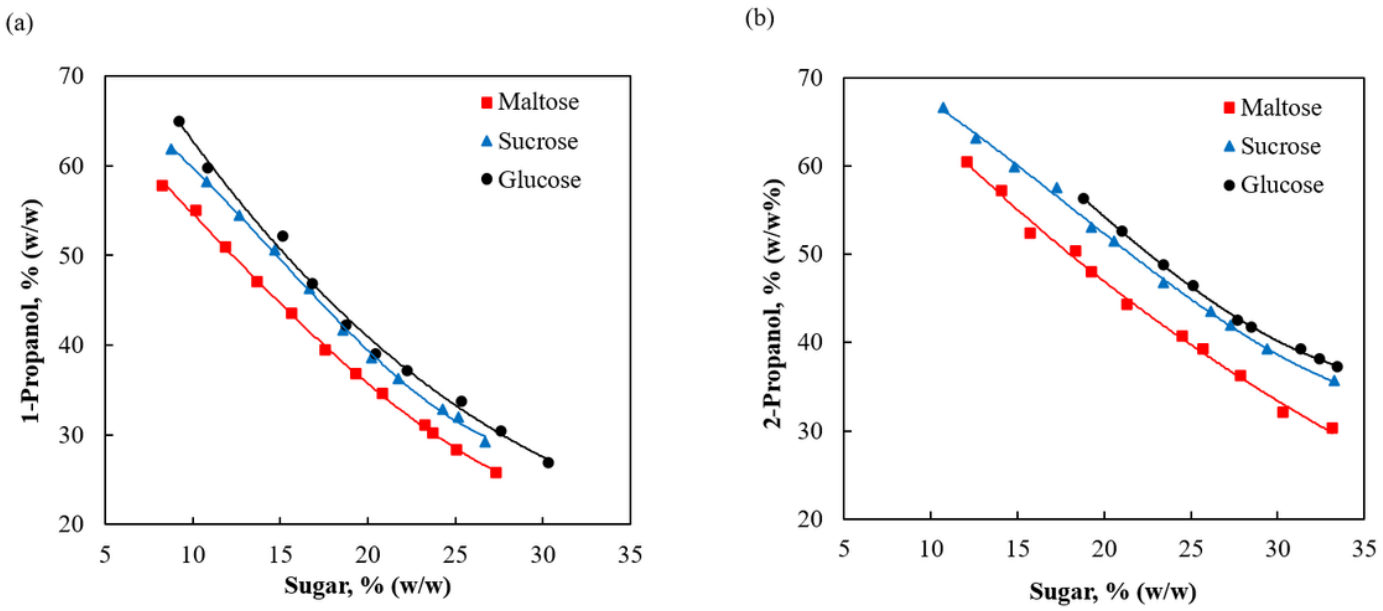

Figure 1

Binodal curves of alcohol/sugar ABS. (a) Binodal curves of 1-propanol/sugar ABS and (b) Binodal curves of 2-propanol/sugar ABS. The solid lines of the binodal curves were obtained by connecting the sufficient points experimentally.

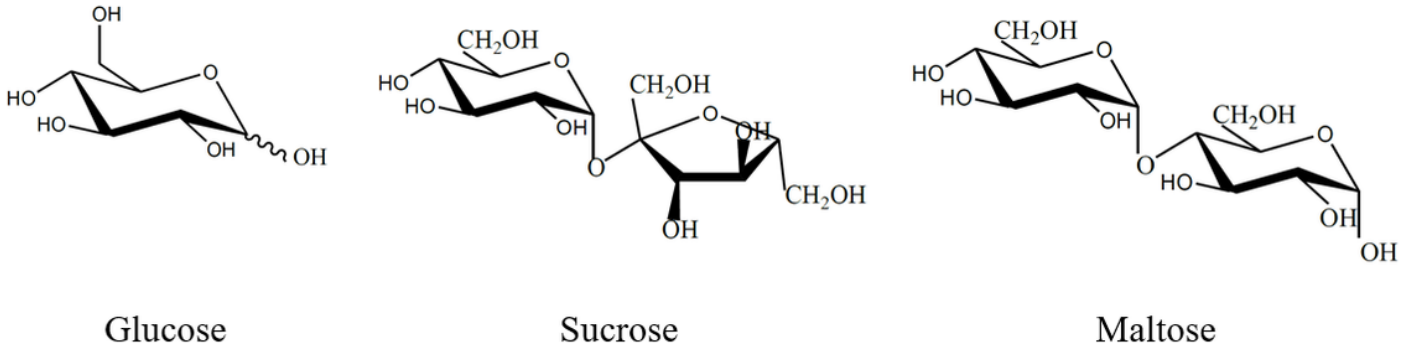

Figure 2

Molecular structures of sugars used to form the alcohol/sugar ABS.

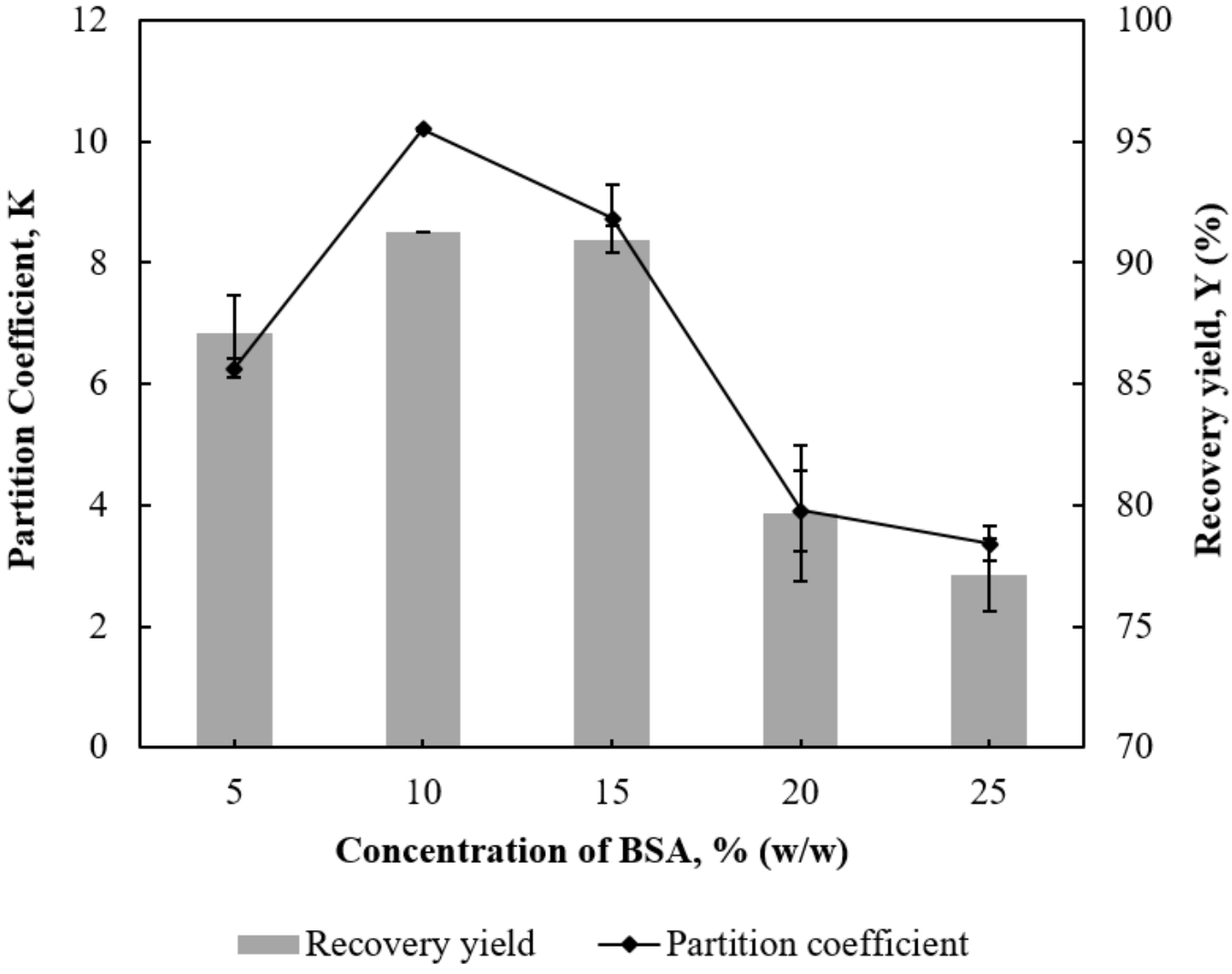

Figure 3

Effect of amount of BSA added to the ABS on the partition efficiency of BSA. The amount of BSA was varied at a range of 5-25% (w/w) to evaluate the K and Y of BSA in the 35% (w/w) 1-propanol/22% (w/w) maltose ABS. The results were expressed as mean ± standard deviation of triplicate readings.

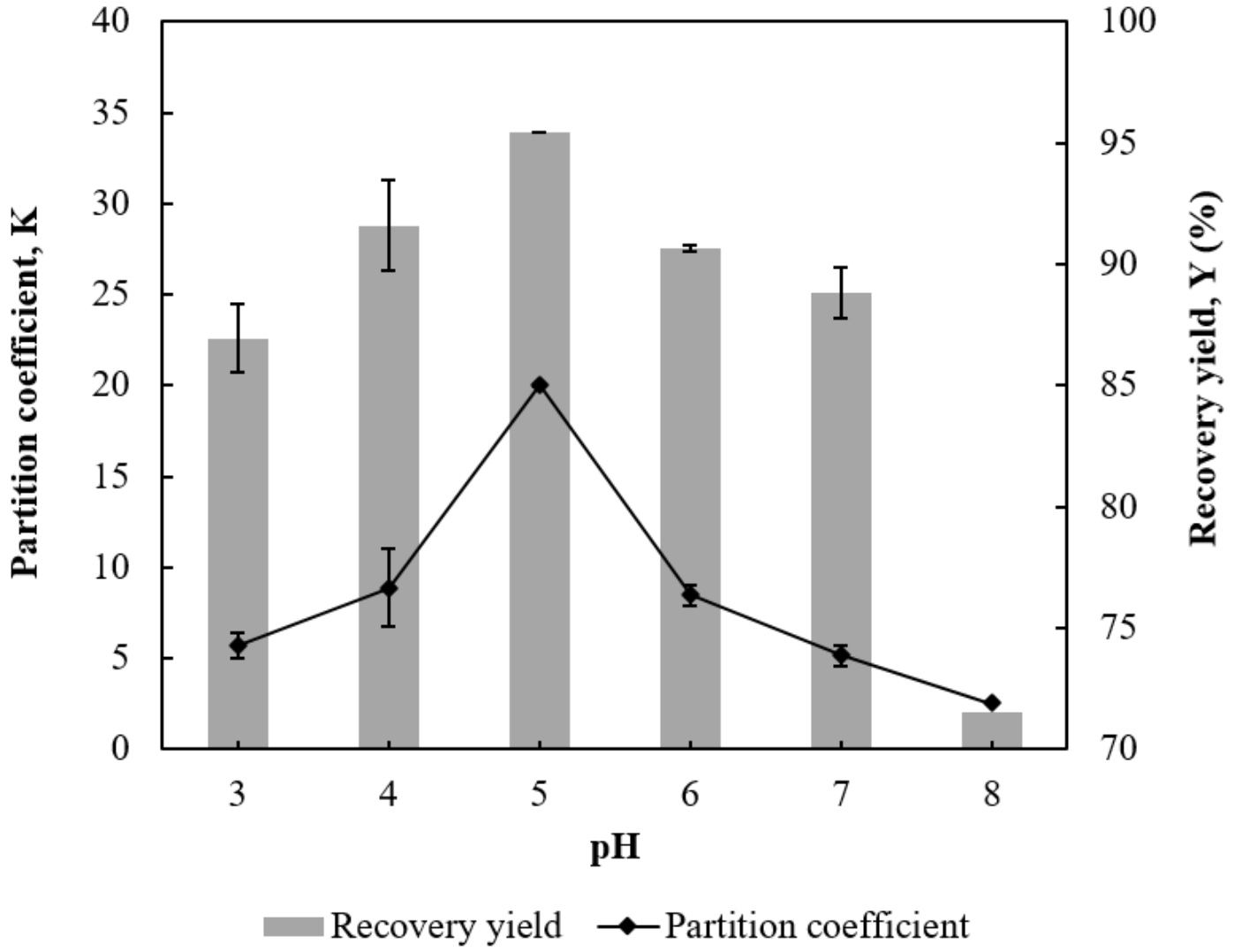

Figure 4

Effect of pH on the partition efficiency of BSA. The pH of the 35% (w/w) 1-propanol/22 % (w/w) maltose ABS with 10% (w/w) BSA was varied between pH 3.0 and pH 8.0 to investigate the effect of pH on the K and Y of BSA. The results were expressed as mean ± standard deviation of triplicate readings.

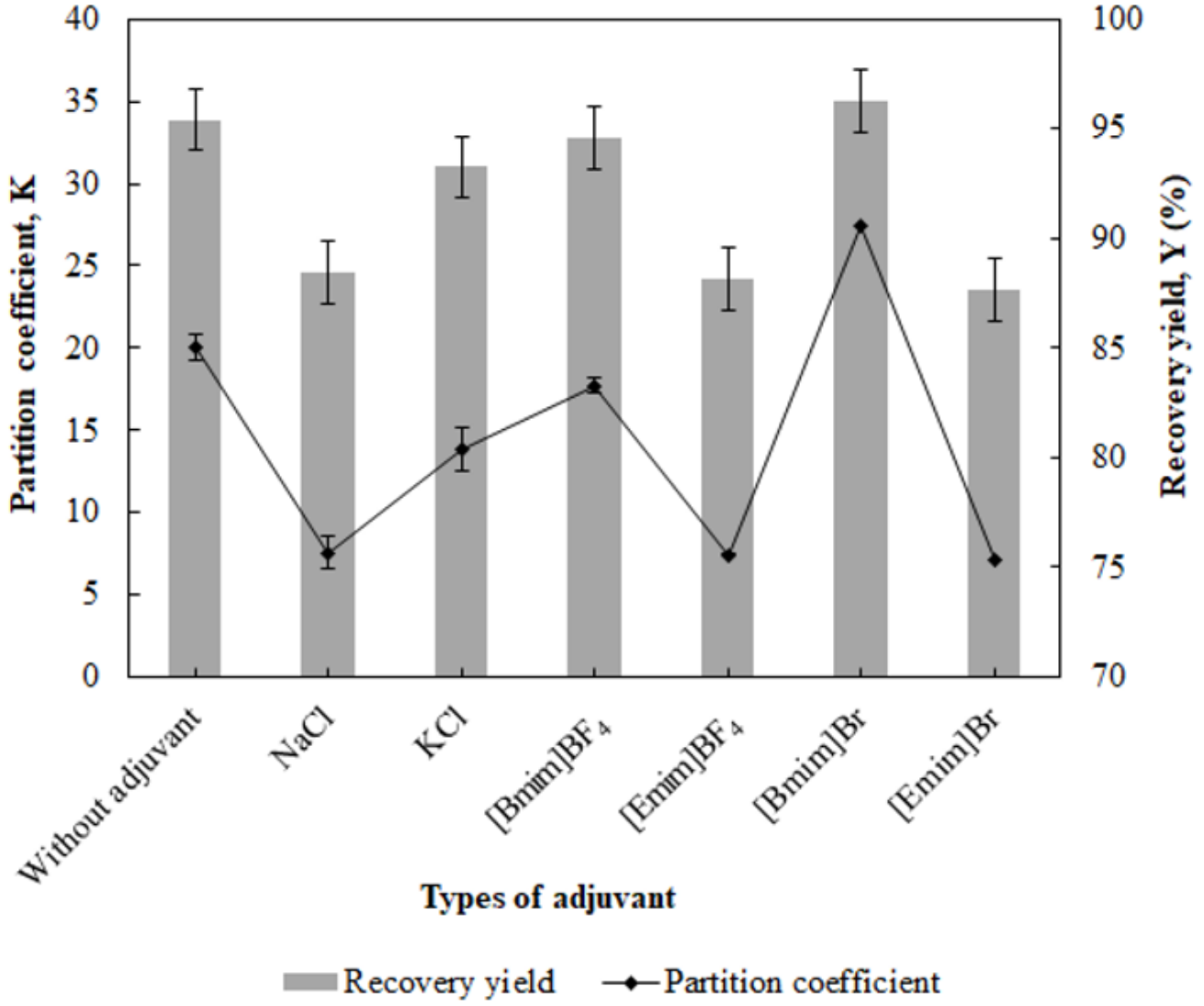

Figure 5

Effect of types of adjuvants on the partition efficiency of BSA. Neutral salts, such as NaCl and KCl, and ILs, such as [Bmim]BF4, [Emim]BF4, [Bmim]Br and [Emim]Br, were added at a fixed concentration of 1% (w/w) to the 35% (w/w) 1-propanol/22% (w/w) maltose ABS at pH 5.0 as adjuvants to evaluate the effect of without and with adjuvant on the K and Y of BSA. The results were expressed as mean ± standard deviation of triplicate readings.

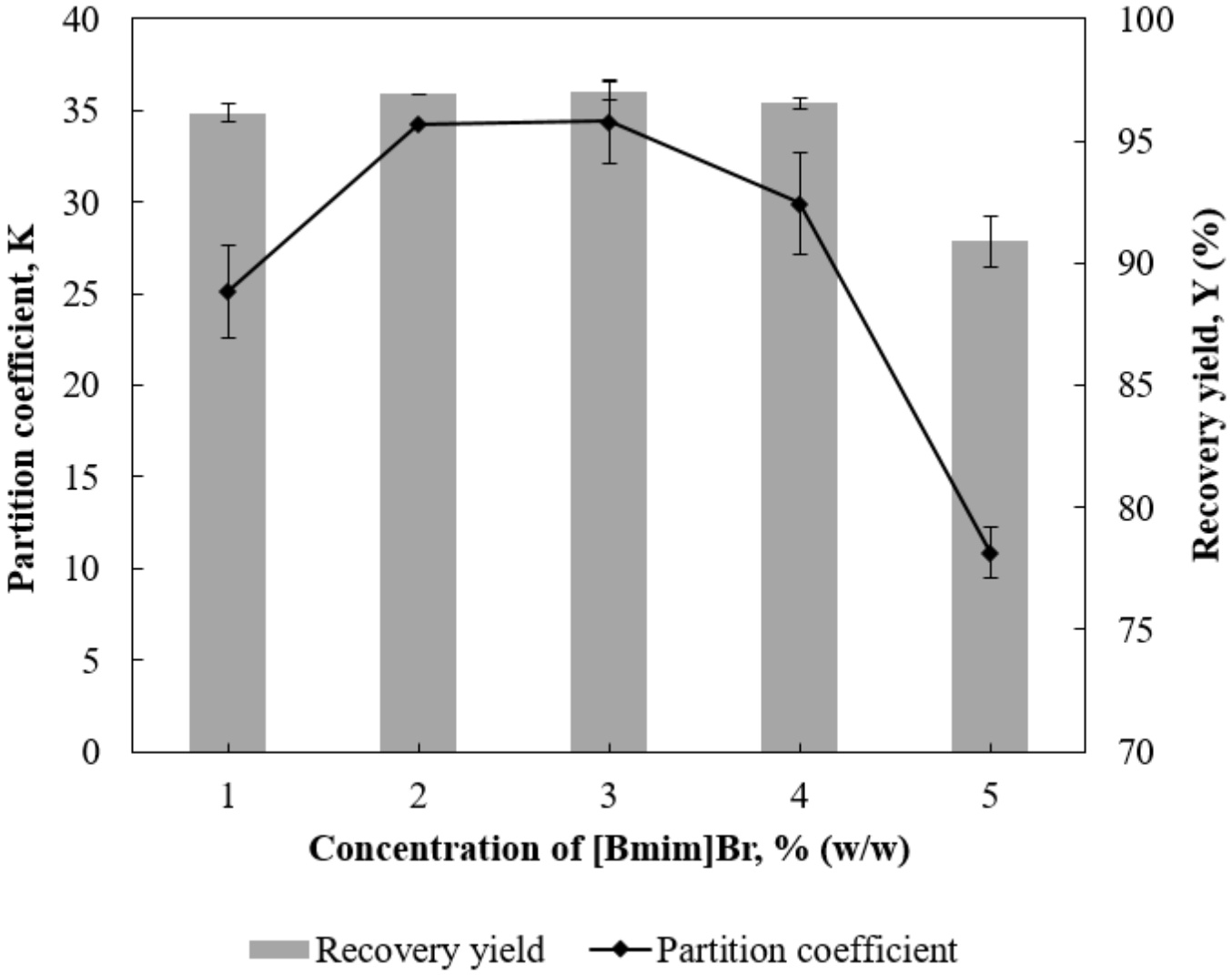

Figure 6

Effect of concentration of adjuvant [Bmim]Br on the partition efficiency of BSA. The effect of [Bmim]Br's concentration ranging from 1.0% (w/w) to 5.0% (w/w) on the K and Y of BSA was evaluated. The results were expressed as mean ± standard deviation of triplicate readings.

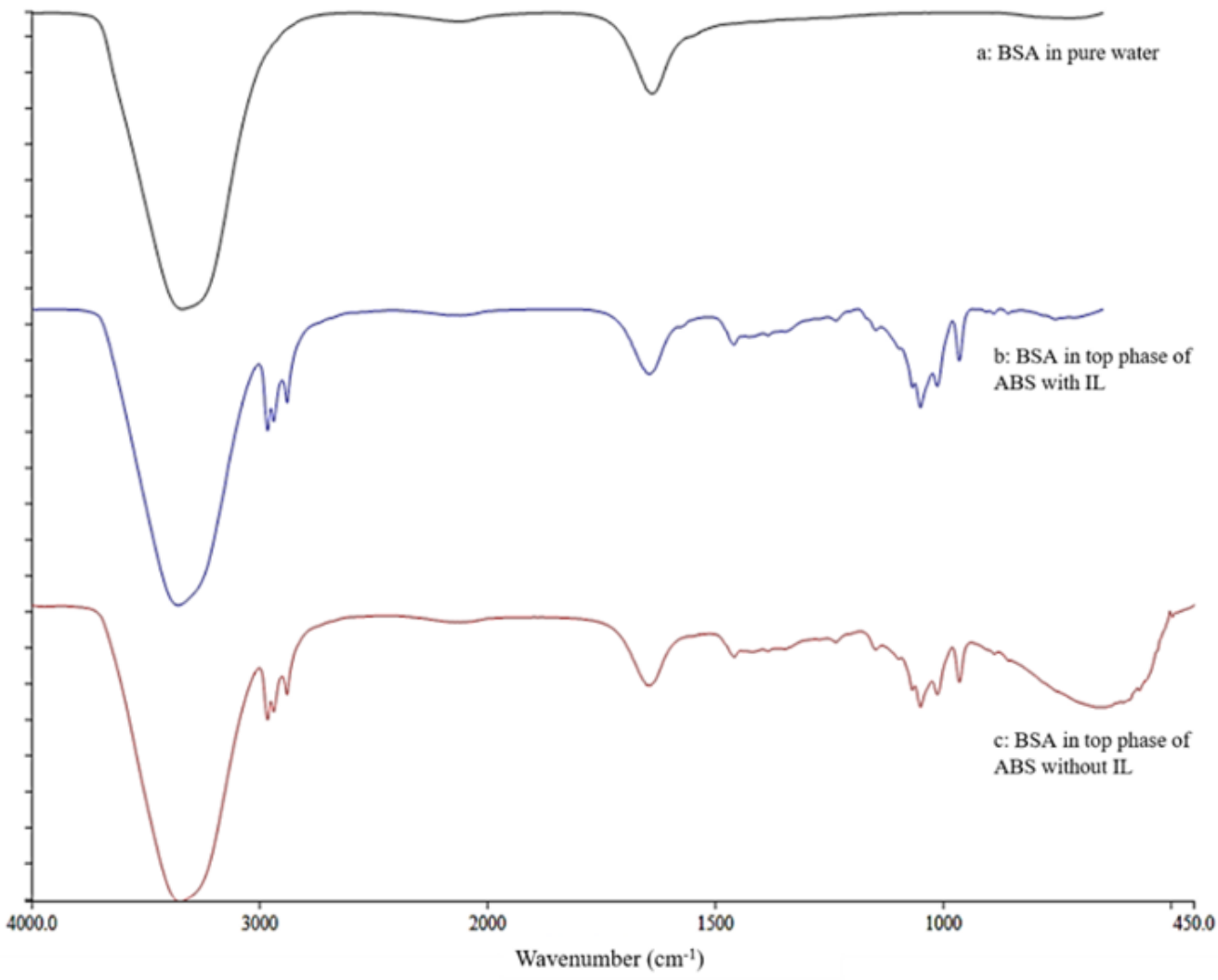

Figure 7

FTIR spectra of BSA in various solution. a) BSA in pure water; b) BSA partitioned in the alcohol-rich top phase of the 35% (w/w) 1-propanol/22% (w/w) maltose ABS at pH 5 and added with 3% (w/w) [Bmim]Br; c) BSA partitioned in the alcohol-rich top phase of the 35% (w/w) 1-propanol/22% (w/w) maltose ABS at pH 5.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SugaringoutABSgraphicalabstract.docx