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Research Article

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Polymer and alcohol-based three-phase partitioning systems for separation of polysaccharide and protein

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Abstract

BACKGROUND: Natural polymers are macromolecules produced by living organisms, and they have a wide range of applications and relevance for the development of a circular economy. However, large-scale production continues to be hindered by several factors, such as downstream processing. In this work, three-phase partitioning (TPP) systems were investigated for separation of model polysaccharide (dextran, alginate, and gum arabic) from protein [Bovine serum albumin (BSA) and lysozyme]. The recyclability of the phase-forming compounds used to form the extractive platform was assessed by ultrafiltration (UF). This study contributes to the development of production processes for biopolymers from fermented waste by proposing an effective separation technique for fractionation of biopolymers. Such biopolymers are often collected as mixtures, but with the studied approaches, fractionation of polysaccharides from proteins may also be employed. With the chosen systems, the scope of TPP systems is expanded by using another class of phase-forming compound (polymers); in addition, UF was studied as a versatile regeneration approach.

RESULTS: Within the TPP approach, the best separation of dextran from BSA was achieved using TPP systems composed of 25 wt% polyethylene glycol (PEG) + 25 wt% $K_3C_6H_5O_7$ and 36 wt% EtOH + 10 wt% K_3PO_4, in which more than 95% of dextran and BSA were found as precipitate and partitioned to top phase (PEG or EtOH-rich), respectively. By using other model compounds, it was found that the molecular weight and charge of the biopolymer play a key role in the yield and selectivity of TPP systems. Finally, by using ultrafiltration/diafiltration, about 99% of the ethanol and phosphate salt used to form the extractive platform could be retrieved in the permeate stream.

CONCLUSION: The high extraction yields, good selectivity, and recyclability of phase-forming compounds confirm the potential of polymer-based and alcohol-based TPP systems to fractionate biopolymer mixtures.
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Keywords: dextran; bovine serum albumin (BSA); biopolymer separation; three-phase partitioning (TPP); aqueous two-phase system (ATPS); ultrafiltration (UF)

INTRODUCTION

Biopolymers are an important class of natural molecules with multiple applications in a range of fields, such as food, medical, cosmetics, and pharmaceuticals, due to their compositional (chemical structure) diversity.[1,2] These natural polymers play an important role in promoting sustainability due to their biodegradability and high availability from natural renewable sources. However, large scale use of biopolymers is still hindered by the costs of downstream processing.[3,4] Biopolymers are often obtained as a highly complex crude mixture, leading to a challenging purification process. Downstream processing of biopolymers generally involves three main steps: extraction, fractionation, and concentration.[4,5] In particular, the fractionation step aims at separating the different classes of biopolymer (i.e., polysaccharide and proteins) present in the crude extract.[4–6] Besides improving purity, fractionation also allows for the complete valorization of

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biopolymer crude extract, thus paving the path towards a circular economy.

Several techniques have been suggested for the fractionation of biopolymers, such as precipitation,[7–9] membrane processes,[10,11] and liquid chromatography,[12,13] among others. However, specifically for fractionation of biopolymers with limited value, these approaches have some disadvantages. For the precipitation, only a single fraction is recovered, and the process requires large amounts of organic solvent. Even though chromatography presents high purification factors, it is typically expensive due to low throughput and complex scale-up.[13–15] In addition, membrane separations for the highly viscous streams will result in a very low throughput. An effective downstream process for biopolymers requires low cost and complexity, along with high yields and purity. Aqueous two-phase system (ATPS) is a potential technique that can fulfill those requirements. ATPS is a type of separation technique in which a two-phase system is formed by mixing two water-soluble compounds at certain concentrations and, due to incompatibility between these compounds, phase splitting takes place.[16–18] As the two phases are mostly composed of water, ATPS can provide a mild compatible media for water-soluble biopolymers. ATPS has received considerable attention for separation of polysaccharide/proteins.[17–19] Several studies have demonstrated the outstanding ability of ionic liquid-based ATPS for the fractionation of biopolymers.[20–23] Polymer-based[24–27] and alcohol-based[28–30] ATPS have also led to promising results regarding the fractionation of biopolymers.

While it was studied ionic liquid-based ATPS for the separation of the biopolymers in previous investigation,[22] it was decided to conduct a broad scoping study that also included Three Phase Partitioning (TPP), a relevant fractionation technique for natural polymers.[31–33] This relatively simple technique makes use of organic solvent (often butanol) combined with an aqueous solution of salt [$(NH_4)_2SO_4$] to promote precipitation of biopolymers at the interface between the two liquid phases.[6,32,34] TPP systems can be understood as a combination of salting out and alcohol precipitation techniques. The main advantage of the TPP system is that it allows for the integration of concentration and partial purification steps.[6,32,34] [6,32,34] Most research on TPP systems is regarding the purification of proteins,[35] for which TPP systems were originally described.[34]

Much less investigated for this application, TPP systems can also be used for fractionation of biopolymer mixtures.[36,37] Some promising results for fractionation of biopolymers have been reported by Wang et al,[38] who demonstrated that separating microbial polysaccharide and protein can be achieved using $(NH_4)_2SO_4$/butanol TPP system. In this case, polysaccharide mostly migrated to the salt phase, with an extraction yield of 52% and less than 5% protein content. A similar system was employed for fractionation of marine polysaccharide and protein mixtures, and relatively high extraction yields (>85%) of polysaccharide and protein to salt phase and precipitate, respectively, were obtained.[39] Sarkar et al[40] studied the fractionation of microalgae biopolymer extract using $(NH_4)_2SO_4$/butanol TPP system. Under optimized conditions, 71% and 40% yields for protein and carbohydrates, respectively, were reported. Other authors[41] have also reported effective fractionation of microalgae polysaccharide and protein using $(NH_4)_2SO_4$/butanol TPP. In this case, 79% of protein fraction was obtained as precipitate, while 91% of polysaccharide fraction partitioned to the salt phase.

Despite the recent progress in the field of TPP systems for biopolymer separation, most of the research still deals with systems composed of butanol and ammonium sulfate. As a result, much less is known about the possibility of using other chemicals to form such systems. The development of novel TPP systems, especially those based on environmentally friendly compounds, has the potential to expand the range of applications for this relatively simple separation technique. A promising approach to develop new TPP systems is exploiting the ability of certain compounds conventionally used to form ATPS to reduce the solubility of biopolymers in aqueous phases, thus leading their enrichment at the interface.

A few researchers have already exploited the TPP/ATPS concept, mostly relying on the IL-based ATPS/TPP approach. Pereira et al[42] demonstrated that 97% of protein in honey can be obtained as precipitate, with a purity level of up to 90%, while most carbohydrates were found in the top phase in IL-based ATPS/TPP. Separation of lactoferrin from other whey proteins was carried out using $BmimBF_4$/NaH_2PO ATPS/TPP and 74% of lactoferrin was obtained at the interface.[43] Even fewer studies have exploited other types of ATPS (for instance, polymer-based) for the formation of TPP systems and one of them is the work of Belchior and Freire.[44] The authors studied the fractionation of egg white proteins by ATPS/TPP system composed of polyethylene glycol (PEG) 2000 and phosphate salt. This approach led to 82% recovery yield of ovalbumin in the PEG-rich phase and 77% of lysozyme was obtained as precipitate at interface. Some advantages of the polymer-based ATPS/TPP approach are the lower cost and environmental impact when compared to its IL-based counterparts. Another alternative to IL-based ATPS/TPP is the use of alcohol-based systems. This type of system is also attractive for the ATPS/TPP approach because it is composed of relatively inexpensive phase-forming compounds, which are characterized by lower viscosity and reduced settling times. Another advantage of alcohol-based ATPS is that it is often easier to regenerate, since distillation can be used to recover the alcohol for reuse.

Another knowledge gap in this field concerns the isolation of the target molecule and the recycling of phase-forming compounds after fractionation step. Even though these aspects are essential for the process feasibility, they are often not addressed in TPP systems studies.[43,44] Since the compounds to be separated differ greatly in molecular weight, ultrafiltration (UF) represents a suitable technique to separate biopolymers from the phase-forming compounds in TPP systems. In UF, a pressure gradient pushes the fluid through a porous media, selectively allowing small molecules to pass while blocking larger ones.[45] The potential to separate macromolecules from small molecules using UF has been extensively explored in the dairy industry,[45] and in this work, the technique was also explored for fractionation of the biopolymers from all smaller molecules after the initial ATPS-based TPP fractionation of the biomolecules.

Hence, polymer-based and alcohol-based ATPS were investigated regarding their ability to form TPP systems and fractionate a mixture of model polysaccharide and protein. Combined with PEG or ethanol, salts like citrate, phosphate, and sulfate were used as phase-forming compounds. Solutions of model compounds were used to mimic crude extract of natural polymers. Dextran was selected as model compound because it is a polysaccharide with commercial relevance for applications in the pharmaceutical, food, agricultural, and fine chemical industries.[46] Besides this, crude extract of dextran is also known to contain protein as one of the main impurities.[9,47] The model biopolymer solution also contained bovine serum albumin (BSA) in order to investigate the ability of the studied TPPs to separate polysaccharide from protein. The effects of operational conditions (namely, the type of phase-forming compound and concentration) were investigated. Finally, the isolation of fractionated biopolymer and the regeneration of phase-forming compounds

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were studied by UF. Although both dialysis and distillation have been used as regeneration techniques for TPP systems, UF-based diafiltration is the most valuable secondary fractionation technique that allows for the isolation and concentration of the high boiling point compounds after TPP.

In summary, this study makes a significant contribution by proposing an efficient, high-throughput separation technique that minimizes chemical usage and reduces costs for fractionation of biopolymers. Additionally, the research expands the potential applications of TPP systems by replacing t-butanol with polymers and offers a proof-of-concept for a more versatile regeneration method.

MATERIALS AND METHODS

Materials

Polyethylene glycol with molecular weight of 400 $g.mol^{-1}$ (PEG 400) was purchased from Sigma-Aldrich (Schnelldorf, Germany). Ethanol (EtOH) was obtained at VWR (Amsterdam, The Netherlands; purity ≥99.8%). The salts used to form ATPS/TPP systems were ammonium sulfate [$(NH_4)_2SO_4$, VWR, Amsterdam, The Netherlands, purity ≥99.0%], tripotassium citrate monohydrate ($K_3C_6H_5O_7$, Alfa Aesar, Karlsruhe, Germany, purity ≥99.0%), and di-potassium hydrogen phosphate (K_2HPO_4, VWR, Amsterdam, The Netherlands, purity = 98.0%). The model biopolymers used were sodium alginate, bovine serum albumin (purity ≥98.0%), dextran from *Leuconostoc spp*., gum arabic from acacia three, and lysozyme from hen egg white. All the model biopolymers were acquired from Sigma-Aldrich (Schnelldorf, Germany). The main properties of the model biopolymers investigated are summarized in Table 1. The water used to make the systems was ultrapure (Milli-Q, with a resistance of 18 μΩ cm at 25 °C).

ATPS Selection and phase composition

The TPP systems investigated in this work were based on ATPS. Phase diagrams at 25 °C were obtained from literature[51,52] and were used as estimates to select mixture point at the biphasic region for the experiments in this work, which were conducted at room temperature (21 ± 1 °C). Diverse authors have reported no significant changes on the binodal curve of PEG400-salt[53–56] and alcohol-salt systems[52,57] with changes of temperature. Therefore, it is anticipated that these data are valid to represent the systems investigated in this work with acceptable accuracy. The equilibrium phase composition was obtained using the approach proposed by Merchuk et al.[58]

Extraction procedure

The TPP systems were prepared in 15 mL tubes by weighting appropriate amounts of the phase-forming compounds and aqueous solution containing biopolymers. The tubes were mixed thoroughly by a vortex mixer. Complete phase separation was achieved by centrifugation (4500 rpm for 10 min). Using such tubes, the volumes of the top and bottom phases could be noted with an accuracy of +/−0.13 mL (half the smallest division) and the phases were carefully separated using a syringe. Samples of the two separated phases were collected and analyzed for protein and polysaccharide concentration, as described in the Analytical analysis section. The concentration of biopolymers at interface was estimated by mass balance. All experiments were carried out at room temperature (21 ± 1 °C). The experiments were performed in duplicate, and the results were reported as the average of two independent assays with their respective standard deviations.

Table 1. Molecular weight (MW), isoeletric point (pI) of the proteins investigated, and acid dissociation constant (pKa) of the charged polysaccharide investigated

Biopolymer	MW (kDa)	p.I./pKa
Lysozyme	14[48]	10.5–11.0[48]
BSA	65[48]	4.7[48]
Gum arabic	200	2.2[49]
Sodium alginate	300	3.4–4.4[50]
Dextran	450–650	Not applicable

Abbreviation: BSA, bovine serum albumin.

Regeneration procedure

Regeneration of the phase-forming compounds used to form the extractive platform was conducted by ultrafiltration operated in diafiltration mode. In a stirred cell module (10 mL), dead-end filtration was carried out at 3 bar and using polysulfone membranes. For the bottom phase, a membrane of 100 kDa molecular weight cut-off (MWCO) (Mycrodin Nadir) was used, while a membrane of 20 kDa MWCO (Alfa Laval) was used for the top phase. Operation in diafiltration mode consisted of a discontinuous addition of solvent (ultrapure water) to the retentate. The diavolume (DV), also known as diafiltration volume, is defined in Eqn 1, where $V_{solvent}$ and $V_{initial}$ are the volume of solvent (water) added to the retentate and the initial feed volume, respectively. Diavolume can also be understood as the washing volume necessary to reduce the concentration of phase-forming in the retentate to the desired concentration.

$$DV = \frac{V_{solvent}}{V_{initial}} \quad (1)$$

Analytical analysis

Colorimetric assays were chosen to quantify polysaccharides and proteins, as this approach is widely employed in the biopolymer field. This choice ensures that the obtained results can be easily compared with existing literature. Pierce™ BCA Protein Assay Kit (Thermo Fischer™) was used to measure protein concentration in samples from both phases. The absorbance of the mixture was measured at 562 nm using a microplate spectrophotometer (Victor3 1420 Multilabel Counter, Perkin Elmer). Calibration curve was obtained using Bovine serum albumin (BSA), which was employed as standard.

The alginate concentration was determined using a modified m-phenylphenol uronic acid assay,[59] using alginate as standard for the calibration curve. The concentration of the other model polysaccharides was determined by phenol-sulfuric acid assay,[60] using glucose as standard for the calibration curve. For both assays, the absorbance of the mixture was measured at 490 nm using a microplate spectrophotometer. Due to interference caused by phase-forming compounds, samples were submitted to ultrafiltration prior to analysis, using Amicon ultra centrifugal filters (MWCO 10 kDa, Merck Millipore) to remove those compounds.

For the regeneration experiments, the alcohol concentration in the permeate was estimated through the total organic carbon concentration (TOC), using a total organic carbon analyzer (TOC-L, Shimadzu, Japan). To estimate the concentration of salt in the retentate and concentrate stream, the concentration of

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phosphate (PO_4^{3-}) was determined by ion chromatography using a Metrohm Compact IC Flex 930, which was equipped with an anion column (Metrohm Metrosep A Supp5–150/4.0), a guard column (Metrohm Metrosep A Supp 4/5 Guard), and a conductivity detector. The mobile phase consisted of an aqueous solution of 3.2 mM sodium carbonate, 1 mM sodium bicarbonate, and 1% (v/v) acetone. The injection volume of the sample was 100 µL.

Calculation methods

The performance of each system was evaluated by calculating the yield, as shown Eqn 2, where m_{phase} represents the mass of polysaccharide or protein in one of the phases (phase = bottom, precipitate, or top) and $m_{initial}$ is the mass of polysaccharide or protein present in the initial solution. The purity was calculated by Eqn 3, where $m_{target\ biopolymer}$ is the mass of the desired biopolymer in one of the phases and $m_{total\ biopolymer}$ is the total mass of biopolymer present in the same phase. For the regeneration experiments, the recovery of phase-forming compounds was estimated as shown in Eqn 4, where m_{phase} represents the mass of phase-forming compounds in the permeate and $m_{initial}$ is the mass of phase-forming compound before filtration.

$$\text{Yield}\% = \frac{m_{phase}}{m_{initial}} \quad (2)$$

$$\text{Purity}\% = \frac{m_{target\ biopolymer}}{m_{total\ biopolymer}} \quad (3)$$

$$\text{Recovery}\% = \frac{m_{phase}}{m_{initial}} \quad (4)$$

RESULTS AND DISCUSSION

Fractionation by polymer-based ATPS/TPP

Effect of type of phase-forming compounds

For this investigation, various systems were investigated that were composed of 25 wt% PEG400 and 25 wt% of a salt, the salt being K_3PO_4, $(NH_4)_2SO_4$ or $K_3C_6H_5O_7$. Figure 1 shows the partition behavior of BSA and dextran over the three phases (salt-rich, PEG-rich, and precipitate), and for each of the biopolymers, the purity is given for the phase in which that biopolymer is predominant. The detailed data on the yields and purity levels are given in the Supplementary Material (Table S1). It follows from Fig. 1, that separation of dextran from BSA was achieved for all systems since the purity of the phases (i.e., composition based on biopolymer fraction) ranged from 98% to 100%. This means that the dextran and BSA had a preferential affinity for different phases. For instance, BSA mostly partitioned to the PEG-rich phase (79–98%). The yield of BSA in the PEG-rich phase was not highly affected by the nature of the salt and was as follows: $K_3PO_4 < (NH_4)_2SO_4 \approx K_3C_6H_5O_7$. On the other hand, the extraction of dextran was more significatively affected by different salts. 84% of dextran was extracted to the salt-rich phase in PEG400/$(NH_4)_2SO_4$ system, while 99% of dextran was found as precipitate in the PEG400/$K_3C_6H_5O_7$ system. Significant precipitation of dextran, but to a lesser extent (34%), also occurred for the PEG400/K_3PO_4 system. The pH of the system also depended on the type of salt used. Use of $(NH_4)_2SO_4$ as phase-forming compound led to pH = 6, while $K_3C_6H_5O_7$ resulted in a pH of 9 and K_3PO_4 resulted in a pH = 13.

Besides causing different pH levels, the salts used to form the extractive platform also had different affinities for water, which influences the solubility of polysaccharide in the salt-rich phase. There was a competition between the salt ions and polysaccharide molecules to be solvated by water and, with respect to polysaccharide, this competition was more unfavorable in the presence of citrate than sulfate and phosphate salt. As shown in Figure 1, this unfavorable competition led to the highest precipitation yields of dextran PEG/citrate among the studied systems. These results are in line with those of Du et al,[25] who also reported the preferential affinity of dextran for the salt-rich phase in PEG/$(NH_4)_2SO_4$ ATPS. Overall, considering the TPP approach, PEG400/$K_3C_6H_5O_7$ had the best performance and was further investigated regarding the effect of the concentrations of polymer and salt on the fractionation of dextran and BSA.

Effect of concentration of phase-forming compounds

Experiments with the PEG400/$K_3C_6H_5O_7$ systems were done at concentrations of the phase-forming compounds ranging from 20 to 40 wt% and the results are given in Fig. 2. The detailed data on the yields and purity levels are given in the Supplementary Material (Table S2). As shown in Figure 2, the extraction yield of BSA to PEG-rich phase was affected by the changes in the concentration of citrate and PEG. Reducing the PEG concentration from

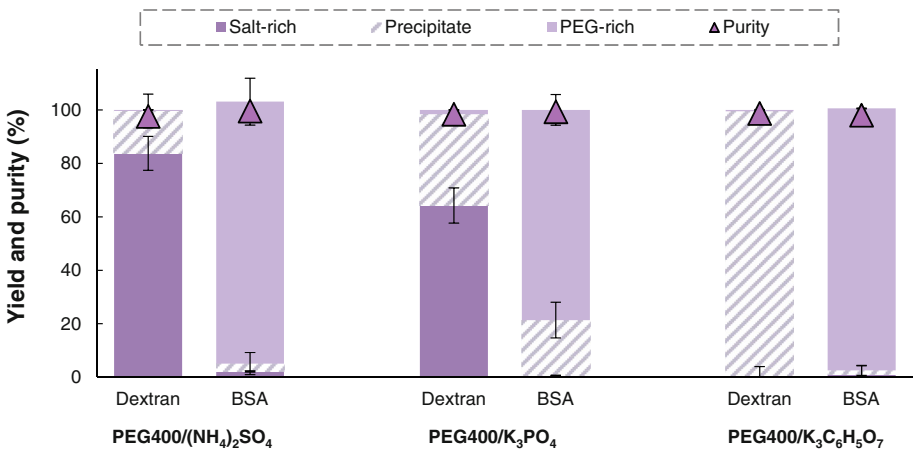

Figure 1. Partitioning behavior of dextran and bovine serum albumin (BSA) in polyethylene glycol (PEG)400 based three-phase partitioning systems composed of 25 wt% PEG400, 25 wt% of different salts, and 50 wt% aqueous solution of model biopolymer (dextran 2.5 g/L and BSA 2.5 g/L). Purities are given for the phase in which that biopolymer is predominant. The results presented are the average of two independent assays and the error bars represent standard deviation.

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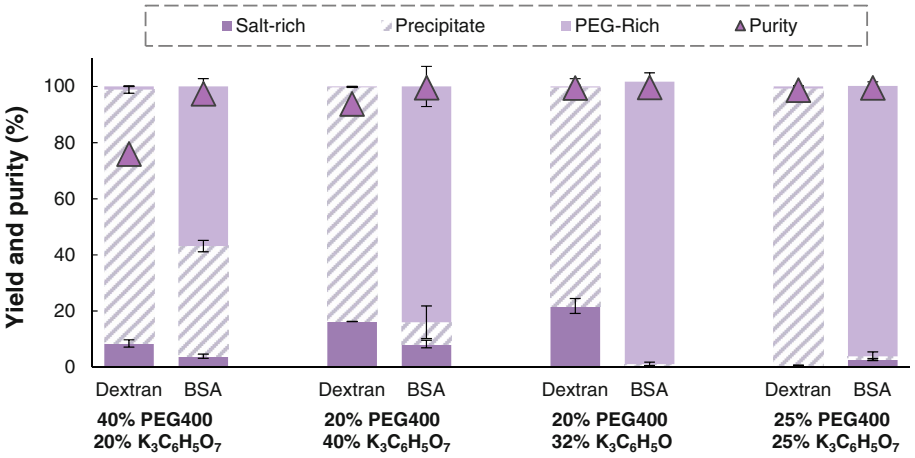

Figure 2. Partitioning behavior of dextran and bovine serum albumin (BSA) in polyethylene glycol (PEG)400 based three-phase partitioning systems composed of different concentrations of PEG400 and $K_3C_6H_5O_7$ (based on weight percentage); the remaining percentage to complete 100% was aqueous solution of model biopolymer (dextran 2.5 g/L and BSA 2.5 g/L). Purities are given for the phase in which that biopolymer is predominant. The results presented are the average of two independent assays and the error bars represent standard deviation.

40 to 20 wt% led to an increase of extraction yield of BSA to PEG-rich from 57% to 84%. Even higher extraction yields of BSA (96–100%) were obtained as the concentration of both PEG and citrate were reduced. Dextran was mostly found to be an interfacial precipitate and it was observed that less precipitation occurred for systems composed of relatively lower concentrations of PEG.

These results can be related to the equilibrium concentration of the phases, as shown in Table 2. In a polymer/salt system, the partitioning of biopolymers is driven by a combination of factors, including size-dependent, hydrophobicity, electrostatic interactions, specific molecular interactions, and conformational changes.[61] In particular, factors like size-dependent (volume exclusion) and hydrophobicity seemed to play a major role in this case. The volume exclusion implies that the higher the polymer concentration, the higher the volume occupied by the polymer. That reduces the available space for biopolymers in the polymer phase and causes the partitioning of the biopolymer towards the opposite phases (salt-rich or precipitation).[61,62] The results in Fig. 2 agree with this effect, as the highest extraction yield of BSA (to PEG-rich phase) was obtained for the system with the lowest concentration of PEG in the top phase based on Table 2 (25 wt % PEG400 + 25 wt% $K_3C_6H_5O_7$). The decrease of partitioning of proteins to the PEG-rich phase as PEG's concentration increases has also been reported by other researchers.[44,63]

Two well-known effects are involved in hydrophobic interactions: the phase hydrophobicity and the salting-out effect.[64] Although PEG is, in principle, hydrophilic, the relative hydrophobicity of top phase (PEG-rich) varies accordingly with PEG's concentration. As PEG concentration decreases, more water molecules are available in the top phase, which decreases the phase relative hydrophobicity. This favors the partition of BSA proteins towards the top phase. The salting-out effect is regarding the reduction of the solubility of biopolymers in the salt-rich phase as concentrations of salt increase in this phase.[64] Given the high amount of water necessary to dissolve the salts in the system, the biopolymers to be partitioned would experience only partial hydration. As a result, partitioning toward the oppositive phase (PEG-rich) is favored in such conditions. This would explain the decrease of partitioning of dextran to salt-rich phase as the citrate concentration increased from 32 to 40 wt%, while keeping the same concentration of PEG. Other authors have reported that excessive concentration of salt caused a reduced solubility of polysaccharides in the bottom phase.[26]

Effect of feed composition

It was also investigated how the performance of PEG400/$K_3C_6H_5O_7$ ATPS/TPP (polymer-based system with the best performance in this study) was dependent on the model biopolymer's structure, and for this, three polysaccharides and two proteins were applied. Figure 3 shows the extraction yields and purity for separation of the polysaccharides (alginate, dextran, and gum arabic) from proteins (BSA and lysozyme). The detailed data on the yields and purity levels are given in the Supplementary Material (Table S3). There were no relevant changes for the protein fraction, as both BSA and lysozyme were enriched (96%) in the

Table 2. Equilibrium phase composition (mixture, top or PEG-rich, and bottom or salt-rich phases, in weight fraction) of different PEG400 based ATPS used to study fractionation of dextran and BSA at 21 ± 1 °C

	Mixture		Top phase		Bottom phase	
System	PEG	Salt	PEG	Salt	PEG	Salt
PEG400/$K_3C_6H_5O_7$	0.400	0.200	0.709	0.054	0.014	0.526
	0.200	0.399	0.526	0.100	0.027	0.501
	0.200	0.319	0.413	0.143	0.037	0.451
	0.250	0.250	0.360	0.168	0.094	0.376

Abbreviation: PEG, polyethylene glycol.

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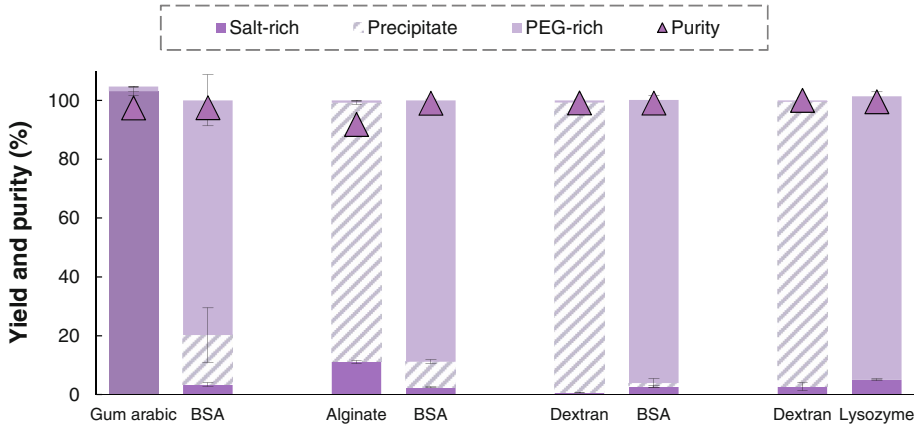

Figure 3. Partitioning behavior of different model polysaccharides (gum arabic, alginate, and dextran) and model protein [bovine serum albumin (BSA) and lysozyme] in three-phase partitioning system composed of 25 wt% PEG400, 25 wt% $K_3C_6H_5O_7$, and 50 wt% biopolymer aqueous solution (2.5 g/L each biopolymer). Purities are given for the phase in which that biopolymer is predominant. The results presented are the average of two independent assays and the error bars represent standard deviation.

PEG-rich phase. A more relevant effect on extraction yield was observed for different model polysaccharides. For instance, 100% gum arabic was extracted to salt-rich phase, while 11% of alginate was found in the salt-rich phase. This happened because a large amount of alginate (88%) precipitated at interface, much like dextran (99%).

Both alginate and gum arabic are anionic polysaccharides, while dextran is a neutral polysaccharide. The similar extraction trends between alginate and dextran suggest that, regardless of the charge, these model polysaccharides still establish similar intermolecular interactions and that other factors, such as the molecular weight, play a more relevant role. The molecular weight of model polysaccharide was as follows: gum arabic <alginate<dextran; this indicates that the higher the molecular weight of model polysaccharide, the higher the precipitate amount at interface, which is beneficial within the TPP approach.

The effect of molecular weight of biopolymers on their partitioning behavior in ATPS has also been studied by other authors.[26,44,53] Some studies[26,27] have shown that relatively small polysaccharides (<100 kDa) also tended to migrate to the salt-rich phase in PEG/phosphate ATPS. For the partitioning behavior of proteins, a similar trend has been reported for systems composed of PEG400 and salt. For instance, Suarez Ruiz et al[53] reported that protein also mostly (>85%) partitioned to the PEG-rich phase using PEG400/citrate ATPS. As PEG's molecular weight increased, as shown in literature,[44,65] more protein precipitated at the interface instead of partitioning to the polymer-rich phase.

Fractionation by alcohol-based ATPS/TPP

Effect of type of phase-forming compounds

The effect of salt type on the partition of dextran and BSA was investigated using biphasic systems composed of 25 wt% EtOH + 20 wt % salt, except for the citrate system since a biphasic system could not be formed at such a mixture point. Instead, a system with similar composition (23 wt% EtOH and 23 wt% $K_3C_6H_5O_7$) was selected. Figure 4 shows that TPP systems were formed and that separation of dextran from BSA was achieved for all cases investigated. The detailed data on the yields and purity levels are given in the Supplementary Material (Table S4). BSA mostly migrated to the alcohol-rich phase and the extraction yield to alcohol-rich phase was as follows: $(NH_4)_2SO_4 < K_3C_6H_5O_7 < K_3PO_4$. Dextran was either preferentially extracted to the salt-rich phase or precipitated at the interface. Similar to what was observed for polymer-based ATPS/TPP, the fraction of dextran in the precipitate depended on the type of salt used. Using ethanol combined with different salts, the precipitation yield of dextran was as follows: $(NH_4)_2SO_4 < K_3PO_4 < K_3C_6H_5O_7$. The pH of the system also depended on the type of salt used, as sulfate salt led to pH = 6, while the pH was 9 and 13 when citrate and phosphate salts were used, respectively.

The formation of precipitate seems to be related to the salting-out strength of the investigated salts. Citrate and phosphate anions exhibit stronger interaction with water molecules when compared to sulphate, thus reducing the water molecules available to interact with dextran molecules. As result, dextran precipitated at the interface more in such systems. Other researchers[39,66] have also reported that polysaccharide is mostly extracted to the salt-rich phase in EtOH/$(NH_4)_2SO_4$ ATPS. Studies using salts other than sulfate, such as phosphate,[67,68] also reported little extraction yield of polysaccharide to the salt-rich phase. The high partition of BSA towards alcohol-rich phase is likely due to salting-out and, since BSA has hydrophobic regions on its surface, such hydrophobic regions favored its migration to the alcohol-rich phase. Amid et al[69] reported a similar partition trend using different salts for the extraction of serine protease. The authors obtained the highest partition coefficient for the system composed of EtOH and phosphate salt ($K = 22$), followed by citrate ($K = 18$), and sulfate ($K = 12$) based systems.[69]

Overall, TPP composed of ethanol and citrate/phosphate salts had the best performance as three-phase partitioning systems, with the phosphate-based system having a slightly higher extraction yield and purity when compared to the citrate-based system. Therefore, EtOH/K_3PO_4 will be further investigated in the following studies when it comes to the effects of concentration of phase-forming compounds and feed composition.

Effect of concentration of phase-forming compounds

The effect of the concentrations of phosphate and ethanol was studied by varying them from 10 to 25 wt% (K_3PO_4) and from 20 to 36 wt% (EtOH). As shown in Figure 5, the extraction yield of BSA to alcohol-rich phase was not significatively affected by the changes in the concentration of phosphate and ethanol. BSA preferentially partitioned to the alcohol-rich phase as its

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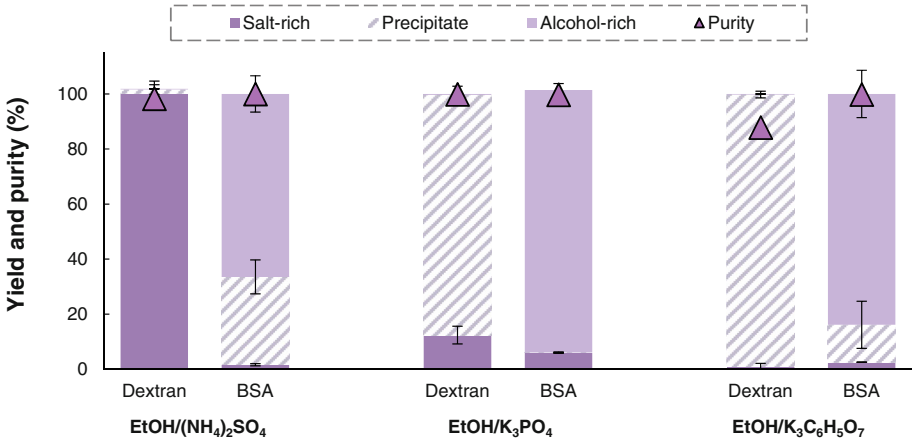

Figure 4. Partitioning behavior of dextran and bovine serum albumin (BSA) in ethanol (EtOH) based three-phase partitioning (TPP) systems composed of 25 wt% EtOH, 20 wt% of different salts, and 55 wt% aqueous solution of model protein and polysaccharide (2.5 g/L dextran and 2.5 g/L BSA), except citrate-based TPP (23 wt% EtOH, 23 wt% $K_3C_6H_5O_7$). Purities are given for the phase in which that biopolymer is predominant. Results presented are averages of two independent assays and the error bars represent standard deviations.

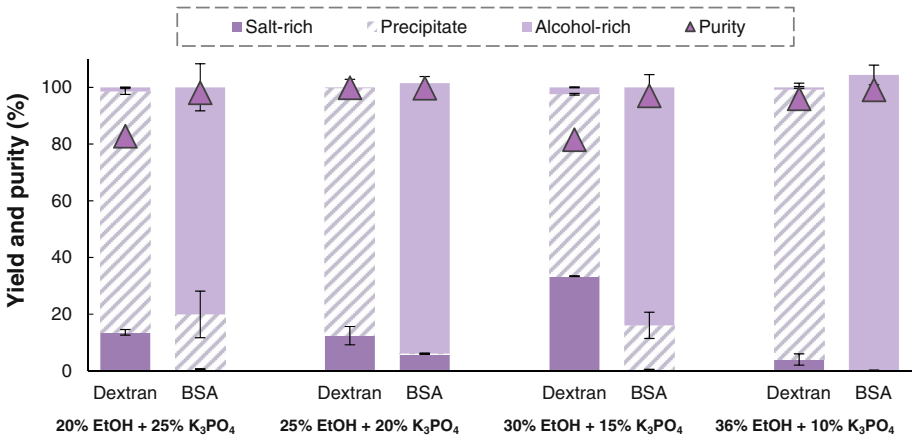

Figure 5. Partitioning behavior of dextran and bovine serum albumin (BSA) in ethanol (EtOH) based three-phase partitioning systems composed ethanol and phosphate salt (K_3PO_4) in different concentrations (based on weight percentage). Purities are given for the phase in which that biopolymer is predominant. Results presented are averages of two independent assays and the error bars represent standard deviations.

extraction yield ranged from 85% to 100%. The highest extraction yield of BSA occurred for the mixture point of 36 wt% EtOH and 10 wt% K_3PO_4. On the other hand, dextran mostly precipitated at the interface for the different mixture points studied. The system composed of 30 wt% EtOH + 15 wt% K_3PO_4 displayed the lowest precipitation yield of dextran, as the polysaccharide also partitioned to the salt-rich phase. Similar to BSA, the system composed of 36 wt% EtOH + 10 wt% K_3PO_4 had the best performance for separating dextran since 95% dextran was obtained as interfacial precipitate. The detailed data on the yields and purity levels are given in the Supplementary Material (Table S5).

The equilibrium phase composition seems to play a key role in the yield and selectivity of the systems. As shown in Table 3, the system with the best performance on separating dextran and BSA had the lowest concentration of EtOH in the top phase and the highest concentration of phosphate in the bottom phase. Increasing the concentration of both ethanol and phosphate leads to the precipitation of biopolymer (dextran or BSA) as these compounds compete for water molecules and, consequently, reduce the solubility of biopolymer in either the bottom or top phase. This trend was observed for the mixture point 30 wt% EtOH + 15 wt% K_3PO_4 since it had the lowest phosphate fraction in the bottom phase; consequently, the highest extraction of dextran to the bottom phase was also observed for this system. Within the TPP approach, the best performance was achieved for the system with the highest concentration of salt in the bottom phase and lowest concentration of ethanol in the top phase (36 wt% EtOH + 10 wt% K_3PO_4). This ensured nearly complete precipitation of dextran (95%), while BSA was completely partitioned to the alcohol-rich phase (100%).

Effect of feed composition

It was also investigated how the feed composition affected the performance of EtOH/K_3PO_4 TPP systems for the fractionation of polysaccharide and protein mixtures. Figure 6 shows the separation of different polysaccharides (alginate, dextran, and gum arabic) from protein (BSA and lysozyme). The detailed data on the yields and purity levels are given in the Supplementary Material (Table S6). EtOH/K_3PO_4 TPP was more affected by the feed composition, in comparison to PEG400/$K_3C_6H_5O_7$ TPP. For the partition of proteins, 100% BSA was in the ethanol-rich phase, while nearly 100% lysozyme precipitated at interface. For the

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Table 3. Equilibrium phase composition (mixture, top or alcohol-rich, and bottom or salt-rich phases, in weight fraction) of different Ethanol based ATPS used to study separation of dextran and BSA at 21 ± 1 °C

	Mixture		Top phase		Bottom phase	
System	EtOH	Salt	EtOH	Salt	EtOH	Salt
EtOH/K_3PO_4	0.202	0.250	0.543	0.008	0.005	0.388
	0.250	0.200	0.449	0.014	0.005	0.387
	0.300	0.150	0.529	0.014	0.012	0.349
	0.362	0.100	0.436	0.024	0.001	0.473

Abbreviation: EtOH, Ethanol.

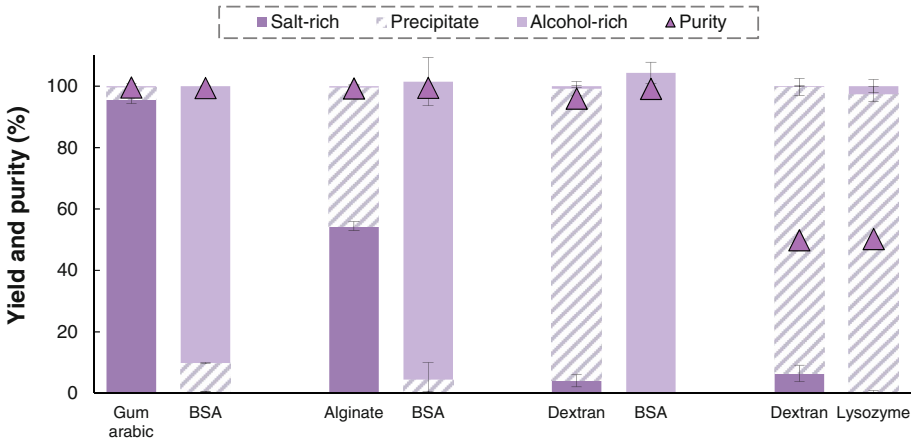

Figure 6. Partitioning behavior of different model polysaccharides (gum arabic, alginate, and dextran) and model protein [bovine serum albumin (BSA) and lysozyme] in three-phase partitioning system composed of 36 wt% EtOH, 10 wt% K_3PO_4, and 54% biopolymer aqueous solution (2.5 g/L each biopolymer). Purities are given for the phase in which that biopolymer is predominant. Results presented are averages of two independent assays and the error bars represent standard deviations.

polysaccharides, it was found that large fractions of alginate (45%) and dextran (95%) were found as precipitates at the interface, while 96% of gum arabic partitioned to the salt-rich phase. Similar to polymer-based TPP, molecular weight is likely to be responsible for the different partitioning behaviors among the model polysaccharides. BSA was mostly extracted to the ethanol-rich phase, while lysozyme, a much smaller protein (14 kDa), was mostly found as precipitate. Besides having a different molecular weight, lysozyme is a relatively more hydrophobic protein,[70] suggesting that factors other than molecular weight are driving the partitioning of proteins in EtOH/K_3PO_4 systems.

Performance comparison

In this study, ethanol and PEG-based TPP systems were investigated for the fractionation of model biopolymer mixture. Table 4 shows a comparison among these two types of systems regarding relevant aspects for the feasibility of the separation technique: performance (in terms of yield and purity), compatibility, cost, and regeneration. In general, both PEG and ethanol systems presented high yields (>80%) and purity levels in single stage extractions under most of the investigated conditions. Regarding compatibility, PEG-based TPP systems are a more suitable option as they demonstrated high yields and selectivity for a larger variety of model biopolymer solutions. For instance, for the feed composed of dextran and lysozyme, ethanol-based TPP was not able to selectively separate these biopolymers, while the PEG-based system could fractionate this mixture. Regarding cost, alcohol-based TPP systems are likely a more suitable option since the phase-forming compounds are less costly and used in a lower concentration. Finally, regarding regeneration, alcohol-based systems are more advantageous because the phase-forming compounds can be regenerated by diverse techniques, such as membrane processes and distillation, while PEG-based systems cannot be recovered by evaporation means due to the high boiling point of polymers.

The findings also suggest that the main advantages of the three-phase partitioning systems studied in this work are the amount of chemicals required and energy demand. As shown by Xu et al,[71] ethanol precipitation is the most common technique to separate polysaccharides from other classes of biopolymers. The authors carried out a comprehensive literature review

Table 4. Comparison of the potential of EtOH-based and PEG-based three-phase partitioning systems for fractionation of biopolymer mixture taking in account relevant aspects for the feasibility of the separation technique

Aspects	EtOH-based	PEG-based
Performance (yield and purity)	✓	✓
Compatibility	×	✓
Cost	✓	×
Regeneration	✓	×

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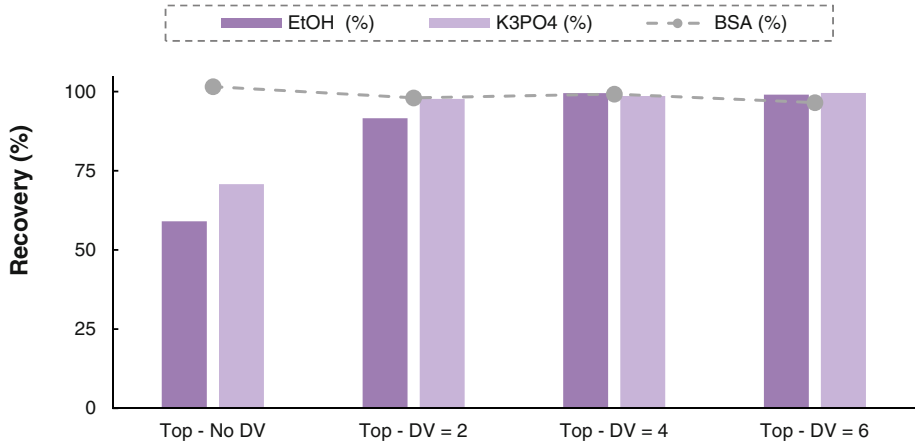

Figure 7. Performance of ultrafiltration (UF) for separating phase-forming compounds (ethanol and K_3PO_4) from bovine serum albumin (BSA) present in the top phase. Top phase obtained after fractionation by three-phase partitioning system composed of 36 wt% EtOH + 10 wt% K_3PO_4 + 54% biopolymer aqueous solution (2.5 g/L dextran and 2.5 g/L BSA). UF Operational conditions: T = 21 ± 1 °C, 3 bar. DV, diafiltration volume.

regarding the utilization of ethanol precipitation for the separation of polysaccharides and found that most of the publications used ethanol concentration within the range of 70–80% and at low temperatures (4 °C). Alternatively, the TPP systems investigated in this work are operated at room temperature and require a much lower total concentration of phase-forming compounds (46% and 50% for ethanol and PEG-based TPP systems, respectively). In addition, the investigated TPP systems also showed improvements in relation to traditional TPP systems. Diverse studies[40,72–74] reported that the optimized concentration of salt in butanol/$(NH_4)_2SO_4$ TPP systems ranged from 30 to 70% w/w, while the amount of salt required in our investigation ranged from 10 to 25%w/w.

Regeneration of phase-forming compounds by ultrafiltration

To demonstrate the feasibility and effectiveness of UF as a regeneration technique, the phases obtained after fractionation using alcohol-based TPP systems were used as feed. This selection was initially based on its lower cost and reinforced by this investigation, which indicated that the alcohol/salt system exhibited a promising performance in fractionating biopolymer mixtures and required smaller amounts of phase-forming compounds, making it the ideal choice for a proof of concept.

Based on results discussed in the previous section within the TPP approach, dextran (model polysaccharide) can be retrieved by resolubilizing the precipitated fraction. As BSA was mostly extracted to the alcohol-rich phase, ultrafiltration/diafiltration was used to separate BSA from phase-forming compounds (EtOH and K_3PO_4), as shown in Fig. 7. By adding water (amount equal to six times the volume of the feed stream), it was possible to increase the recovery of K_3PO_4 and ethanol (>99%) while keeping an insignificant loss of BSA during the filtration.

These findings suggest that UF/diafiltration is a potential alternative to dialysis, which is one of the most common techniques in the literature for recovery of phase-forming compounds.[31,75,76] [31,75,76] Some advantages of this approach are that it is less time consuming and uses less water, thus reducing the extent to which the target biopolymer and recovered phase-forming compounds are diluted during filtration. UF/diafiltration is also an alternative for distillation, which is often used to recover the alcohol used in traditional TPP systems.[40,72,73] The main advantage is that UF/diafiltration is a more versatile technique because it also allows for the recovery of high boiling point compounds (such as salts and polymers). Future process design approaches may make use of UF (possibly with diafiltration) and potentially also produce extra concentrated polymer solutions from which precipitation may be induced to isolate the product.

CONCLUSION

This work has shown that the technique of TPP systems based on ATPS is an interesting separation technique for natural polymers separation because it allows fractionation and concentration of biopolymer mixtures in a single step. High yields of protein were obtained in the polymer or alcohol-rich phase, whereas the polysaccharide fraction could be easily retrieved as interfacial precipitate and dissolved in an adequate volume of aqueous solution. For polymer-based TPP, it was found that using PEG400 combined with $K_3C_6H_5O_7$ in equal mass percentage (25 wt%) resulted in the best fractionation performance: 99% dextran was obtained as precipitate, while 89% BSA partitioned to PEG-rich phase. For alcohol-based TPP, about 100% BSA migrated to the alcohol-rich phase while 95% dextran was found as precipitate. Since complete fractionation was not achieved in this single stage extraction, a multistage countercurrent extraction approach may be useful to increase the extraction yields and purity of the biopolymers in the streams.

It was also demonstrated that the performance of TPP systems depended on the composition of feed. Therefore, a tailored selection of phase-forming compounds, considering the composition of the stream to be purified, should be considered. Finally, the recovery of phase-forming compounds (after fractionation) was achieved by ultrafiltration/diafiltration since about 99% ethanol and salt was retrieved in the permeate stream, while the target biopolymer molecule was obtained (concentrated) in the retentate stream.

This research contributes to the development of biopolymer separation technology for applications in polysaccharide and protein separation from natural biopolymers cultivated in waste fermentations, which helps purifying these polymers using relatively inexpensive chemicals with processes that can be carried out at room temperature.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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