

Controlling the L-asparaginase extraction and purification by the appropriate selection of polymer/salt-based aqueous biphasic systems

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Abstract

BACKGROUND: L-Asparaginase (ASNase) is an important biopharmaceutical for the treatment of acute lymphoblastic leukemia (ALL); however, with some restrictions due to its high manufacturing costs. Aqueous biphasic systems (ABS) have been suggested as more economical platforms for the separation/purification of proteins, but a full understanding of the mechanisms behind the ASNase partition is still a major challenge. Polymer/salt-based ABS with different driving-forces (salting-out and hydrophilicity/hydrophobicity effects) were herein applied to control the partition of commercial ASNase.

RESULTS: The main results showed the ASNase partition to the salt- or polymer-rich phase depending on the ABS studied, with extraction efficiencies higher than 95%. For systems composed of inorganic salts, the ASNase partition was controlled by the polyethylene glycol (PEG) molecular weight used. Cholinium-salts-based ABS were able to promote a preferential ASNase partition to the polymer-rich phase using PEG-600 and to the salt-rich phase using a more hydrophobic polypropylene glycol (PPG)-400 polymer. It was possible to select the ABS composed of PEG-2000 + potassium phosphate buffer as the most efficient to separate the ASNase from the main contaminant proteins (purification factor = 2.4 ± 0.2), while it was able to maintain the enzyme activity for posterior application as part of a therapeutic.

CONCLUSION: Polymer/salt ABS can be used to control the partition of ASNase and adjust its purification yields, demonstrating the ABS potential as more economic platform for the selective recovery of therapeutic enzymes from complex broths.

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

Keywords: enzymes; aqueous biphasic systems (ABS); bioseparations; liquid–liquid extraction; purification; separation

INTRODUCTION

Biopharmaceuticals are produced by engineered biological sources or biotechnological processes, exhibiting further advantages, such as high potency and specificity. However, the structural complexity, large size, and high susceptibility of the biomolecules make the manufacturing processes of biopharmaceuticals quite challenging.¹ Among these, the biopharmaceutical L-asparaginase, L-asparagine amidohydrolase, EC.3.5.1.1 (ASNase), a therapeutic enzyme largely used in the treatment of childhood acute lymphoblastic leukemia (ALL), has been intensively studied.^{2–4} As other biopharmaceuticals, the high purity demands increases the ASNase high commercial price.⁵ The design of alternative and stabilizing downstream platforms is thus of utmost importance and fundamental in reducing the overall ASNase manufacturing costs.⁶

Considering that biopharmaceuticals are quite unstable proteins, conventional liquid–liquid extraction methods using volatile, hazardous and non-biocompatible solvents are here

avoided. In this context, due to their high water content, aqueous biphasic systems (ABS) have been studied as biocompatible and environmentally friendly alternatives for the recovery and purification of different bioproducts,^{7,8} particularly the ABS using: (i) inert, biocompatible and biodegradable polymers, such as polyethylene glycol (PEG)⁹ and polypropylene glycol (PPG);¹⁰ (ii) buffers that

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simulate the cellular environment; and (iii) biocompatible salts derived from renewable sources with lower toxicity and costs, such as cholinium-based-salts.¹¹ Moreover, the application of these systems and solvents obey the principles of Green Chemistry¹² and the concerns of biocompatibility and selectivity needed on the downstream processing of biopharmaceuticals.¹³

ABS can be used as an initial (low resolution) downstream stage by reducing contaminants and sample volume, and consequently the complexity and cost of further biopharmaceutical purification steps.¹⁴ As an example, these systems were successfully used on fractionation of biomolecules like proteins and carotenoids by separating them according to their intrinsic properties, namely the solubility, ionic strength, molecular mass, polarity and surface charge.^{15,16} ABS composed of polymers and salts were also successfully applied in the purification of proteins, biopharmaceuticals, antibiotics and other biomolecules,^{17–20} due to the proper manipulation of the different interactions between the phase forming components and the solutes. Asenjo and Andrews²¹ highlighted that the control of the protein partition in polymer/salt-based ABS can be achieved exploring the properties of the coexisting phases, i.e. the relative hydrophobicity and electrochemical potential, or the proteins properties like their molecular size, conformation, and biospecificity.

Recently, polymer/salt-based ABS using cholinium ([Ch]⁺)-based salts (or ionic liquids, ILs) were proposed as platforms for solutes partition,^{22–25} particularly, due to the relative hydrophobicity/hydrophilicity balance between the coexisting phases.²⁶ Furthermore, it was demonstrated that [Ch]⁺-based salts can promote specific protein-ions interactions, acting directly on the protein partitioning capacity, as shown for beta-lactoglobulin, A and B, lysozyme, and ribonuclease A and B,²⁷ as well as on protein stabilization,¹¹ as previously observed for monoclonal antibodies,²⁸ cytochrome *c*,²⁴ rubisco,¹⁹ bovine serum albumin (BSA)²⁹ and α -chymotrypsin.³⁰

These previous studies demonstrated that polymer/salt ABS can provide biocompatible environments to maintain the protein structural conformation^{11,19,24,28–30} and to enlarge the range of protein partition driving mechanisms,²⁷ but a careful selection of the phase forming agents is crucial. Nevertheless, only a few works have investigated the mechanisms behind the purification of ASNase using ABS.^{18,31,32} Exceptions are a work reporting its purification by using micellar ABS,³¹ or other more recent work in which PEG-based ABS were applied to recover recombinant ASNase from *Escherichia coli*.¹⁸ Considering the lack of ASNase partition studies and the plethora of properties [molecular weight (MW), type and concentration of polymers, and/or ionic strength and nature of salts] that can be properly adjusted for each ABS, new studies are required to allow for the deep understanding of these mechanisms.

The aim of this work was the study of the partition of ASNase using a series of polymer/salt-based ABS to understand the mechanisms behind the protein partition. Afterwards, some systems were selected and applied on the purification of ASNase directly from the fermentation broth after the lysis of the *E. coli* cells. The best system operating the purification of the enzyme was selected, considering the results of purification factor (PF) and enzyme activity.

EXPERIMENTAL

Materials

ASNase (EC.3.5.1.1) was purchased from ProSpec-Tany[®] (ENZ-287, Rehovot, Israel). PEG polymers with average MW of 300 g mol⁻¹

(PEG-300, pure), 600 g mol⁻¹ (PEG-600, pure), 1000 g mol⁻¹ (PEG-1000, pure), 1500 g mol⁻¹ (PEG-1500, pure) and 2000 g mol⁻¹ (PEG-2000, pure), and PPG polymer with average MW of 400 g mol⁻¹ (PPG-400, pure) were acquired from Sigma-Aldrich[®] (São Paulo, Brazil) and used as received. Protein assay dye reagent was obtained from Bio-Rad (Hercules, CA, USA). Sodium sulfate (Na₂SO₄), monopotassium phosphate (KH₂PO₄), dipotassium phosphate (K₂HPO₄), potassium citrate (C₆H₅K₃O₇) and citric acid (C₆H₈O₇, > 95 wt%), cholinium chloride ([Ch]Cl, with purity \geq 98%), Trizma-HCl buffer base, L-asparagine (> 99%), hydroxylamine (> 99%), iron(III) chloride (FeCl₃), trichloroacetic acid (TCA), hydrochloric acid (HCl) and acid L-aspartic β -hydroxamate acid were also acquired from Sigma-Aldrich[®] and used as received. Cholinium acetate ([Ch][Ac], with purity of \geq 98%) was purchased from lolitec[®] (Germany) and used as received. Ultrapure water double distilled, passed through a reverse-osmosis system and further treated by filtration through a Millipore Milli-Q ion-exchange system (18 M Ω cm) (Brazil), was used.

ASNase partition in polymer/salt ABS

For each polymer/salt ABS, different ternary mixtures (polymer + salt + water) at the biphasic region were prepared adopting the phase diagrams information previously reported, detailed in Table 1. All phase forming components and the potassium phosphate buffer (20 mol L⁻¹, pH 7.4) solution of ASNase (at 1 mg mL⁻¹) were added to Eppendorf flasks (2.0 mL) by weighing (\pm 10⁻⁴ g) to obtain a total mass of 1.0 g. The ASNase concentration in each system was fixed as 0.2 mg mL⁻¹. Afterwards, the mixtures were vigorously stirred using a vortex tube mixer, and then equilibrated at 25 (\pm 1) °C during 1 h. After the equilibrium, to guarantee the complete separation of the phases, the systems were centrifuged at 2000 \times g for 30 min at 25 (\pm 1) °C. After centrifugation, both top and bottom phases were carefully separated, using Pasteur-pipettes, and weighed (\pm 10⁻⁴ g). All the assays were performed in duplicate, and the respective standard deviations determined. The interference of the ABS components was ascertained with the corresponding blank control samples, which were prepared with ultrapure water instead of ASNase aqueous solution.

The partition behavior in each ABS under study was assessed in terms of ASNase partition coefficient (*K*) and ASNase extraction efficiency [EE (%)], both determined according to Eqns (1) and (2), respectively:

$$K = \frac{[\text{ASNase}]_{\text{top-phase}}}{[\text{ASNase}]_{\text{bottom-phase}}} \quad (1)$$

$$\text{EE}(\%) = \frac{[\text{ASNase}]_{\text{ASNase-rich}} \times V_{\text{ASNase-rich}}}{([\text{ASNase}]_{\text{ASNase-rich}} \times V_{\text{ASNase-rich}}) + ([\text{ASNase}]_{\text{ASNase-poor}} \times V_{\text{ASNase-poor}})} \times 100 \quad (2)$$

where [ASNase] and *V* are, respectively, the ASNase concentration (in mg mL⁻¹) and volume of the phase (in milliliters), while the subscripts 'top-phase', 'bottom-phase', refer to the top and bottom phases, respectively. In Eqn (2), the subscripts 'ASNase-rich' and 'ASNase-poor' correspond to the phases (top or bottom) with high or low ASNase content, respectively. To facilitate the lecture of the ASNase partition between the top and bottom-phases, the *K* is represented in logarithmic function (log₁₀), to facilitate the interpretation. Thus, log *K* > 0 corresponds to a preferential partition of ASNase towards the top phase, while log *K* < 0 refers to its preferential partition towards the bottom phase. Note that

Table 1. Physicochemical properties and extraction parameters [partition coefficient (*K*) and extraction efficiency (*EE* (%)) of polymer/salt-based aqueous biphasic systems (ABS) used for the L-asparaginase (ASNase)

Ternary system	Weight composition (wt%)			pH		Conductivity (mS)		Viscosity (mPa.s)		Density (g/cm ³)		Water Content (%)		Partition parameters		Relative activity (%)	
	Polymer	Salt	Water	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	log (<i>K</i>)	EE (%)	Top	Bottom
<i>Polyethylene glycol (PEG) + citrate buffer</i> ^{33,34}																	
PEG-600	15	20	65	7.04	6.91	2.36	19.1	26.75	a	1.13	a	40.00	60.58	∞	100	101 ± 2	0
PEG-1000	15	20	65	6.96	6.82	1.818	28.9	23.71	3.66	1.12	1.27	47.52	62.48	0.51 ± 0.05 ^a	69 ± 2.81 ^a	70.25 ± 3.24	0
PEG-1500	15	20	65	7.02	6.79	1.116	24.5	41.97	3.58	1.12	1.27	44.15	62.66	0.42 ± 0.09 ^a	61 ± 1 ^a	71 ± 2	0
PEG-2000	15	20	65	7.02	6.82	0.918	20.9	60.81	5.287	1.11	1.34	44.73	54.32	-0.4 ± 0.1 ^a	23 ± 2 ^a	30 ± 2	0
<i>PEG + phosphate buffer</i> ³³																	
PEG-300	15	20	65	6.92	6.97	17.63	117.8	4.86	2.906	1.11	1.24	63.41	71.26	∞	100	103 ± 2	0
PEG-600	15	20	65	7.05	6.75	8.57	134.9	6.85	2.514	1.10	1.25	55.71	69.69	∞	100	105 ± 2	0
PEG-1000	15	20	65	7.00	6.71	5.02	140.1	10.49	2.358	1.09	1.24	57.57	71.91	-0.47 ± 0.09	84 ± 2	2 ± 1	97 ± 2
PEG-1500	15	20	65	6.98	6.69	3.595	142.7	15.68	2.308	1.09	1.24	57.98	72.58	-0.73 ± 0.06	92 ± 0.7	0	98 ± 3
PEG-2000	15	20	65	7.01	6.70	2.926	142.8	22.41	2.216	1.09	1.24	58.97	72.03	-1.37 ± 0.06	99 ± 0.2	0	98 ± 3
<i>PEG + sodium sulfate (Na₂SO₄)</i> ³⁵																	
PEG-300	15	10	75	6.69	7.11	12.02	23.5	5.82	3.148	1.10	1.20	65.17	72.46	0.59 ± 0.03	93.8 ± 0.2	105 ± 1	0
PEG-600	15	10	75	7.02	7.18	3.43	29.4	6.62	2.603	1.08	1.19	63.98	77.07	0.37 ± 0.01	72.8 ± 0.8	100.9 ± 0.1	0.4 ± 0.1
PEG-1000	15	10	75	4.29	4.68	14.3	98.51	5.99	2.082	1.08	1.15	70.49	81.06	-1.96 ± 0.04	97 ± 2	0	100.2 ± 0.4
PEG-1500	15	10	75	6.64	6.65	8.168	106.3	9.47	1.859	1.08	1.15	66.61	79.75	-1.97 ± 0.03	98 ± 2	0	97.5 ± 0.6
PEG-2000	15	10	75	6.40	6.21	6.294	111.8	13.14	1.818	1.07	1.15	63.89	82.97	-1.98 ± 0.02	98 ± 1	0	99.6 ± 0.8
<i>PEG-600 + cholinium ([Ch]⁺)-salt</i> ²⁶																	
[Ch]Cl	50	30	20	7.75	7.21	29.36	0.603	18.98	123.12	1.10	1.13	31.19	13.28	-0.12 ± 0.06	65 ± 3	0	88.0 ± 0.3
[Ch]IAc	50	30	20	9.61	9.72	14.71	0.614	19.15	101.17	1.10	1.12	38.37	16.70	-0.6 ± 0.1	89 ± 3	0	73.0 ± 0.3
<i>PPG-400 + [Ch]⁺ salt</i> ^{23,25}																	
[Ch]Cl	30	16	54	5.64	6.39	0.023	60.63	58.71	2.806	1.02	1.05	15.91	64.70	∞	100	0	109 ± 2
[Ch]IAc	30	16	54	7.50	8.04	0.019	36.06	65.73	3.067	1.03	1.05	16.59	69.69	∞	100	0	116.9 ± 0.1

^a In the citrate-based ABS it occurred ASNase precipitation in the interface (formation of a three-phase partition, TPP). Thus, the determination of the *K* and *EE* (%) values considered the precipitation, where it represents the protein amount in the precipitate or in the polymer-rich phase.

unlike the K , the EE (%) was solely determined for the phase where the ASNase was more concentrated.

For the ASNase partitioning experiments using *E. coli* cell lysates the mass balance (MB) and PF were also determined using Eqns (3) and (4), respectively:

$$\text{MB (\%)} = \frac{([\text{TP}]_{\text{top-phase}} \times V_{\text{top-phase}}) + ([\text{TP}]_{\text{bottom-phase}} \times V_{\text{bottom-phase}})}{(V_{\text{top-phase}} + V_{\text{bottom-phase}}) \times [\text{TP}]_{\text{initial}}} \times 100 \quad (3)$$

$$\text{PF} = \frac{SA_{\text{ASNase rich-phase}}}{SA_{\text{initial}}} \quad (4)$$

where [TP] and V are, respectively, the total protein concentration (in mg mL^{-1}) and volume of the phase (in milliliters), while the subscripts 'top-phase', 'bottom-phase' and 'initial', refer to the top, bottom phases and initial amount added in the ABS, respectively. $SA_{\text{ASNase rich-phase}}$ and SA_{initial} are, respectively, the specific ASNase activity (in U mg^{-1}).

Physicochemical characterization of polymer/salt ABS coexisting phases

After the separation of the coexisting phases, considering the different properties of each polymer/salt ABS the pH, conductivity, viscosity, density and water content of all the polymer and salt-rich phases were determined to obtain further insights about their intrinsic physicochemical characteristics (Table 1). To reduce the associated uncertainties, all properties were measured, and the respective standard deviations determined.

The conductivity ($\pm 1.0\%$ mS cm^{-1}) and pH (± 0.003) of each phase were measured at $25 (\pm 1) ^\circ\text{C}$ using a portable pH meter and conductometer Metrohm®/Model 914. The calibration of conductometer was carried out with a potassium chloride (KCl) standard solution ($1408 \mu\text{S cm}^{-1}$ and 0.1 mol L^{-1}), while the pH meter was calibrated using two standard buffers (pH values of 7.00 and 4.01 ± 0.02).

Viscosity and density values were experimentally determined at $25.00 \pm 0.02 ^\circ\text{C}$ and atmospheric pressure using an automated SVM 3000 Anton Paar® rotational Stabinger viscometer-densimeter (Anton Paar GmbH, Graz, Austria). The dynamic viscosities have a relative uncertainty of $\pm 0.35\%$ while the absolute uncertainty for the density is $5 \times 10^{-4} \text{ g cm}^{-3}$. The viscosimeter-densimeter was previously calibrated using standard solutions.

The water content of top and bottom phases was measured by volumetric Karl–Fischer titration at $25 ^\circ\text{C}$, using a Karl–Fischer 852 Titrand from Metrohm® ($\pm 0.5\%$), HYDRANAL-methanol rapid (reagent for accelerated volumetric one component Karl–Fischer titration) and HYDRANAL-Composite 5 (reagent for volumetric one-component Karl–Fischer titration methanol free), both reagents supplied by Sigma-Aldrich®, as titrants.

Quantification of asparaginase concentration

After the phase separation, the ASNase concentration (in mg mL^{-1}) in each phase was determined by size exclusion high performance liquid chromatography (SEC-HPLC) analysis, using a Chromaster HPLC system (VWR Hitachi®) equipped with a binary pump, column oven, temperature controlled autosampler coupled with an UV-visible diode array detector (DAD). SEC-HPLC was performed on an analytical column Shodex Protein KW-802.5 (8 mm ID \times 300 mm). Each phase was diluted 1:6 (v/v) in potassium

phosphate buffer 100 mmol L^{-1} (pH 7.4), and $25 \mu\text{L}$ of the diluted sample was then injected in the system. The mobile phase consists of 100 mmol L^{-1} of potassium phosphate buffer pH 7.0 with 0.3 mol L^{-1} of sodium chloride (NaCl) run isocratically at a flow rate of 0.5 mL min^{-1} . The column oven temperature was kept constant at $25 ^\circ\text{C}$ as well as the autosampler temperature. The detection wavelength was set at 280 nm. The quantification of ASNase was carried out by an external standard calibration method in the range of 0.001 to 1 mg mL^{-1} of protein.

For the samples obtained after the cell lysate, the total proteins were quantified by the Bradford method using the Bio-Rad Protein Assay Dye reagent for the protocol using 96-well microplates. Briefly, the reagent was diluted 1:4 in ultrapure water and then filtered. In each well were added $10 \mu\text{L}$ of the corresponding sample and $200 \mu\text{L}$ of the filtered reagent, immediately homogenized and kept at room temperature for 5 min. Absorbance was measured on a PerkinElmer® plate reader at 595 nm. The calibration curve was performed by varying the concentration of the commercial ASNase standard (ProSpec) from 0.05 to 0.5 mg mL^{-1} .

Quantification of ASNase enzymatic activity

The ASNase activity was measured by L-aspartic β -hydroxamate acid method, which was adapted from the protocol established by Drainas et al.³⁶ and previously validated by our group.³⁷ The absorbance was measured at 500 nm (Plate Reader, PerkinElmer® Enspire Multimode). A calibration curve was performed ranging from 0.01 to $3 \mu\text{mol}$ of ferric β -hydroxamate mL^{-1} , through multiple dilutions of L-aspartic β -hydroxamate stock solution and the addition of $\text{FeCl}_3/\text{TCA}/\text{HCl}$ solution. Furthermore, 1 U is defined as the amount of the enzyme that catalyzes the conversion of $1 \mu\text{mol}$ of L-asparagine per milliliter per minute of reaction at $37 ^\circ\text{C}$. The ASNase activity is defined by enzyme units per milliliter (U mL^{-1}), 1 U is defined as the amount of the enzyme that catalyzes the conversion of $1 \mu\text{mol}$ of L-asparagine per milliliter per minute of reaction at $37 ^\circ\text{C}$. The relative activity (%) represented by the ratio between the ASNase activity after purification and the initial ASNase activity (i.e. allowing to indicate possible variations of ASNase activity during the ABS partitioning) was determined through Eqn (5).

$$\text{Relative Activity (\%)} = \frac{A_{\text{sample}}}{A_{\text{initial}}} \times 100 \quad (5)$$

where, A_{sample} corresponds to the enzymatic activity at each sampling point, and A_{initial} corresponds to the initial enzymatic activity (time 0 h). All tests were performed in triplicate, being the results represented by the average of three independent experiments and respective standard deviation errors.

Maintenance of microorganism, culture media and conditions

Escherichia coli BL21 + ASNase1 was kindly provided by Dr Gisele Monteiro of the Departamento de Tecnologia Bioquímica-Farmacêutica, Faculdade de Ciências Farmacêuticas - Universidade de São Paulo (USP, SP - Brazil). The microorganism was kept in a Petri dish for a maximum of 15 days. For reactivation, a colony contained in the plate was incubated in 5 mL of Luria–Bertani (LB) culture medium (10 g tryptone, 5 g NaCl and 5 g yeast extract per liter) enriched with $50 \mu\text{g mL}^{-1}$ of carbenicillin in 15 mL flasks, and kept at constant agitation on an orbital shaker (12 h at $37 ^\circ\text{C}$, 200 rpm). After this period, the cultivation was discontinued and 10% (w/v) of sterile glycerol was added for

cryoprotection. Then, the fermented medium was aliquoted into 50 μL samples each and preserved at $-80\text{ }^{\circ}\text{C}$.

For preculture, transformed *E. coli* BL21 + ASNase was grown (12 h, $37\text{ }^{\circ}\text{C}$) in 100 mL of fresh sterile LB medium containing carbenicillin at the same concentrations used for microorganism up to an optical density (OD) at 600 nm = 0.7 ± 0.1 . ASNase (PDB:3ECA) expression was induced by the addition of 1 mmol L^{-1} isopropyl β -D-1-thiogalactopyranoside (IPTG) after 3 h growth at $37\text{ }^{\circ}\text{C}$. After 24 h of culture time, the cells were separated by centrifugation ($4000 \times g$ at $4\text{ }^{\circ}\text{C}$, for 20 min) and the cell pellet obtained was used for further cell disruption assays.

ASNase cell disruption and storage

The cell disruption was performed by an osmotic shock procedure, where the cells were exposed to a hyperosmotic solution and then to a hypoosmotic solution. Initially, for each 50 mL of fermented culture after centrifugation, the resulting cell pellet (2 g) was treated with 160 mL of hyperosmotic buffer [100 mmol L^{-1} Tris-HCl, pH 8.0, 500 mmol L^{-1} sucrose, 0.5 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA)], incubated on ice for 5 min and then centrifuged (20 min, $4500 \times g$ at $4\text{ }^{\circ}\text{C}$). After, 120 mL of hypotonic solution of 0.5 mmol L^{-1} magnesium chloride (MgCl_2) was added to the pellet formed, followed by incubation on ice for 5 min to break the outer membrane of the microorganism that was previously exposed to the hypotonic solution. After centrifugation (40 min, $18\,000 \times g$ and $4\text{ }^{\circ}\text{C}$), the resulting supernatant is the periplasmic fraction. To this fraction, it was added 6 mL of Tris-HCl (1 mol L^{-1}), to reach the final concentration of 50 mmol L^{-1} Tris-HCl pH 8.8, being thus the sample kept frozen at $-20\text{ }^{\circ}\text{C}$. Before use, the periplasmic fraction was submitted to a buffer exchange. Briefly, the samples were ultrafiltered ($8000 \times g$, 30 min, $25\text{ }^{\circ}\text{C}$) using the Vivaspin 20 (GE Healthcare Life Sciences, São Paulo, Brasil) ultrafiltration concentrator with 10 kDa MW cut-off membrane, and subsequently, the buffer was exchanged for 20 mmol L^{-1} of potassium phosphate buffer pH 7.4. Figure S1 (in Supporting Information) shows the illustrated scheme of the protocol applied to obtain ASNase from cell lysate. Concentrated samples were then used in subsequent ASNase purification assays using ABS.

ASNase recovery from cell lysate

For ASNase partition systems using *E. coli* cell lysate, the conditions and compositions of the ternary mixtures were the same as those used for commercial ASNase partition systems (Table S1 in Supporting Information). After complete partition, the samples were pre-filtered to remove all system forming components, such as polymers and salts, to avoid interference with quantitative protein and activity protocols. The analysis of the samples obtained from the cell lysate were not performed in HPLC due to the high protein load and possible residues of the components of the systems present in the samples, as they could damage the chromatographic column. In addition, the presence of phase components may interfere with quantification methods, as well as to change the ASNase activity; therefore, the phase forming components were removed from each sample and loaded with potassium phosphate buffer.

Thus, the phases of each system were ultrafiltered using the Vivaspin[®] 6 (Sartorius) ultrafiltration concentrator with 10 kDa MW cutoff membrane to remove the phase forming components (Fig. S1). The samples were then washed and loaded with 20 mmol L^{-1} of potassium phosphate buffer pH 7.4 as follows:

$150\text{ }\mu\text{L}$ of the phases of each system were diluted in 5 mL of 20 mmol L^{-1} potassium phosphate buffer pH 7.4 and centrifuged ($7000 \times g$ at $25\text{ }^{\circ}\text{C}$, 20 min). The recovered volume was washed (5 mL of 20 mmol L^{-1} phosphate buffer pH 7.4) and the final volume was measured and considered for concentration/dilution calculations. To ensure that ultrafiltration did not cause protein loss in the systems, the total proteins present in the permeate were quantified, and no protein traces were observed.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

After the separation process, the presence of contaminants in the samples was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was prepared according to pre-established protocol.³⁸ Briefly, the initial crude extract used for the extractions and each phase of each system were evaluated by this technique. For systems where the precipitate was observed at the interface, the suspended interface material was also analyzed. For sample preparation, 30 μL of each sample was added to 60 μL of dissociation buffer [62.5 mmol L^{-1} Tris-HCl buffer, pH 6.8, 10% (v/v) glycerol, 2% SDS % (v/v), 5% (v/v) 2-mercaptoethanol and 0.05% (v/v) bromophenol blue). The mixture was heated ($100\text{ }^{\circ}\text{C}$, 10 min) to complete protein denaturation. After cooling to room temperature, 20 μL of the samples were applied to the gel and run was set at 20 A. The gel had two different compositions, the upper phase with 3.7% (v/v) acrylamide/bis-acrylamide solution (29:1), 123 mmol L^{-1} Tris, 1.08% (v/v) SDS, 0.23% (v/v) tetramethylethylenediamine (TEMED) and 0.1% (w/v) of ammonium persulfate in ultrapure aqueous solution; and the lower phase also in an ultrapure aqueous solution with 12.5% (v/v) acrylamide/bis-acrylamide solution (1, 29), 0.37 mmol L^{-1} Tris, 1% (v/v) SDS, 0.01% (v/v) TEMED and 0.05% (w/v) ammonium persulfate.

RESULTS AND DISCUSSION

Control of ASNase partition by selecting the nature of polymer/salt ABS

The partition of a target protein using polymer/salt ABS is directly dependent on the physicochemical properties of the coexisting phases, allowing thus the manipulation of the ASNase migration between phases. To understand and obtain further insights of the partition mechanisms of ASNase in polymer/salt ABS, the protein *EE* values were determined at $25\text{ }^{\circ}\text{C}$ and the physicochemical properties of the phases characterized. Initially, ABS composed of five different PEG polymers (300, 600, 1000, 1500, and 2000 g mol^{-1}) and salts (buffers, inorganic and organic) were prepared, and used to infer on how the increase of the PEG MW affects the partition of ASNase at $25\text{ }^{\circ}\text{C}$. The partition parameters [*EE* (%) and $\log K$], relative enzyme activity, pH, conductivity (in mS cm^{-1}), viscosity (in mPa s), density (in g cm^{-3}) and water content (in wt%) values for each PEG/phosphate buffer ABS were determined, as detailed in Table 1. It is important to note that with exception of potassium citrate buffer-based ABS, all systems allowed the maintenance of the enzymatic activity (> 97%) at the end of the partition process (as shown by the relative activity values shown in Table 1).

To facilitate the understanding of the partition trends, the *EE* values of each system are compared in Fig. 1. The results of PEG/phosphate buffer-based ABS showed an inversion of the ASNase partition with the increase of the PEG MW, where using PEG-300 and PEG-600, the ASNase was preferentially partitioned into the top phase (PEG-rich phase) (Fig. 1(a)), while using

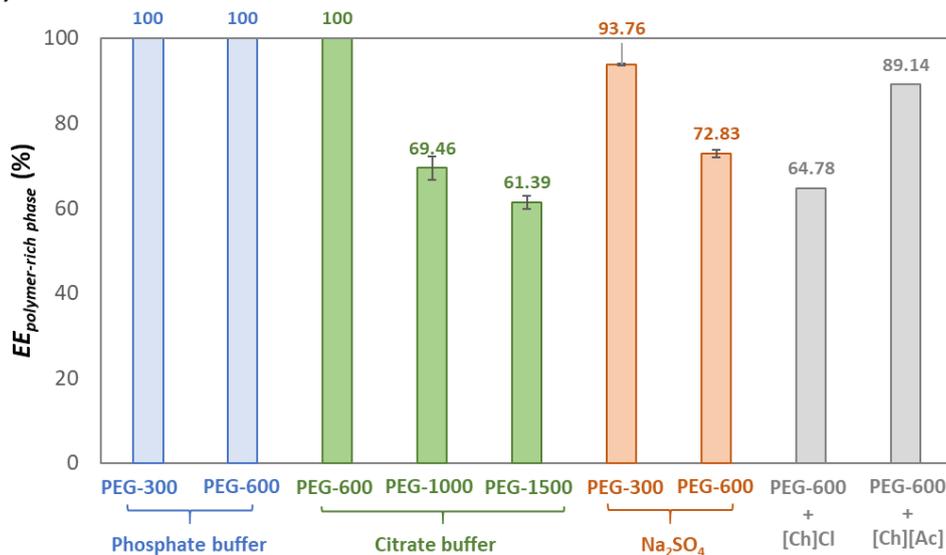
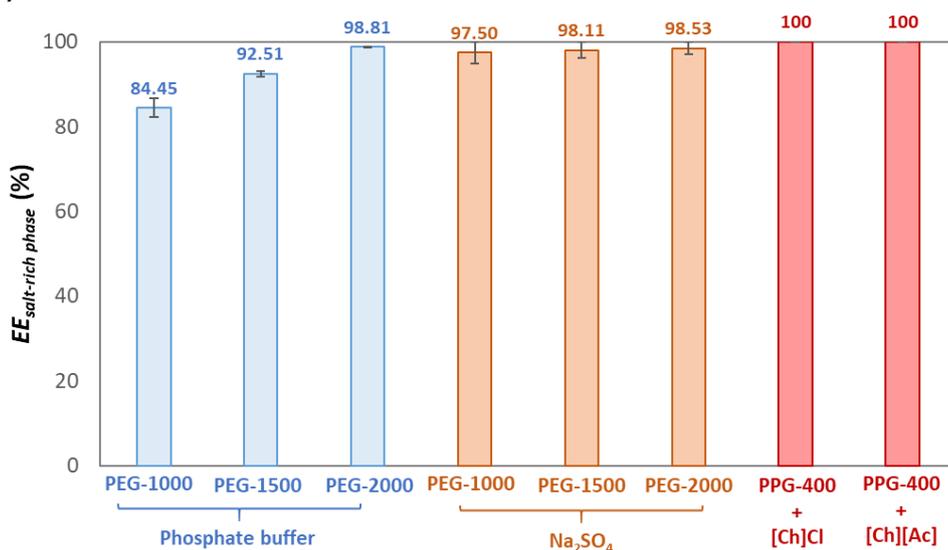
(a) POLYMER-RICH PHASE

(b) SALT-RICH PHASE


Figure 1. L-Asparaginase (ASNase) extraction efficiency (EE (%)) into the (a) polymer-rich or (b) salt-rich phases, at 25 °C and atmospheric pressure, using the following polymer/salt aqueous biphasic systems (ABS): PEG/phosphate buffer; PEG/citrate buffer; PEG/sodium sulfate; PEG/[Ch]⁺-salts; PPG/[Ch]⁺-salts.

PEG-1000, PEG-1500 and PEG-2000 the protein preferentially migrated to the bottom phase (salt-rich phase) (Fig. 1(b)). Interestingly, while both PEGs with low MW extract approximately 100% of the ASNase in a single extraction step, the ASNase EE (%) for the salt-rich phase increased with the PEG MW, i.e. PEG-1000 ($84 \pm 2\%$) < PEG-1500 ($92.5 \pm 0.7\%$) < PEG-2000 ($98.8 \pm 0.2\%$). The inversion of the ASNase partition in PEG/phosphate buffer ABS reveals that, through the proper selection of the PEG MW, a full control of the ASNase migration can be achieved, maintaining always high EE values (> 84%) independently of the protein being concentrated in the PEG or salt-rich phase.

To confirm if the inversion of the partition could be observed in other polymer/buffer-based ABS, the ASNase partition in four citrate buffer-based systems, using PEG-600, PEG-1000, PEG-1500 and PEG-2000 as phase forming agents, was carried out (results in Fig. 1 and Table 1). Note that ASNase extraction using

PEG-300/citrate buffer ABS was not determined, since it does not form an ABS. In addition, as result of the high entropy of hydration of the citrate anion (strong salting-out aptitude), a three-phase partition (TPP) system was observed for the systems composed of PEG with high MW (PEG-1000, PEG-1500 and PEG-2000). The corresponding amount of ASNase present in the precipitate $EE_{\text{precipitate}}$ (%) is shown in Fig. 2.

From the results of Fig. 1(a) we observe that, except for PEG-2000/citrate buffer ABS, the ASNase preferentially partitioned to the PEG-rich phase (> 61%). The ASNase full extraction was achieved with the ABS composed of PEG-600. However, by increasing the PEG MW (PEG-1000 and PEG-1500), the EE values decreased. Although both PEG/citrate and PEG/phosphate ABS exhibit similar partitions, in the citrate-based systems, part of the ASNase was precipitated in the interphase and thus, it was not contaminating the salt-rich phase, which is actually another way

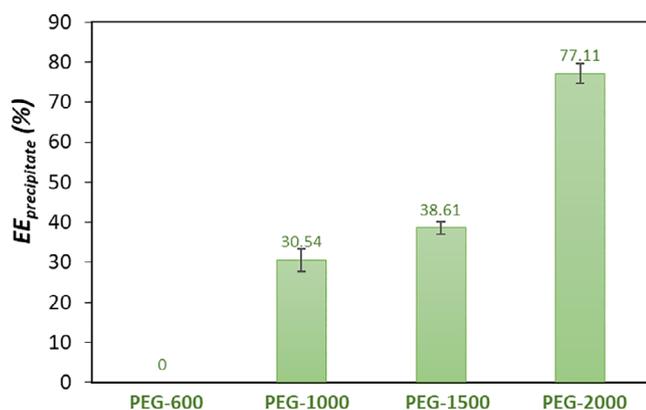


Figure 2. Percentage of L-asparaginase (ASNase) extracted in the precipitate (interface), named as extraction efficiency in precipitation ($EE_{\text{precipitate}}$ (%)) for the citrate-based aqueous biphasic systems (ABS) with PEG-1000, PEG-1500 and PEG-2000, at 25 °C and atmospheric pressure.

to do the separation of ASNase from the contaminant proteins. Indeed, as shown in Fig. 2, the enzyme precipitation increased with the PEG MW, being observed approximately 77% of ASNase precipitation with the PEG-2000/citrate buffer-based ABS. Considering that in all buffered-ABS (citrate and phosphate) the pH of the coexisting phases remained between 6.69 to 7.05 (Table 1), the ASNase precipitation was only resultant from the nature of the citrate ions and not from any pH change. Due to the higher basicity of the citrate ions (in comparison with phosphate, according to the Hofmeister series^{39,40}), these are strongly solvated, dehydrating more the PEG-rich phase (where the ASNase was preferentially concentrated). The higher dehydration of the PEG-rich (top) phases was confirmed by their lower water content (%) values (Table 1) in the citrate-based ABS (from 40 to 48%) when compared with the phosphate-based ABS (from 55 to 63%). Therefore, in PEG-1500/citrate buffer and PEG-2000/citrate buffer ABS, and contrarily to the corresponding phosphate buffer-based ABS, the partition of ASNase towards the salt-rich phase was limited by the strong salting-out effects of the citrate anions, which caused the enzyme precipitation at the interface, as previously observed for other proteins.^{21,35,41}

Although it was clear the limitations imposed by the salting-out mechanisms on the partition of ASNase to the salt-rich phase, the partition inversion and high EE values towards the salt-rich phase using phosphate buffer-based ABS and PEG-1000, PEG-1500 or PEG-2000 were still not fully understood. Then, to evaluate if the inversion of the ASNase partition also occurred in other polymer/salt ABS, the ASNase partition using ABS composed of different PEGs (PEG-300, PEG-600, PEG-1000, PEG-1500 and PEG-2000) and Na_2SO_4 was assessed. Similarly to what was shown in Fig. 1, also for systems composed of Na_2SO_4 -based ABS with PEG-300 and PEG-600, the ASNase was preferentially concentrated into the polymer-rich phase (EE of $93.8 \pm 0.2\%$ and $72.8 \pm 0.8\%$, respectively), while using PEGs with MW higher than 1000 g mol^{-1} , the protein almost fully partitioned in the salt-rich phase (EE (%) > 97%).

The preferential partition of the ASNase to the polymer-rich phase using PEG-300 and PEG-600-based ABS seems to be the result of the predominance of the salting-out effects. ASNase had a higher affinity to the low ionic strength (PEG-rich) phases, exhibiting conductivities at least one-order of magnitude lower than the coexisting salt-rich phases (Table 1), which is again in close agreement with previous works.^{42,43} However, using ABS composed of

PEG-1000, PEG-1500 and PEG-2000, the preferential separation of ASNase in the salt-rich phase seems to be a result of a more complex balance of interactions. The increase of PEG MW affects directly the solutes' partition and indirectly the phases' composition (changes in the binodal curves⁴⁴). In general, the effect of the polymer MW in the protein partition is commonly associated with at least one of the two following phenomena: (i) steric exclusion of the protein;^{45,46} (ii) increase of the relative hydrophobicity of the polymer rich-phase (reduction of hydrophilic groups/hydrophobic area ratio).^{19,27,47,48} The excluded volume effect implies that interactions between polymer and protein are limited to the absence of steric overlap, where small polymer molecules can occupy the entire volume fraction not filled with protein,⁴⁹ while the increase of relative hydrophobicity of the polymer-rich phase favors the partition of hydrophobic proteins into it (due to the hydrophobic interaction between the non-polar amino acid residues of the proteins and the ethylene groups of the PEG).⁵⁰ However, note that the most hydrophobic PEG can be excluded from hydrophilic domains of the proteins, decreasing its solubility in the PEG-rich phase.^{43,44} Herein, because of the hydrophilic character of ASNase,⁵¹ more than the entropic and steric effects, the complete extraction to the salt-rich phase seems to be a result of the hydrophobicity/hydrophilicity balance between the phases.⁵² According to the water content values detailed in Table 1, the ASNase was preferentially partitioned to the water-richer phases, i.e. more hydrophilic, following the tendencies already observed for other hydrophilic enzymes.⁵³

Subsequently, for a deeper understanding of the partition mechanisms, the systems composed of [Ch]Cl- and [Ch][Ac]-based ABS were tested. Among the different species available, this study was performed using [Ch]⁺-based salts due to their capacity to maintain the stability of proteins.²⁹ The hydrophobic/hydrophilic balance of this new set of ABS was adjusted by mixing aqueous solutions of both [Ch]⁺-salts with two polymers of low MW with different polarities, namely PEG-600 as the most hydrophilic and PPG-400 as the most hydrophobic (Table 1). Similar to the previous systems, all [Ch]⁺-salts-based systems exhibited pH values higher than the ASNase pI of 4.9,⁵⁴ guarantying its negative overall surface charge. From the physicochemical properties, contrarily to the common polymer/salt ABS (where both phases are rich in water), it was observed that the water content of PEG-600/[Ch]⁺-salts and PPG-400/[Ch]⁺-salts ABS was quite low, particularly in the polymer-rich phases (ranging from 13 to 17%, approximately), i.e. a more hydrophobic environment was formed. Moreover, due to the low hydration energies of the [Ch]⁺-salts, the coexisting phases of these two types of ABS exhibited low conductivity values ($\leq 60.63 \text{ mS cm}^{-1}$). As shown in Fig. 1, despite their different physicochemical characteristics, all [Ch]⁺-salts-based systems were effective for the extraction of ASNase (EE (%) > 65%). The results demonstrated that, by changing the relative hydrophobicity of the polymers, the systems induce the inversion of the ASNase partition. In this case, the enzyme has a preferential partition to the polymer-rich phase using ABS [Ch]Cl/PEG-600 and [Ch][Ac]/PEG-600 ABS and a full partition ($EE \approx 100\%$) to the [Ch]Cl or [Ch][Ac]-rich phase using PPG-400-based ABS.

Considering the moderate hydrophilicity of ASNase, the complete extraction for the most hydrophilic (salt-rich) phase using both more hydrophobic PPG-400-based ABS seems to be simply a result of protein water affinity. However, the ASNase partition in the PEG-600-based ABS seems to be a result of a more complex mechanism. In these systems, despite the ASNase hydrophilicity, the protein was preferentially concentrated in the phase

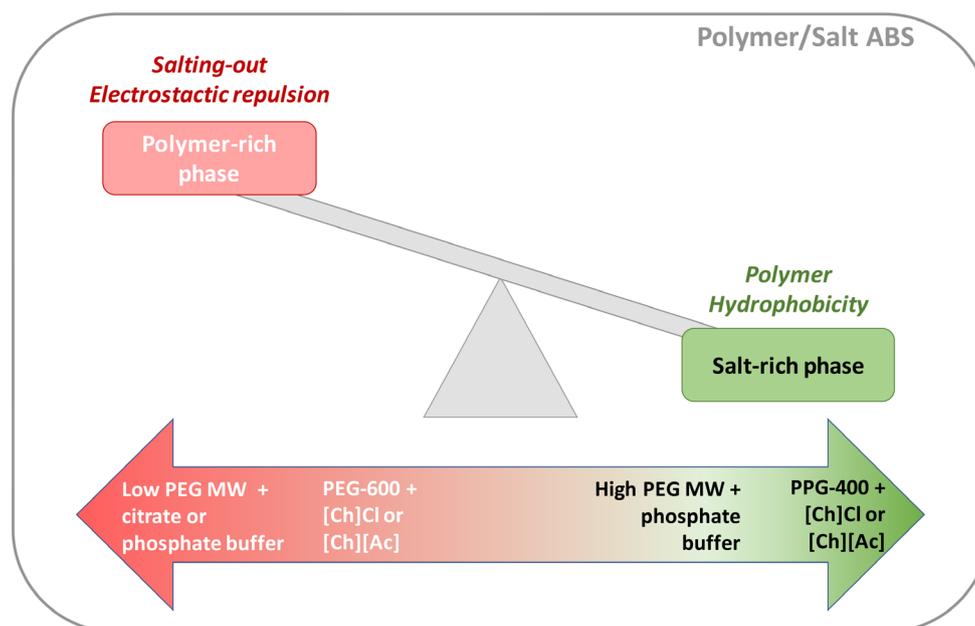


Figure 3. Driving-forces controlling the L-asparaginase (ASNase) partition using different polymer/salt-based aqueous biphasic systems (ABS).

Table 2. Experimental data obtained for the study of extraction of L-asparaginase (ASNase) from fermentation broth after cell lysis applying the aqueous biphasic systems (ABS) selected^a

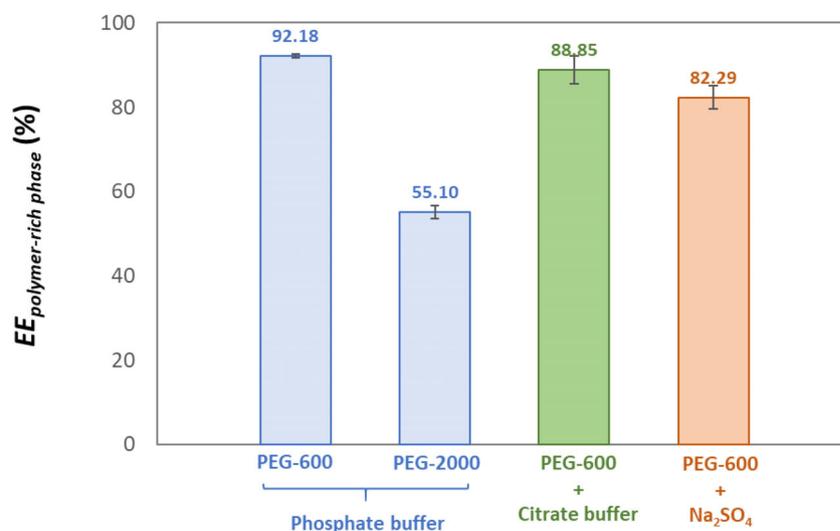
Ternary systems	Proteins (mg mL ⁻¹)		Mass balance (%)	ASNase activity (U mL ⁻¹)		Purification factor (PF) (AE _{final} /AE _{initial})	
	Top	Bottom		Top	Bottom	Top	Bottom
<i>PEG + phosphate buffer</i>							
PEG-600	2.8 ± 0.3	0.170 ± 0.001	110 ± 5	49 ± 5	0.14 ± 0.05	2.11 ± 0.09	0.1 ± 0.1
PEG-2000	1.5 ± 0.2	0.5 ± 0.1	71 ± 12	11 ± 4	10.6 ± 0.4	0.9 ± 0.3	2.4 ± 0.2
<i>PEG + sodium sulfate (Na₂SO₄)</i>							
PEG-600	1.7 ± 0.2	0.56 ± 0.08	106 ± 9	6.4 ± 0.2	2.4 ± 0.2	0.47 ± 0.07	1.7 ± 0.1
PEG-2000	0.53 ± 0.09	1.54 ± 0.3	95 ± 15	1.1 ± 0.2	4.0 ± 0.3	0.3 ± 0.1	1.1 ± 0.1
<i>PEG-600 + cholinium ([Ch]⁺)-salt</i>							
[Ch]Cl	2.1 ± 0.3	0.13 ± 0.03	113 ± 14	1.4 ± 0.2	0.00	0.080 ± 0.001	0.00
<i>PPG-400 + [Ch]⁺-salt</i>							
[Ch]Cl	0.14 ± 0.08	1.4 ± 0.3	122 ± 2	0.3 ± 0.3	2.82 ± 0.07	0.00	0.9 ± 0.2
[Ch][Ac]	0.09 ± 0.01	1.04 ± 0.02	81 ± 1	0.16 ± 0.03	11.45 ± 0.04	0.20 ± 0.02	1.24 ± 0.02
<i>PEG + citrate buffer</i>							
PEG-600	1.0 ± 0.2	0.13 ± 0.03	88 ± 14	5.8 ± 0.8	0.02 ± 0.02	0.8 ± 0.1	0.1 ± 0.2
PEG-2000	1.6 ± 0.1	0.8 ± 0.2	89 ± 9	15 ± 7	4.2 ± 0.2	1.1 ± 0.5	2.2 ± 0.4

^a Initial protein concentration in cell lysate was standardized at 1.07 ± 0.15 mg total protein and specific activity 5.69 ± 2.10 U mg⁻¹.

with low water content (polymer-rich phase), demonstrating that the hydrophilic/hydrophobic balance is not fully governing the ASNase partition. Further evidences can be obtained by comparing the anion effect of both PEG-600/[Ch]⁺-salts-based ABS and the corresponding *EE* (%) values: [Ch][Ac] 89 ± 2 > [Ch]Cl 65 ± 3. The highest *EE* (%) was obtained with ABS based on the more hydrophilic [Ac]⁻ anion (high Gibbs free energy of hydration). Thus, in addition to the hydrophobic/hydrophilic balance, a salting-out effect of [Ch]⁺-salts also influenced the ASNase partition, similarly to the PEG-600/buffer and PEG-600/Na₂SO₄ ABS. These results show that the protein partition is a complex balance of several cumulatively contributing factors. To sum up, a simple and representative illustration schematizing the different mechanisms acting in the ASNase partition is depicted in Fig. 3.

For the adequate use of the different ABS to promote the purification of ASNase, in addition to the high *EE* (%), it is also fundamental to guarantee the maintenance of the enzyme structure and activity. Thus, the enzyme catalytic activity was evaluated after the extraction stage. As shown in Table 1, after the partition experiments, the ASNase relative activity was around 100% for most of the ABS studied, demonstrating thus the high biocompatibility of these systems. The exceptions were the systems composed of citrate buffers that caused ASNase precipitation and PEG-600/[Ch]⁺-based salts, which led to a decrease in the ASNase biocatalytic activities (relative losses varying from 12 to 70%). The results obtained in this work are thus quite promising, i.e. high *EE* values, complete control of the partition and maintenance of the biocatalytic activity of the ASNase.

(a) POLYMER-RICH PHASE



(b) SALT-RICH PHASE

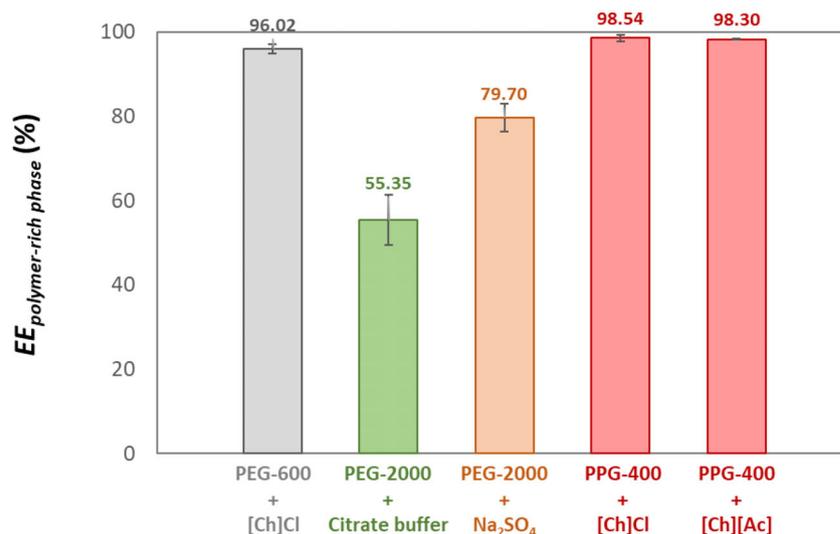


Figure 4. Extraction efficiency, EE (%) of total proteins obtained for each aqueous biphasic systems (ABS) studied on the purification of L-asparaginase (ASNase) from the *Escherichia coli* cell lysate in the polymer-rich phase (a) and salt-rich phase (b), at 25 °C, by applying PEG/phosphate buffer; PEG/citrate buffer; PEG/sodium sulfate; PEG-600/[Ch]⁺-salts; and PPG-400/[Ch]⁺-salts ABS. The error bars represent the standard deviation of three independent assays.

Purification of a recombinant ASNase from *E. coli* cell lysate

After the comprehension of the main mechanisms acting in the ASNase partition, some ABS were selected to further study the purification of a recombinant ASNase (PDB:3ECA) after production by bioprocess, and directly from the *E. coli* cell lysate. The ABS tested were the ones able to promote the complete ASNase partition towards the polymer-rich phase and others allowing the concentration of the enzyme on the salt-rich phase. The set includes systems consisting of PEG-600 and PEG-2000 + potassium phosphate buffer and citrate buffer and Na₂SO₄. Additionally, the ABS composed of PEG-600 + [Ch]Cl, PEG-600 + [Ch][Ac], PPG-400 + [Ch]Cl and PPG-400 + [Ch][Ac] were also selected. All selected systems were prepared at the same concentrations and conditions previously studied with the commercial ASNase, except the PEG-2000/citrate buffer system. In

this ABS, some adjustments in terms of compositions were done to guarantee the formation of the biphasic region after adding the cell lysate (Table 2). Thus, to prevent modifications of pH after the addition of the cell lysate, a buffer exchange step was performed for all samples using the same buffer used in this work to dilute the commercial ASNase (potassium phosphate buffer 20 mmol L⁻¹ pH 7.0). The main results of EE (%) of total proteins are shown in Fig. 4.

The results represented in Fig. 4 show that all systems present EE (%) > 50% highlighting the similar behavior of these ABS to extract the ASNase when the commercial sample is replaced by a more complex mixture, ASNase directly recovered from the after-lysis *E. coli* fermentation broth (although describing different values when compared). Contrarily to what occurred with the commercial enzyme, with the addition of ASNase from cell

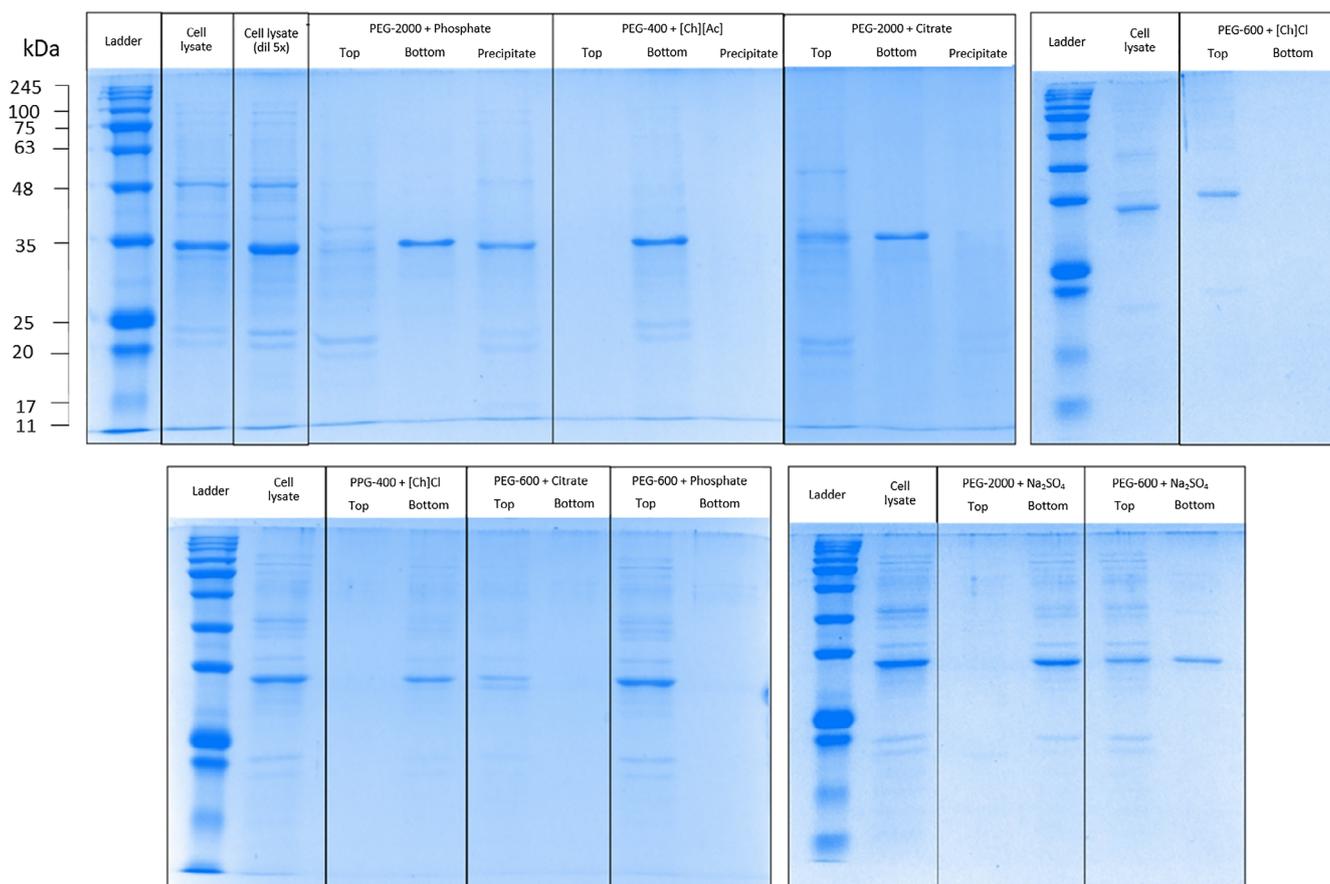


Figure 5. SDS-PAGE electrophoresis of after-lysis *Escherichia coli* fermentation broth and the extracts after the L-asparaginase (ASNase) purification using the different aqueous biphasic systems (ABS). The ASNase band is observed at approximately 34 kDa.

lysate, a precipitation occurred in the PEG-2000/phosphate buffer and PPG-400/[Ch][Ac] systems, probably instigated by the presence of contaminants with higher capacity to destabilize the ASNase. Moreover, and regarding the *EE* values determined, only for the case of PEG-2000/buffer systems (phosphate and citrate) a decrease of 20% in the *EE* (%) values for the non-commercial ASNase (60%) was observed when compared with the commercial sample (80%).

Although the *EE* (%) values for PEG-2000/phosphate buffer and PEG-600/ Na_2SO_4 ABS, were 55% and 80%, these systems were highly selective, i.e. the ASNase partitioned from the cell lysate to the salt-rich phase (bottom phase), while the contaminant proteins migrated to the opposite phase (polymer-rich phase) (as shown by the PF in Table 2). It is also observed that for the PEG-600/ Na_2SO_4 system the ASNase was present in both phases, although, predominantly concentrated in the bottom (polymer rich) phase, a result slightly different from the one obtained with the commercial enzyme. After a cell-disruption step, a diversified plethora and high content of contaminant periplasmatic proteins is present in the lysate, e.g. nucleosidases, β -lactamase, alkaline phosphatase, ribonuclease, endonuclease I, carboxypeptidase II, cytochrome c, hydrogenase and nitrite reductase.⁵⁵ Although the presence of these contaminants can change the migration tendencies of ASNase,^{17,25} these will correspond to less than 2.5 wt% of the total ABS, exhibiting a minor impact on the thermodynamic balances of the liquid–liquid systems. Moreover, there are also some differences on both enzyme samples used in this work, such as the MW,

volume and balance of polar and non-polar regions on the proteins surface (Table S2, in Supporting Information), which may also explain some changes in the results.

Interestingly, the ASNase partition was reversed for the system composed of PEG-600/[Ch]Cl, being the non-commercial ASNase partitioning to the polymer-rich phase contrarily to what happened with the commercial sample. Despite the inversion, the PF obtained was low and the ASNase specific activity decreased, demonstrating the poor performance of this system to purify the enzyme. For systems composed of PPG-400/[Ch]⁺-salts, the PF obtained with [Ch][Ac] were higher than those of [Ch]Cl, probably due to some parallel salting-out effects imposed by the presence of contaminants, since this behavior was not verified for the commercial ASNase.

It is noteworthy that the sequential use of different ABS using polymers, salts, or buffers (phosphate and citrate) can be combined to concentrate the enzyme in different phases, going beyond a simple low-resolution purification platform. Systems where ASNase selectively migrates to the polymer phase may subsequently be used to constitute a buffer-containing system, thus retrieving the sample from the polymer phase in a simplified manner. After isolation of ASNase, removal of the polymers and salts of each phase, and a new buffer exchange (phosphate buffer pH 7.4 20 mmol L⁻¹) step, the samples were monitored for the presence of contaminant proteins by SDS-PAGE (Fig. 5). The results obtained with SDS-PAGE are in agreement with the PF obtained for each system, presented in Table 2. By analyzing the electrophoresis data,

it was possible to confirm not only the preferential migration of ASNase for each phase, but also the decrease of the contaminant proteins in ABS, thus proving the accuracy of *EE* (%) and PF results.

For the ABS based in PEG-2000/phosphate systems, the SDS-PAGE results showed that, despite the absence of ASNase in the precipitate formed and concentrated in the interphase when using the commercial enzyme (Fig. 5), the same was not verified in the precipitate formed after addition of after-lysis fermentation broth. Nonetheless, the same was not observed for the PPG-400/[Ch][Ac], for which no protein was found in the precipitated material. For PEG-2000/Na₂SO₄, PEG-600/phosphate, PEG-600/[Ch]Cl, PEG-600/citrate and PPG-400/[Ch]Cl systems with PF ≤ 1, no increase in ASNase purification was observed, as demonstrated by the presence of contaminant bands represented in Fig. 5.

To demonstrate the viability of the ABS used, the enzyme activities of each phase were measured at the end of the process (Table 2). The results show that the ASNase activity was maintained. An increase of the specific activity was verified which may indicate a higher purity of ASNase after purification demonstrated by the PF and SDS-PAGE data obtained for the best systems: PEG-2000/phosphate buffer PF = 2.4 ± 0.2 > PEG-2000/citrate buffer PF = 2.2 ± 0.4 > PEG-600/Na₂SO₄ PF = 1.7 ± 0.1 > PPG-400/[Ch][Ac] PF = 1.24 ± 0.02.

In the end, behind the comprehension of the partition phenomena, it was possible to select the ABS based in PEG-2000/phosphate buffer as the most efficient system (PF = 2.4 ± 0.2) to purify this therapeutic enzyme directly from the after-lysis *E. coli* fermentation broth.

CONCLUSIONS

In this work, a simple picture of the main mechanisms controlling the ASNase partition in polymer/salt-based ABS was developed and comprehended. The strength of two distinct effects was evaluated, namely the hydrophobic/hydrophilic balance and the salting-out aptitude of the salt ions. Here, the polymer with high hydrophobicity favored the enzyme partition towards the salt-rich phase, while ions with high hydration free energies allowed the migration of the protein towards the polymer-rich phase. The set of results obtained in this work shows that the polymer/salt-based ABS can be effective alternatives for the recovery of therapeutic enzymes, since through the control of the proteins partition, these can be recovered in the most favorable environment. After understanding the main mechanisms behind the ASNase partition, the best ABS to promote the purification of ASNase from the fermentation broth after cell lysis was selected. The ABS composed of PEG-2000/phosphate buffer was chosen as the most efficient system, because, while maintaining the enzyme activity, it had the high PF value (PF = 2.4 ± 0.2). In the end, it was demonstrated that ABS represent a very useful tool for the development of improved downstream processes applicable to recover and purify therapeutic enzymes like ASNase. In this sense, we believe that the use of ABS may reduce the number of chromatography steps currently used in ASNase purification, also allowing (if needed) the integration of upstream and downstream processes in a continuous regime. Nevertheless, further studies to evaluate the recycling of the phase-forming components, ASNase polishing, and economic and sustainability evaluation are still recommended before a future industrial application.

ACKNOWLEDGEMENTS

This work was developed in the scope of the project FAPESP 2014/16424-7 and within the scope of the project CICECO-Aveiro Institute of Materials, Portuguese Foundation for Science and Technology (FCT) Ref. UID/CTM/50011/2019, financed by national funds through the FCT/MCTES. A. Magri acknowledges the financial support from Brazilian National Council of Technological and Scientific Development (CNPq) 163292/2015-9. The authors are grateful for the financial support of FCT for the doctoral grant of SFRH/BD/102915/2014 of João H. P. M. Santos and the contract under Investigator FCT 2015 contract number IF/00402/2015 of S.P.M. Ventura. This work was also supported by FAPESP/Brazil through the grants 2013/08617-7, 2014/19793-3, 2015/07749-2, 2018/25994-2 and 2018/15104-0. G. Monteiro and C. Rangel-Yagui received a Productivity Fellowship from the CNPq (309595/2016-9 and 301832/201-0)). A. Magri, M. V. Pimenta, G. Monteiro and J. F. B. Pereira also thank the support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) finance code 001.

Supporting Information

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

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