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Partitioning features of bovine trypsin and α-chymotrypsin in polyethyleneglycol-sodium citrate aqueous two-phase systems

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Abstract

The partitioning of bovine trypsin and α-chymotrypsin – proteases of similar physico-chemical properties – in different polyethyleneglycol/sodium citrate aqueous two-phase systems was investigated. The effect of different factors such as polyethyleneglycol molecular weight, pH, tie line length, temperature and the presence of an inorganic salt on the protein partition coefficient were analysed. Both a decrease in PEG molecular weight and an increase in pH led to a higher partition coefficient for both enzymes. Aqueous two-phase systems formed by PEG of molecular weight 3350 and citrate pH 5.2 showed the best separation capability which was enhanced in presence of sodium chloride 3%. The transfer of both proteins to the top phase was associated with negative enthalpic and entropic changes.

Keywords: Trypsin; α-chymotrypsin; Pancreatic proteases; Aqueous two-phase systems

1. Introduction

Bovine trypsin is an enzyme that is widely used for commercial purposes to digest or process other proteins, including some therapeutic proteins. Traditional purification methods isolate trypsin from bovine pancreas where it is synthesized and stored in the form of its inactive precursor, the trypsinogen. α-chymotrypsin, another well-known pancreatic serine protease, represents the principal trypsin contaminant since both proteases exhibit chemical similarities [1]. Excellent protocols for the separation of trypsin from chymotrypsin which employ affinity and ionic exchange chromatography [2] are available; however, it is desiderable to develop new methods that improve and may replace totally or partially any stages of the current purification procedures.

The liquid–liquid extraction principle applied to aqueous two-phase systems (ATPSs) offers a potentially attractive method for obtaining industrial enzymes, due to its ease of scale-up and low material cost [3,4]. To form the two phases, aqueous solutions of either two polymers or a polymer and an inorganic salt are required. For industrial purposes, polymer/potassium phosphate systems are the most commonly used but the high salt concentration required represents a waste disposal problem. Recently, we employed sodium citrate as a substitute for potassium phosphate since the citrate anion is biodegradable and non toxic [5].

The quantitative modelling of protein partitioning in two-phase aqueous polymer systems is an extremely complex problem since such behaviour depends on a broad array of factors, including protein size, conformation, and surface structure; and the interactions between different components as well. A composition for the ATPS has to be selected to quantitatively extract the target protein from one of the phases with minimal concentration of contaminant molecules. At present, no model has yet been developed which allows the *a priori* calculation of protein partitioning, therefore, the ATPS which provides the optimal separation can only be selected after experimental work [6,7].

In this study, we describe the partitioning features of bovine trypsin and α-chymotrypsin in polyethyleneglycol/citrate ATPSs in order to evaluate the applicability of partitioning as

Abbreviations: PEG600, PEG1000, PEG1450, PEG3350 and PEG8000, polyethyleneglycols of average molecular masses: 600, 1000, 1450, 3350 and 8000, respectively; TRP, bovine trypsin; ChTRP, α-chymotrypsin; ATPS, aqueous two-phase system

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a putative method to isolate TRP from pancreas and to obtain any information about their partitioning mechanism.

2. Materials and methods

2.1. Chemicals

Trypsin (TRP), α-chymotrypsin (ChTRP) from bovine pancreas, polyethyleneglycols of average molecular masses: 600; 1000; 1450; 3350 and 8000 (PEG600, PEG1000, PEG1450, PEG3350, PEG8000), α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-benzoyl-L-tyrosine ethyl ester (BTEE) and 1 anilino-8-naphthalene sulfonate (ANS) were purchased from Sigma Chem. Co. and used without further purification. All the other reagents were of analytical quality.

2.2. Fluorescence, absorbance and circular dichroism spectra of TRP and ChTRP in the presence of PEGs

Absorption spectra were performed on a Jasco V-550 spectrophotometer with a slit of 2 nm and a scan rate of 200 nm min^{-1}. Fluorescence measurements were performed on an Aminco Bowman S2 spectrofluorometer using a thermostatized cuvette of 1 cm of optical pathway. The proteins were excited at 280 nm and the emission was recorded from 300 to 400 nm. Circular dichroism spectra (CD) were performed in a Jasco J-810 spectropolarimeter, using a thermostatized cuvette of 1 mm of pathlength, the scan rate was of 50 nm min^{-1} and the bandwidth was of 1 nm. Repetitive scanning of three cycles was used.

2.3. Measurements of the protein relative surface hydrophobicity (So)

The relative surface hydrophobicity of the protein was determined by applying the optical method previously reported [8]. Aliquots (from 4 to 40 μL) of the protein solution (1.5 mM) were added to a sample containing 3 mL of ANS (final concentration 40 μM) in 50 mM sodium phosphate buffer, pH 5.5. The fluorescence emission intensity at 484 nm (while exciting at 365 nm) for each protein concentration was measured on an Aminco Bowman S2 spectrofluorometer at 20 °C. The initial slope of the fluorescence intensity versus protein concentration plot has been shown to be correlated to the relative surface hydrophobicity (So).

2.4. Enzyme assays

Trypsin activity was determined through a method modified from Gildberg and Overbo [9]. The substrate, BAPNA, was used in the assay at a final concentration of 0.85 mM in 100 mM buffer Tris–HCl pH 8.2. The reaction was followed by measuring the absorbance of the released reaction product, *p*-nitroanilide, which absorbs at 400 nm (molar absorptivity of 10,500 M^{-1} cm^{-1}) for 4 min.

The α-chymotrypsin assay is based on the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester (BTEE) [10]. The reaction velocity is determined by measuring the increase in absorbance at 256 nm resulting from the substrate hydrolysis over a period of 4 min. BTEE was used in the assay at a final concentration of 0.6 mM in 100 mM buffer Tris–HCl pH 8.2 containing 100 mM $CaCl_2$.

Both enzyme assays were performed at a constant temperature of 22 °C. The activities were calculated from the initial linear portion of the absorbance versus time curve.

2.5. Preparation of the aqueous biphasic system

To prepare the biphasic aqueous systems, stock solutions of the phase components: PEG of different molecular weight 40% (w/w) and sodium citrate 20% (w/w) of a given pH were mixed according to the binodal diagram previously obtained in our laboratory [4]. The desired pH (5.2 or 8.2) of the sodium citrate solution was adjusted by the addition of sodium hydroxide. Low-speed centrifugation to speed up phase separation was used after a thorough gentle mixing of the system components, then 0.5 mL of each phase was mixed to reconstitute several two-phase systems in which the protein partition was assayed. The total system compositions were selected according to the binodal diagrams obained in our laboratory and are shown in Table 1.

2.6. Determination of the partition coefficient (K_p)

Partitioning behaviour of TRP and ChTRP was analysed by dissolving a given amount of protein (1.5–2.1 μM total system concentration) in the two-phase systems containing 0.5 mL of each equilibrated phase. Small aliquots of the protein stock solution (1500 μM) were added to the systems (10–14 μL) in order to make the change of the total volume of each phase negligible. After mixing by inversion for 1 min and leaving it to settle for at least 120 min, the system was centrifuged at low speed for the two-phase separation. Samples were withdrawn from separated phases and after the appropriate dilution (with the equilibrated phase free from protein) the protein activity in each phase was determined. The partition coefficient was calculated according to:

$$K_{\mathrm{p}} = \frac{[\mathrm{Act}]_T}{[\mathrm{Act}]_B} F \quad (1)$$

Table 1
Composition of aqueous two-phase systems formed by PEGs of different molecular weight and sodium citrate

System[a]	PEG (% w/w)	Citrate (% w/w)
PEG600-5.2	19.91	15.50
PEG1000-5.2	17.05	13.25
PEG1450-5.2	16.30	12.26
PEG 3350-5.2	13.24	10.61
PEG8000-5.2	13.32	9.22
PEG600-8.2	19.47	11.40
PEG1000-8.2	19.31	10.29
PEG1450-8.2	18.42	9.67
PEG 3350-8.2	14.26	7.77
PEG8000-8.2	12.65	7.34

[a] Each system is named with the PEG molecular weight followed by the mean pH value.

where $[Act]_T$ and $[Act]_B$ are the enzyme activities of the partitioned protein in the PEG and citrate-rich phases, respectively. The effect of phase composition on the enzyme activity was considered. A correction factor (F) was calculated as the ratio between the activities of the reference solutions (known concentration) of the enzyme in each phase. Temperature was maintained constant and controlled to within ±0.1 °C. All the measurements were developed by triplicate.

2.7. Determination of thermodynamic functions associated with the protein partitioning

The TRP and ChTRP partition coefficients (K_{p_1}, K_{p_2}) were determined at two different temperatures ($T_1 = 20$ °C and $T_2 = 37$ °C) and by applying the van't Hoff equation:

$$\ln \frac{K_{p_2}}{K_{p_1}} = \frac{\Delta H^\circ}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \quad (2)$$

the enthalpic change (ΔH°) associated with the protein partitioning was calculated. The free energy change (ΔG°) was determined from the $\Delta G^\circ = -RT \ln Kr$ and the entropic change (ΔS°) from:

$$\Delta S^\circ = \frac{(\Delta H^\circ - \Delta G^\circ)}{T} \quad (3)$$

3. Results and discussion

3.1. Influence of PEG presence on TRP and ChTRP spectroscopical behaviour

3.1.1. Absorption and fluorescence spectra

The presence of PEGs of different MW (600 to 8000) at 5 and 10% w/w did not produce any modification in both protein absorption spectra (data not shown). Besides, the native TRP and ChTRP fluorescence spectra at 340 nm (when excited at 280 nm) were analysed in the presence of PEG. Neither significant shifts nor height changes of the emission peak position were observed, which suggests the absence of a conformational change in the environment of the tryptophan residues on the proteases. The effect of PEG presence for a long period of time (3 h) on the spectra was also studied. No appreciable modification either on the absorption or in the fluorescence emission was observed.

3.1.2. Circular dichroism spectra

Circular dichroism spectra of proteins are sensitive to protein structure. Both TRP and ChTRP can be classified as β_{II} proteins. They have predominantly β-sheet but their CD spectra resemble those of unfolded proteins. Fig. 1 shows the far UV CD spectrum of ChTRP (in absence of PEG) with its characteristic features: a local minimum at 230 nm and a global minimum at 202 nm [11]. Presence of PEGs (5% w/w) of 600, 1000 and 1450 molecular weights practically did not affect the ChTRP spectrum while PEG3350 and PEG8000 induced both a blue shift of the global peak and less negative ellipticities. The presence of PEGs of different molecular weight also caused a moderate decrease in the negative elipticities of TRP around 197 nm (data not shown). No

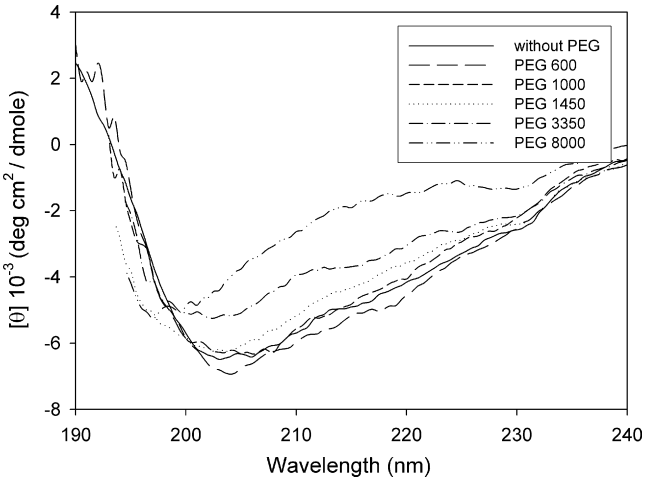

Fig. 1. Effect of PEG presence (5% w/w) on the far CD spectrum of ChTRP. Temperature 20 °C.

effect was observed in the near UV region (270–300 nm) of the TRP and ChTRP CD spectra (data not shown), which suggests that PEG does not induce microarragements at the tryptophan residues accessible to the solvent. This finding agrees with the negligible effect of PEG presence on the fluorescence emission spectrum.

3.2. Partition behaviour of TRP and ChTRP in PEG/sodium citrate ATPSs

The effect of PEG molecular weight and medium pH on the TRP and ChTRP partition coefficients (K_ps) was analysed.

Fig. 2 shows the effect of the molecular weight of PEGs on the partition of TRP and ChTRP. The partition equilibrium was displaced to the bottom phase when the PEG molecular weight increased, which has been shown to be a general rule for many proteins and ATPSs [12]. Highest changes in the K_p values were observed when the PEG molecular weight varied from 600 to 1000. This behaviour may be qualitatively explained on the basis of Flory Huggins' theory for polymers in solution, which

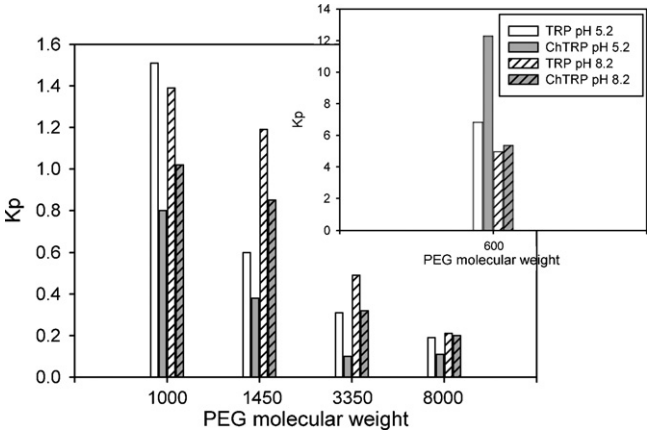

Fig. 2. Effect of PEG molecular weight (from 1000 to 8000) on the K_p value for TRP and ChTRP at pHs 5.2 and 8.2 Inserted figure: K_p values for PEG600/citrate ATPS. Temperature 20 °C. Total system compositions are those of Table 1.

predicts a linear relationship between the ln K_p and the reciprocal of the flexible chain polymer molecular weight.

The pH effect on protein partitioning was analysed. No significant changes of $K_{p_{TRP}}$ and $K_{p_{ChTRP}}$ were observed when pH raised from 5.2 to 6.9 (data not shown). However, when pH increased up to 8.2, both proteases exhibited an important change in their partitioning behaviour. Fig. 2 shows that the pH increase from 5.2 to 8.2 produces an increase of the K_p value for ATPSs formed by PEGs of higher molecular weights. This behaviour could be satisfactorily explained on the basis of the Albertsson equation, which takes into account an electrostatical and a non-electrostatical term [5]:

$$\ln K_p = \ln K_{p^\circ} - \frac{Z_p F \Delta\Psi}{RT} \quad (4)$$

where Z_p is the net protein charge, $\Delta\Psi$ is the interfacial potential and K_{p° is the non-electrostatic term which depends on the protein conformation and the flexible chain polymer characteristics. In PEG/citrate ATPSs, the $\Delta\Psi$ assumes positive values (since the bottom phase is enriched in the citrate anion); therefore, the electrostatic term will assume the opposite sign to the net protein charge. At pH.5.2 and 8.2 media, TRP and ChTRP are positively charged since their isoelectrical points are 9.1 and 10.5, respectively. When pH raises from 5.2 to 8.2, Z_p decreases, thus increasing both protein K_p values. However, an anomalous behaviour – which cannot be only explained on the basis of the Albertsson electrostatic contribution – is observed for ATPSs formed by PEGs of low molecular weights.

Protein partitioning behaviour showed to be sensitive to the surface hydrophobicity [13,14]. A high hydrophobical character of a biomolecule is a factor that favours the partition equilibrium displacement to the PEG-riched phase [15]. The relative surface hydrophobicity (So) measured for ChTRP was nearly ten fold higher than that of TRP (221 and 26 μM^{-1}, respectively). According to this, $K_{p_{ChTRP}}$ values would be expected to be higher than the $K_{p_{TRP}}$ ones, since the other protein physico-chemical properties such as molecular weight and p*I*s are quite similar. However, partition equilibrium for TRP showed to be more displaced to the top phase than ChTRP for most assayed ATPSs, except for PEG600/citrate ATPS.

3.3. Thermodynamics of the TRP and ChTRP partitioning

Fig. 3 shows the ΔH° and ΔS° associated with the TRP and ChTRP partitioning in systems containing PEGs of different molecular weight. The transfer of both proteins from Citrate to the PEG-rich phase is an exothermic process, except for ChTRP in the system with PEG3350, where an endothermic process was observed. Enthalpic change magnitude decreased when the Mw of PEG was increased. Besides, the TRP and ChTRP transfer has negative entropic changes for most assayed systems. Similar behaviour was observed at pH 8.2. For systems formed by PEG of low molecular weight, the free energy changes adopt negative values (the enthalpic changes overcomes entropic changes) while the opposite behaviour ($\Delta G_{transfer} > 0$) occurs for ATPSs formed by PEGs of higher molecular weights. Within the infinite dilution approximation, the solute partition coefficient (without

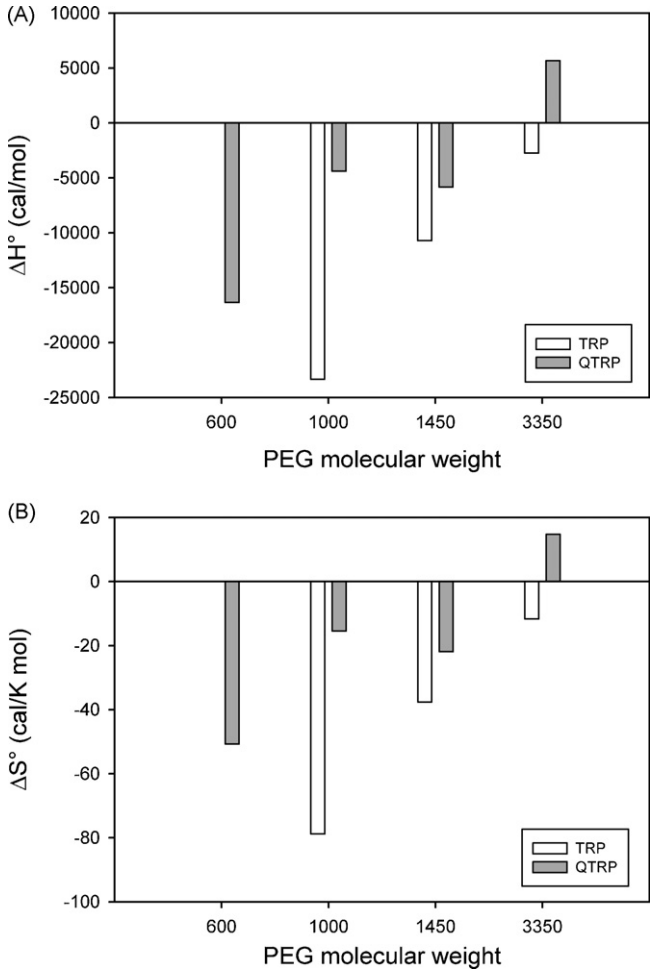

Fig. 3. Enthalpic (A) and entropic (B) changes associated with the transfer of TRP and ChTRP from the citrate-enriched phase to the PEG enriched phase in ATPSs of pH 5.2.

electrostatic component) is given by the sum of two enthalpic and one entropic terms [16]:

$$\ln K_{p^\circ} = \frac{M}{RT}(w_{bs} - w_{ts}) + \frac{M}{RT}(E_t - E_b) + \frac{M}{\rho}\left(\frac{n^t}{V^t} - \frac{n^b}{V^b}\right) \quad (5)$$

where the first is an enthalpic term which describes the direct interaction between the protein (solute) and an average lattice site of the top or bottom phase (w_{ts}, w_{bs}). The second term quantifies the enthalpy required to form the top or bottom phase from the pure components (E_t, E_b). The third one is the entropic term which takes into account the number density (n^t/V^t, n^b/V^b) which is defined as the number of molecules per volume unit in each phase. M and ρ are the protein molecular mass and the number of lattice sites per unit volume, respectively. For partition of low molecular weight proteins (such as TRP and ChTRP) in PEG-salt systems, the salt-rich phase has the higher number density (n^b/V^b) while the polymer-rich phase has the higher self energy (E^t) due to the strong attraction of the salt to water. When systems are formed by PEG of low molecular weight, this enthalpic effect sufficiently compensates the entropic term

Table 2
Effect of tie line length on the TRP and ChTRP partitioning in different ATPSs

$\partial \ln K_p/\partial \Delta w_{PEG}$ (1/%)										
PEG	600		1000		1450		3350		8000	
pH	5.2	8.2	5.2	8.2	5.2	8.2	5.2	8.2	5.2	8.2
TRP	0.04	0.12	0.04	0.03	0.07	0.01	−0.01	−0.04	−0.18	−0.09
ChTRP	0.06	0.11	0.03	0.03	0.02	−0.02	−0.05	−0.05	0.03	−0.03

to make the direct interaction (w_{ts}, $-w_{bs}$) term significant. In this case, if the protein has a strong attraction for a component which is enriched at the top phase, then w_{ts} will be more negative (or less positive), which leads to a more positive (or less negative) value of $\ln K_p$, thus inducing an increase in the tendency for the protein to partition to the top phase. In this way, the high K_p values observed for TRP or ChTRP (>1) in PEG600/citrate ATPSs might be explained by assuming a strong interaction between the positively-charged amino acid residues (pH < p*I*) or the exposed tryptophans in both proteins [17] and the ether groups in the PEG molecule. A similar mechanism was postulated for PEG600 and other small proteins such as lysozyme [18].

According to Diamond and Hsu [19] the slope of the plot $\ln K_p$ versus Δw_{PEG} (difference in the concentrations of PEG between the coexisting phases) is a function of the resulting interaction parameters between phase components such as protein–water, polymer–water and protein–polymer interactions. Positive slopes indicate a good PEG-protein interaction while negative values are associated with an unfavourable interaction. Table 2 shows the $\partial \ln K_p/\partial \Delta w_{PEG}$ values obtained by assaying the protein partitioning at four different tie lines for each PEG and pH. The slope values decrease as the PEG molecular weight increases and become negative for PEGs of higher molecular weight which agrees with the decrease in the protein transfer to the top phase. For most of the assayed sytems, this parameter adopts the highest values for TRP whose partition equilibrium is more displaced to the top phase than that of the ChTRP. However, for PEG600/citrate ATPSs, the $\partial \ln K_p/\partial \Delta w_{PEG}$ for ChTRP is higher than the value for TRP at pH 5.2 (in agreement with their So values) and they adopt similar values at pH 8.2 in good correlation with the observed K_p.

On the other hand, when PEG molecular weight increases, the number density of the top phase (n^t/V^t) decreases, making the last entropic term the dominant contributor (<0) and displacing the partitioning equilibrium to the bottom phase. Partition equilibrium for ChTRP showed to be more displaced to the bottom phase than TRP for most assayed systems. These findings agree with the lower $\partial \ln K_p/\partial \Delta w_{PEG}$ values observed for this protein and with the ChTRP self-association reported by Aune and Timasheff [20] in either the pH ranges 2.8–5.5 or at pHs above 8. The highest average molecular weight of ChTRP would be responsible for both a decrease in the mixing entropy change (entropic term) and a displacement of its partition equilibrium to the bottom phase.

The presence of different salts may affect the K_p value by modifying the electrostatic term of Albertsson equation or by affecting the water structure and hydrophobic interactions differently. The effect of NaCl 3% w/w on the K_p for PEG3350/citrate ATPSs pH 5.2 (which showed the best separation capability) can be visualized from Table 3. For both proteins, the presence of salt induced a displacement of the partitioning equilibrium to the top phase. This agrees with a salting out effect in the bottom phase that displaces the equilibrium to the PEG-riched phase [21]. This displacement is less marked for ChTRP since the high ionic strength due to the salt presence also induces a displacement of the aggregation equilibrium to the dimer state [20]. As a consequence, the ratio $K_{P_{TRP}}/K_{P_{ChTRP}}$, as a measure of the separation capability of this system enhances from 3.6 to 5.7.

Table 3
Effect of NaCl presence on the TRP and ChTRP partitioning behaviour

[NaCl] (% wt/wt)	K_p[a]		$K_{P_{TRP}}/K_{P_{ChTRP}}$
	TRP	ChTRP	
0	0.24	0.067	3.6
3	3.36	0.59	5.7

[a] ATPSs are formed by PEG3350 and sodium citrate, pH 5.2. Temperature 22 °C.

4. Conclusions

TRP and ChTRP partitioning behaviour in PEG/citrate ATPSs showed to be sensitive to polymer molecular weight, medium pH, tie line length and temperature. In spite of their similar physicochemical properties such as molecular weight and isoelectrical point, both proteins showed different partitioning behaviour. ChTRP is more partitioned to the bottom phase (citrate-riched) than TRP for most assayed sytems, except for ATPSs formed by PEG of molecular weight 600 in which ChTRP partitioning equilibrium was more displaced to the top phase. The presence of NaCl 3% enhances the separation capability of PEG3350/citrate ATPSs, being thus a possible strategy to adopt for the separation of both proteins. Although further work needs to be done to choose the most adequate ATPS, these findings suggest that PEG/citrate ATPSs could be employed as a viable and potentially useful first step procedure for the separation of TRP and ChTRP.

On the other hand, a thermodynamic approach suggests that for ATPSs formed by PEG of low molecular weight, the protein transfer to the top phase is enthalpically driven mainly due to a strong interaction between PEG and the protein, which agrees with the observed positive values of $\partial \ln K_p/\partial \Delta w_{PEG}$. On the other hand, PEGs of highest molecular weight exclude protein from the top phase driven by an entropically unfavourable term.

This exclusion effect is more pronounced for ChTRP due to its self-aggregation which mainly occurs in the pH ranges 2.8–5.5 and above 8.

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