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Porcine pancreatic lipase partition in potassium phosphate–polyethylene glycol aqueous two-phase systems

Georgina Bassani, Beatriz Farruggia, Bibiana Nerli, Diana Romanini, Guillermo Picó *

Bioseparation Lab, Physical-Chemistry Department, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, FonCyT, CIUNR and CONICET, Suipacha 570, S2002RLK Rosario, Argentina

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Abstract

This research study examined porcine pancreatic lipase partition in aqueous two-phase systems formed by polyethylene glycol-potassium phosphate at pH 6.0, 7.0 and 8.0, the effect of polymer molecular mass, and NaCl concentration. The enzyme was preferentially partitioned into the polyethylene glycol rich phase in systems with molecular mass 4000–8000, while with polyethylene glycol of 10,000 molecular mass it was concentrated in the phosphate rich phase. The enthalpic and entropic changes found due to the protein partition were negative for all the polyethylene glycol molecular mass systems assessed. Both thermodynamic functions were shown to be associated by an entropic–enthalpic compensation effect suggesting that the water structure ordered in the ethylene chain of polyethylene glycol plays a role in the protein partition. The addition of NaCl increased the lipase affinity to the top phase and this effect was most significant in the system polyethylene glycol 2000–NaCl 3%. This system yielded an enzyme recovery more than 90% with a purification factor of approximately 3.4.

Keywords: Lipase; Aqueous two-phase systems; Partition; Polyethyleneglycol

1. Introduction

Triacylglycerol hydrolases (EC 3.1.1.3), also known as lipases, catalyze both the hydrolysis of esters in largely aqueous solution and their synthesis under conditions of low water activity. During the last decade, lipases have become of great interest to the chemical and pharmaceutical industries with particular interest in the biodiesel area. This is due to their usefulness in both hydrolytic and synthetic reactions. The enzyme purification process presents a significant problem due to the complexity of the protein mixtures and the necessity to retain their biological activity. It has been shown that the 50–90% of the total production cost for a biological product is determined by the degree of purification of the produce. Partitioning in aqueous two-phase systems (ATPS) is a good method to separate and purify mixtures of proteins [1]. ATPS are formed by mixing two flexible chain polymers in water or one polymer and a salt (phosphate, citrate, etc.). This approach provides advantages of biocompatibility, easy processing and high sensitivity in the recognition of ligand–protein interactions [2]. The partitioning of proteins in the standard biphasic aqueous system depends on the concentration and molecular weight of the polymer(s), the concentration and type of the salt, and pH. The protein partition coefficient is defined as the ratio between the protein concentration in the top and bottom phases, respectively; when the enzyme target is in a complex mixture with other proteins, its partition coefficient must be determined only reference to its biological activity in the top and bottom phases. However, there is not enough difference in the partition coefficient of the target protein from other proteins in the crude broth to allow for an efficient separation in a single step.

The protein partition coefficient can be modified by changing the medium experimental conditions such as: pH, salt concentration polymer molecular mass, etc. [3].

Abbreviations: PPL, porcine pancreatic lipase; PEG 2000, polyethylene glycol of average molecular mass 2000; PEG 4000, polyethylene glycol of average molecular mass 4000; PEG 8000, polyethylene glycol of average molecular mass 8000; PEG 10,000, polyethylene glycol of average molecular mass 10,000; ATPS, aqueous two-phase systems.

* Corresponding author at: Facultad de Cs. Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 570, S2002RLK Rosario, Argentina. Fax: +54 341 480 4598.
E-mail address: pico@ifise.gov.ar (G. Picó).

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ATPS have been used as a first purification step since such systems allow removal of contaminants by a simple and economic process. ATPS have a number of advantages with respect to conventional methods for the isolation and purification of proteins: the partition equilibrium is rapidly reached, it can be easily scaled up, and it has the possibility of steady state operation with low cost of the materials which can be recycled. Following a research line to obtain enzymes from the wastes of freezing industry we have applied the liquid–liquid extraction to obtain pancreatic lipase from porcine pancreas. The biotechnological significance of this enzyme is due to the fact that it is used in one of the process steps to obtain biofuels.

In work we determined the partitioning behaviour of porcine pancreatic lipase in aqueous two-phase systems of polyethyleneglycol (PEG)–potassium phosphate and we analysed the effect of medium variables on the partitioning process with the goal of understanding the molecular mechanism of this enzyme partition process.

2. Materials and methods

2.1. Chemicals

Porcine pancreatic lipase (PPL) with a specific activity of 12 units/protein mg, PEG of average molecular weight 2000, 8000 and 10,000 (PEG 2000, 8000 and 10,000), and *p*-nitrophenyl butyrate were purchased from Sigma Chem. Co. (USA), while polyethyleneglycol of average molecular weight 4000 (PEG 4000) was from Riedel-de Haën (Germany). All chemicals were used without further purification.

2.2. Lipase activity assay

This assay was performed by measuring the increase in the absorbance at 400 nm produced by the *p*-nitrophenol released in the hydrolysis of 5.6 mM of *p*-nitrophenyl butyrate (*p*NPB) at pH 7.0 and 25 °C. To initiate the reaction, 50–500 μL of lipase solution was added to 3.0 mL of substrate solution [4].

2.3. Binodial curves used

The binodial diagram used for the PEG 4000, 8000 and 10,000–potassium phosphate systems at pH 7.0 were as described in Ref. [1]. The binodial phase diagram used for the PEG 2000 potassium phosphate systems at pH 7.0 were obtained from the method of cloud point as described in detail by Tubío et al. [5].

2.4. Preparation of the aqueous biphasic system

To prepare the biphasic aqueous systems, the corresponding amount of PEG and 40% (w/w) potassium phosphate stock solution were mixed according to the binodial partition diagrams previously published in Refs. [2] and [7]. Low-speed centrifugation was used after gentle mixing of the system components to speed up phase separation; then 1.0 mL of each phase were mixed and stored at 8 °C to build up several two-phase systems in which the protein partition was assessed.

2.5. Determination of the partition coefficient (K)

The partition coefficient of the PPL between both phases was determined by dissolving increasing amounts of PPL solution (10 mg/mL) (10–50 μL) in the two phases of the pre-formed system containing 1.0 mL of each equilibrated phase, the change of the total volume of each phase being negligible. After mixing gently by inversion for 1 h and leaving it to settle for at least 30 min, the system was centrifuged at low speed for the two phase separation. Samples were withdrawn from separated phases and the PPL activity in each phase was determined by the *p*NPB reaction. The partition coefficient was defined as

$$K = \frac{[\mathrm{Act}]_{\mathrm{top}}}{[\mathrm{Act}]_{\mathrm{bottom}}} \quad (1)$$

where $[\mathrm{Act}]_{\mathrm{top}}$ and $[\mathrm{Act}]_{\mathrm{bottom}}$ are equilibrium concentrations of partitioned protein in the PEG and phosphate rich phases, respectively. In the protein concentration range assayed, a plot of $[\mathrm{Act}]_{\mathrm{top}}$ vs. $[\mathrm{Act}]_{\mathrm{bottom}}$ showed a linear behaviour, K being its slope. In order to evaluate the purification process the following parameter were calculated:

- the enzymatic yield recovery in the top phase $y(\%)_{\mathrm{top}}$ was also calculated according to the given equation:

$$y(\%)_{\mathrm{top}} = \frac{100}{(R/K_{\mathrm{PPL}})} \quad (2)$$

where $R = V_{\mathrm{T}}/V_{\mathrm{B}}$, V_{B} and V_{T} are the bottom and top phase volume, and K the lipase partition coefficient [3]. The total protein concentration in each phase was determined measuring the absorbance at 230 nm, using bovine serum albumin as reference.

The purification factor (PF) was calculated as

$$\mathrm{PF} = \frac{[\mathrm{Act_{TOP}}]}{[\mathrm{Act_{HOMOGENATE}}]}$$

where $\mathrm{Act_{TOP}}$ and $\mathrm{Act_{HOMOGENATE}}$ are the specific lipase activities in the top phase and in the original mixture.

2.6. Thermodynamic functions determination

The enthalpic change (ΔH°) associated to the protein partition in the ATPS was calculated determining the partition coefficient (K_1 and K_2) at two different temperatures (T_1 and T_2), applying the known equation:

$$\ln\frac{K1}{K2} = \frac{\Delta H^{\circ}}{R}\left[\frac{1}{T2} - \frac{1}{T1}\right] \quad (3)$$

and the free energy change was calculated through the following equation:

$$\Delta G^{\circ} = -RT\ln K \quad (4)$$

where ΔG° is the free energy change. The entropic change (ΔS°) was calculated from the equation:

$$\Delta S^\circ = -\frac{\Delta G^\circ - \Delta H^\circ}{T} \quad (5)$$

2.7. Experimental designs and statistical analysis

The influence of the variables molecular mass of PEG and NaCl concentration on the protein recovery (%) in the top phase was evaluated according to a 2^2 factorial design with four repetitions at the central point as reported previously [6]. Mathematica (Version 5.0) and Sigma Plot (Janel Sc. v. 9 software were used for regression and graphical analyses of the data obtained.

3. Results and discussion

3.1. Top/bottom phase composition effect on the PPL activity

The different composition between the top and bottom phase influenced the enzyme activity, therefore, it was necessary to carry out controls of the PPL activity in the phosphate and in the PEG-rich phases. Table 1 shows the PPL activity in the phosphate rich phase and in the top phases (PEG-rich phases) formed by PEG of different molecular mass.

The relative PPL activity was calculated taking as 100% the activity in a buffer medium of potassium phosphate 50 mM, pH 7.0. It can be seen that in the phosphate rich phase the enzyme activity is increased respect to the control (phosphate buffer 50 mM), while in the top phase for PEG 2000 and 4000 an increase in the activity was observed, but PEG of high molecular mass decreased the activity. Similar results have been found for other enzymes in PEG and phosphate concentrated medium [6]. The medium effect on the enzyme activity is a proof of the medium interaction with the protein, by modifying the tertiary structure of the active site.

3.2. PEG concentration and molecular mass effect on PPL partitioning

Fig. 1 shows the partition data for PPL in PEG–phosphate systems at different tie line length (TLL) calculated as

$$\mathrm{TLL} = (\Delta[\mathrm{PEG}]^2 - \Delta[\mathrm{Pi}]^2)^{1/2} \quad (6)$$

Table 1
Effect of medium composition on the PPL relative activity (%)

Medium	Relative activity
Buffer 50 mM pH 7.0	100.0
Bottom phase	104.1
PEG 2000	112.3
PEG 4000	109.4
PEG 8000	84.7
PEG 10,000	94.7

The PPL activity in buffer sodium phosphate 50 mM, pH 7.0 was taken as reference.

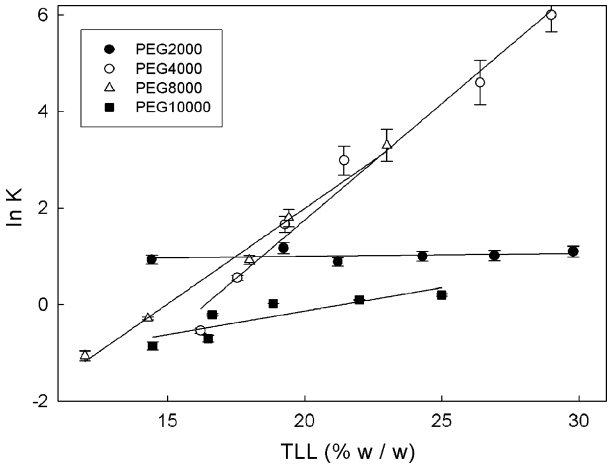

Fig. 1. Dependence of the PPL partition coefficient with the TLL in different aqueous two-phase systems. Temperature 25 °C, pH 7.00.

where Δ[PEG] and Δ[Pi] are the difference between the PEG and phosphate concentration in the top and bottom phases. The general observed trend is a linear relationship between ln K and Δ[PEG]. This behaviour has been noted for the partition of other proteins in phosphate–PEG systems, suggesting that the ln K vs. Δ[PEG] relation follows an equation of the type:

$$\ln K = A + B\Delta[\mathrm{PEG}] \quad (7)$$

The slope of Eq. (7) is a function of protein and polymer molecular mass and the interaction of protein with the polymer and water [6]. From Fig. 1 can be seen that the slope value in the presence of PEG 2000 is very low, while PEG 4000 and 8000 showed significant B value in agreement with a the presence of a strong polymer–protein interaction. The slope in PEG 8000 was lower than the observed for the other two PEGs, as a result of exclusion of the polymer from the protein domain. The system with PEG 10,000 showed significant PEG exclusion due to the high molecular mass of this PEG. This last effect is in agreement with a diminution of the free volume available in the top phase by molecular mass increase of the PEG. However, for PEGs of low molecular mass (2000–4000) the increase of ln K with the TLL is related with a polymer–protein interaction due to the hydrophobic character of the PPL. Some of the drivers for PPL partition are the excluded volume of the protein (given by the free solution available to the protein), and the polymer–protein interaction. Our finding suggests that in systems with PEG 2000–8000 the lipase partition is driven by the enzyme polymer interaction which explains the K coefficient with a value other than unity, while in PEG 10,000 the excluded volume effect over the protein polymer interaction, resulting in a decreasing in the partition coefficient.

Fig. 2 shows the effect of PEG molecular mass (for the third tie line length) on the partition coefficient. In this case the total PEG concentration was approximately similar in all the ATPS, the increase of K value observed for PEG 2000 and 4000, suggest an increase in the PEG–protein interaction, while for PEGs 8000 and 10,000, the opposite effect suggests only the PEG exclude volume effect [7] is driven the PPL partition. Haghtalab et al. [8] presented experimental results for

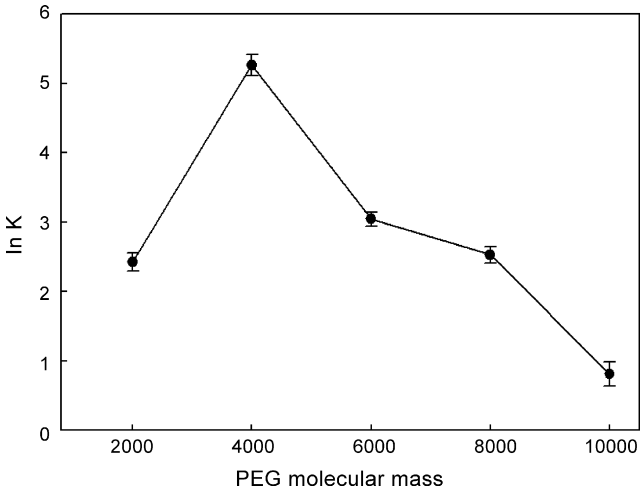

Fig. 2. PEG molecular mass effect (for the third TTL) on the partition coefficient PPL. Temperature 25 °C, pH 7.00.

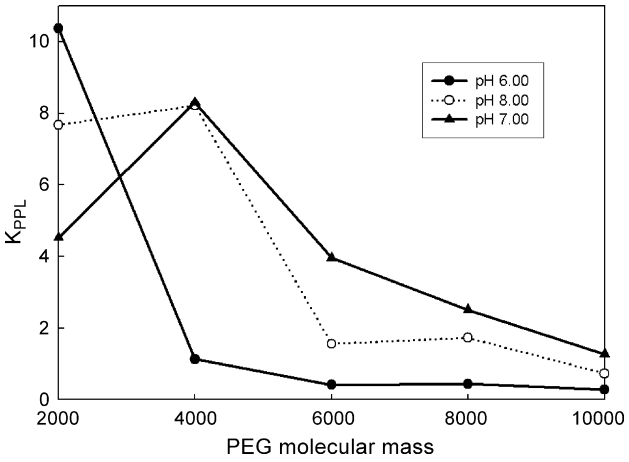

Fig. 3. pH effect on the PPL partition coefficient. Temperature 25 °C.

lysozyme partitioning in PEG–phosphate and PEG–sodium sulphate systems and found that for a given PEG molecular mass, the slope of the $\ln K$ vs. Δ[PEG] curves had both positive and negative values depending on the PEG excluded volume. The excluded volume theory shows that the PEG concentration or an increase in its molecular mass induces a decrease of the protein solubility in the phase where the protein is situated. Previous reports about PEG–protein interaction [9] have demonstrated the presence of a poor interaction between them, when it is compared with other ligands with a specific PEG–protein interaction. Lee and Lee [10] examined the interaction between PEG and betalactoglobulin as a function of PEG molecular mass; the preferential exclusion increases with an increase in the PEG size. This result argues in favour of steric exclusion as the factor that causes the interaction of PEG with proteins. In the case of PEG–betalactoglobulin and PEG–tubulin interactions studied by Timasheff [11], the PEG molecular mass increase induces an exclusion of the polymer from the protein domain, increasing in this way the preferential hydration of the protein. However, at high PEG concentrations, a decrease of the PEG exclusion for the PEG–betalactoglobulin interaction was observed. This opposite behaviour of PEG can be better understood in terms of the PEG solution behaviour. It has been demonstrated that PEG, which is a flexible molecule, it can acquire a compact structure stabilized by intramolecular hydrophobic bonds. The PEG compact structure has a lower interaction with the solvent than the fully extended one; this allows the PEG molecule to interact with the protein.

PEG solutions of high concentration penetrate the hydration layer of the protein allowing the PEG molecule (which is partially hydrophobic) to interact with the hydrophobic region of the protein. Therefore, the observed effect of PEG on a protein can be due to a fine balance between two opposing factors: PEG exclusion, and PEG–protein binding through the hydrophobic area of the protein exposed to the solvent. The latter effect will depend on the chemical structure of the protein. Proteins with a large hydrophobic surface area exposed to the solvent have a greater possibility of interacting with PEG. This interaction has a high affinity and is the only factor which drives the protein partition in favour of the PEG-rich phase [5].

3.3. pH effect on PPL partitioning

Fig. 3 shows the partition coefficient dependence on PEG molecular mass at different pHs. It can be seen that at pH 6.0 there is a strong diminution of the K value at low PEG molecular mass, while this effect was also observed at pH 7.0 and 8.0 in systems with PEG of high molecular mass. PPL has an isoelectric pH about 5.18, therefore at pH 6.0 is slightly negatively charged. According to the Albersson equation [2]:

$$\ln K = \ln K^{\circ} + \frac{F\Delta\Psi Z_{\mathrm{p}}}{RT} \tag{8}$$

protein partition is driven by two effects: the electrostatic component ($F\Delta\Psi Z_{\mathrm{p}}/RT$) determined by the electrical protein charge, and a hydrophobic component (K°) which has a maximal effect when the pH medium is near the isoelectric pH. Under the condition pH 6.0, the hydrophobic component is greater than the electrostatic forces, therefore the PEG of low molecular mass has tends to interacts with the protein. In PEG of high molecular mass the steric hindrance is major decreasing the partition coefficient, being this similar to any assayed pH.

3.4. Temperature effect on PPL partitioning

Because it is impossible to measure in a direct manner the heat when a protein is partitioned between the two phases, we have determined the partition coefficient of PPL at different temperatures. Then by application of the van't Hoff equation the enthalpy change associated to the protein partition was calculated, also the entropic change was calculated by applying Eqs. (3), (4) and (5). Fig. 4 shows negative enthalpy change values obtained for the systems PEG 2000–8000, while for PEG 8000 a positive value was obtained. This agrees with the presence of a strong PEG–protein interaction for these PEG, while for PEG 10,000 the positive change observed suggests a repulsion between them in accordance with an exclusion of the PEG of high molecular mass from the protein domain. Negative entropy changes

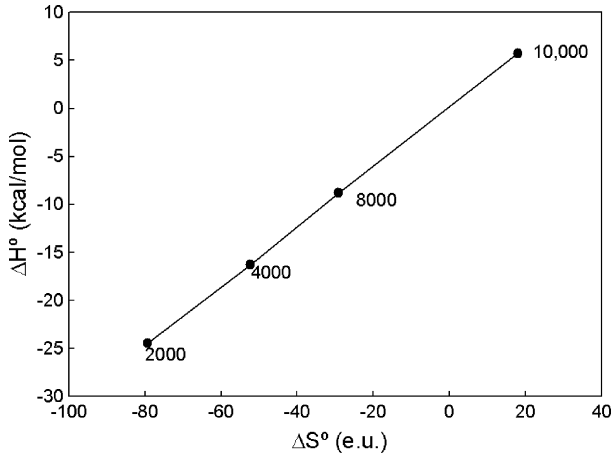

Fig. 4. Entropic–enthalpic compensation plot for PPL partition in aqueous two-phase systems.

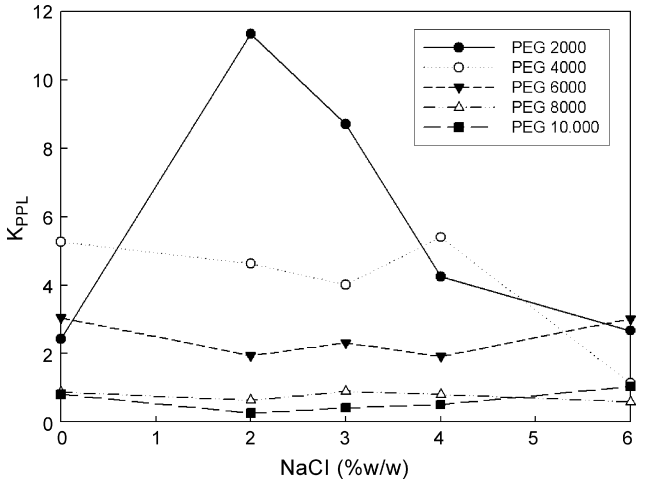

Fig. 5. NaCl effect on PPL partitioning in media of different PEG molecular mass. All the other conditions are as Fig. 1.

observed for all the partitioning (except for PEG 10,000) is suggestive of the formation of an ordered final state when the protein is transferred from the bottom to the top phase in agreement with a protein polymer complex formation. This finding agrees with the negative entropic values observed. The positive thermodynamic values observed for PEG 10,000 could be explained by a lack of interaction between the PEG and the protein domain due to the PEG exclusion from the protein domain by steric hindrance. The ΔH° values were plotted vs. the ΔS° values, taken a ΔH°–ΔS° pair for a PEG of a desired molecular mass. The existence of a linear entropy–enthalpy change relationship suggests that liquid water plays a role in the molecular mechanism of this process. This linear compensation pattern appears to be the thermodynamic manifestation of structure making and structure breaking effects of cosolute on the solvent molecules as was demonstrated earlier by Lumry and Rajender [15]. This behaviour has been reported for the partitioning of other proteins in aqueous two-phase systems [12] associated to a loss of the water ordered around the hydrophobic zone (ethylene chain of PEG and hydrophobic superficial area of the protein exposed to the solvent).

3.5. NaCl effect on PPL partitioning

Fig. 5 shows the dependence of the PPL partition coefficient on the NaCl concentration and PEG molecular mass in the medium. It can be seen there are two different effects according to the PEG molecular mass. PEG 2000 system induced an increasing in the enzyme partition coefficient between 0 and 3% NaCl while a decrease was observed between 3 and 6% of the salt. Systems with the other PEG molecular mass were not affected in a significant manner by the presence of NaCl. A decrease in K is observed at low salt concentration (2% NaCl) while a slightly increase of K was observed with an increase of salt concentration. At the pH values where the partition were assayed, PPL has a negative electrical net charge; ions Na^+ and Cl^- have similar partition coefficient in ATPS [7], they do not affect in a significant manner the interfacial electrical potential. The significant increase in the partition in PEG 2000 cannot be explained regarding a modification on the interfacial electrical potential by salt addition. It is well known that NaCl and other salt have the property of modifying the water structure ordered around the hydrophobic chain, such as ethylene group of PEG or hydrophobic surface area of the protein exposed to the solvent. A loss of this ordered water occurs when a protein is transferred from a salt phase to PEG-rich phase [7], facilitating in this way the interaction between the polymer and the protein and increasing its partition coefficient.

3.6. Determination of the ATPS separation capacity for PPL

The above experiments were performed using a non-pure preparation of PPL which contains other unknown proteins. Most studies about the recovery of a target protein from a natural product are made to begin with the pure target protein. These experiments yield results which in the majority of the cases cannot be compared with those obtained from a complex mixture of proteins containing the target protein. It is well known that the partition behaviour of a pure protein is very different when it is in a complex mixture. Rito Palomares [13] has pointed out that the environment of a protein may induce protein–protein interactions which modify the K of the target protein. The binodial diagram is modified in a significant way inducing a change in the target and the impurities proteins partition.

The selection of the best systems to the recovery of PPL was done in basis to the analysis of the parameter $y(\%)$. When the above experiments were carried out, the total protein concentration in the top and bottom phases were determined and the partition coefficient of the protein was calculated.

Fig. 6A shows the $y(\%)_{top}$ calculated for the top phase (the phase where the PPL has the major affinity) for systems of different molecular mass in the absence and presence of NaCl. The data were expressed as surface plot according to previous reports [14] and methodology used to found the best experimental situation where the recovery of the target enzyme is maximal.

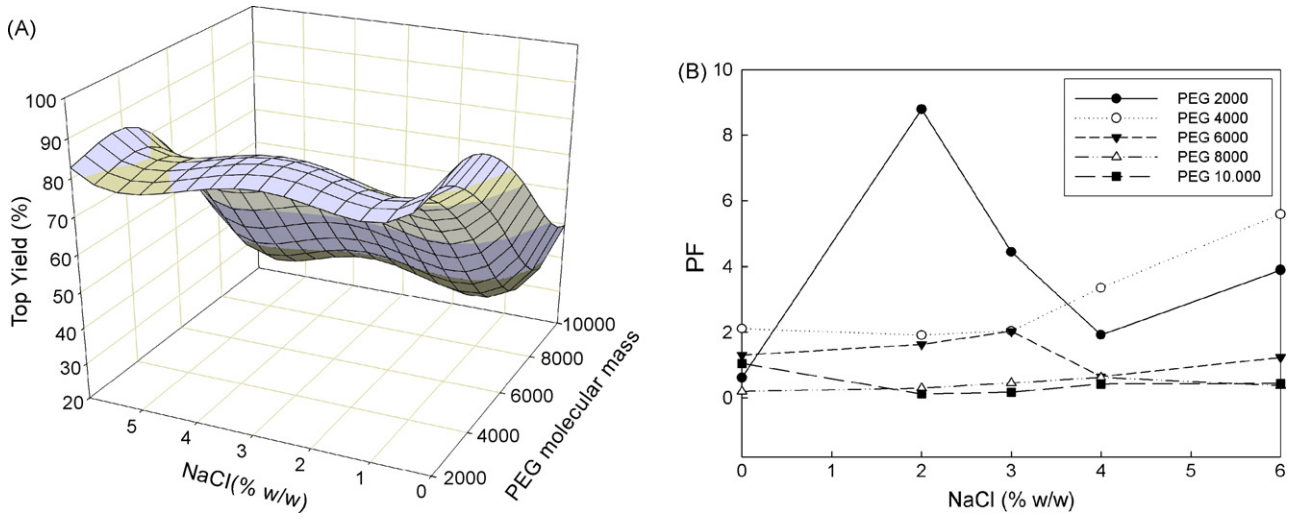

Fig. 6. (A) Top recovery of PPL as a function of PEG molecular mass and NaCl concentration. (B) Top purification factor for PPL as a function of PEG molecular mass and NaCl concentration.

A regression analysis was carried out to fit mathematical model to the experimental data to determine an optimal region for the responses studied. This analysis relates the percentage of recovery $y(\%)_{top}$ with the polymer molecular mass (M_m) and the NaCl concentration. The mathematical model that best fitted the experimental data can be described by the following second order equation:

$$y(\%) = h + aM_m + b[\text{NaCl}] + cM_m{}^2 + d[\text{NaCl}]^2$$

being h, a, b, c, and d fitting constants and Mm the molecular mass of PEG. Fig. 6A shows the response surface for $y(\%)_{top}$ obtained by the mathematical model assayed. The analysis of the variance shows that for the $y(\%)_{top}$ responses, the model adjusted was adequate with high value of $r^2 > 0.98$ and significant values of P (>99%) confidence level, yielded a M_m and [NaCl] values of 2000 and 3%, respectively, which produced the better enzyme recovery.

Fig. 6B shows the purification factor (PF) obtained for the systems in the presence of increase NaCl concentration. This confirms that the best system is PEG2000–NaCl 3%, a purification factor of 8 being observed for this system.

When a pure protein is partitioned in aqueous two-phase systems, the K is independent of the top–bottom volume ratio, however the protein recovery is influenced by the R value as expressed in Eq. (2). Fig. 7 shows the top–bottom phase ratio volume vs. $y(\%)_{top}$ using the system PEG 2000–3% NaCl selected before. It can be seen that an increase in R value from 0.3 to 3.0 induces an increase in $y(\%)_{top}$ value of over 90%.

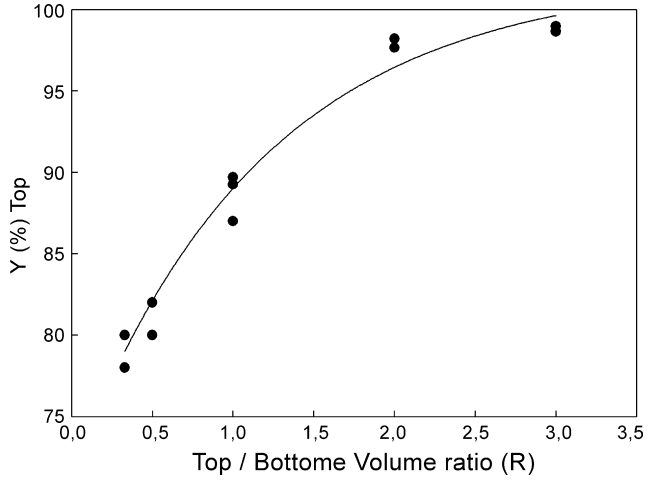

Fig. 7. Top recovery of PPL as a function of top/bottom volume ratio NaCl concentration.

4. Conclusion

PPL is a hydrophobic enzyme with a molecular mass of 54,000 kDa and its isoelectric pH is about 5.4. The high partition coefficient found in the system PEG 2000 is suggesting strong interaction between PEG 2000–PPL due to the low PEG exclusion from the protein domain [13] and the hydrophobic character of PPL. This agrees with the highly exothermic process associated with PPL transfer to the PEG-rich phase which suggests a true interaction between PEG 2000 and PPL. PEG 4000 and higher molecular mass PEGs showed a partition coefficient lower than the unity, according to their molecular mass, as has been found for other enzymes. This finding suggests that, at low PEG molecular mass, the interaction polymer protein is driven the PPL partition, while at high PEG molecular mass, the PEG exclusion from the PPL domain is most important than the polymer protein interaction.

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