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Partition features and renaturation enhancement of chymosin in aqueous two-phase systems

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Abstract

Aqueous two-phase systems of polyethylene glycol (molecular mass 1450, 3350 and 6000)–phosphate and polyethylen-polypropilen oxide (molecular mass 8400)–maltodextrin systems were used in order to study the partition features of recombinant chymosin from inclusion bodies. These systems in the presence of 8 M urea were used for the solubilization of inclusion bodies containing recombinant chymosin and for the oxidative renaturation of this protein. Recombinant chymosin showed to be partitioned in favour of the top phase in all studied systems with a partition coefficient between 4 and 6. The recovery of the chymosin biological activity was 32% in the polyethylen-polypropilen oxide, while in the polyethylene glycol–phosphate the recovery was 50–59%. The results indicate that the liquid–liquid extraction would be an adequate tool able to isolate and concentrate chymosin from inclusion bodies with a yield of biological activity higher than that obtained from the standard method (43%).

Keywords: Chymosin; Maltodextrin; Polyethylene glycol; Polyoxidethylene; Partition; Inclusion bodies

1. Introduction

The use of industrial enzymes has increased in the last few years; therefore, it is necessary to develop new methods for the isolation and purification of proteins with considerably high purity, low cost and industrial applicability. The traditional methods for the isolation and purification of proteins involve some steps such as ammonium sulfate precipitation, ionic and affinity chromatographies, dialysis and final concentration of the product, which require long time and high cost. These operations induce an increase in the unfolding fraction of the macromolecule, with loss of its biological activity and a poor yield of the total process.

Aqueous two-phase systems (ATPS) are formed by mixing two flexible chain polymers in water or one polymer and a salt (phosphate, citrate, etc.) [1]. Proteins are partitioned between the two phases with a partition coefficient that can be modified by changing the experimental conditions of the medium such as pH, salts, ionic strength, among others [2].

ATPS have been used as a first purification step since such systems allow the removal of contaminants by a simple and economic process. They can be generated into a homogenate of a genetically natural or modified product. ATPS have a number of advantages over the conventional methods for the isolation and purification of proteins: the partition equilibrium is reached very fast; it can be applied in scale up; it has the possibility of continuous state operation, low cost and the materials that make up this system are non-expensive and can be recycled.

Chymosin is a neonatal gastric aspartic protease of great commercial importance in cheese industry [3]. The aspartic protease family is widely distributed in many organisms with a molecular mass between 32 and 39 kDa with a different isoelectrical point due to the presence of isoenzymes or autodegradation products. Chymosin is an enzyme with a single polypeptide chain of 323 amino acid residues with a low content of basic residues and it is rich in dicarboxylic acid residues.

In the past years, the bovine chymosin was replaced for recombinant bovine chymosin produced by fermentation. This

Abbreviations: ATPS, aqueous two-phase systems; PEG1450, PEG3350 and PEG6000, as polyethylene glycol of average molecular mass 1450, 3350 and 6000, respectively; QR, recombinant chymosin; QB, bovine chymosin; Pi, phosphate buffer; PEO, poly(ethylene-glycol)-block-poly-(propylene-glycol)-block-poly-(ethylene-glycol); MDX, maltodextrin; IB, inclusion bodies.

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protein has been expressed in different microorganisms such as *Aspergillus niger*, *Kluyveromyces lactis* but in *Escherichia coli* [4] the enzyme is expressed as inclusion bodies, urea is used in order to solubilize them. In general, the refolding process is followed by a low recovery of the enzyme biological activity. The novelty of the modification proposed in this work is to make the dissolution operations of inclusion bodies with urea and the extraction of the target protein using liquid–liquid extraction and aqueous two-phase system in only one step. After solubilization, polyethylene glycol and sodium phosphate are added in order to form an aqueous two-phase system [6]. After phase separation the target protein was extracted. To obtain this PEG, urea, inclusion bodies and sodium phosphate in the adequate concentrations were mixed. We have studied the partitioning features of recombinant chymosin (QR) in a preformed (PEG–phosphate) and more economic and disposable system: PEO/MDX. Temperature, PEG molecular mass and salt concentration effect on the partition were also assayed.

2. Materials and methods

2.1. Chemicals

Bovine chymosin (QB) (EC 3.423.4), and bovine serum bovine (BSA) polyethylene glycol of the average molecular weight 1450 and 3350 (PEG1450 and PEG3350) and the ionic exchange resins Dowex IR 50 were purchased from Sigma Chem. Co. (USA); the polyethylene glycol of the average molecular weight 6000 (PEG6000) was purchased from Merck and used without further purification. Poly(ethylene glycol)-block-poly-(propylene glycol)-block-poly-(ethylene glycol), of average 8400 molecular mass (PEO) was purchased from Sigma Chem. Co. Maltodextrin (MDX) of average molecular mass 1900 was purchased from Kasdorf S.A. (Argentine). It contains 90% of the polyose; the impurities being: glucose, dioses and treoses. The molecular mass of maltodextrin was determined by viscosimetry using a Brookfield DV-II+ programmable viscosimeter. All the other reagents were of analytical quality.

Inclusion bodies obtained from *E. coli* were kindly granted by Drs. H. Menzella, H. Gramajo and E. Ceccarelli. They were washed twice with phosphate buffer (Pi) 50 mM, pH 6.3 and kept at −70 °C until use.

Recombinant chymosin (QR) extraction from inclusion bodies was carried out following the method of Menzella et al. [5]. Inclusion bodies were solubilized by incubating with pH 10.5, 50 mM phosphate and 50 mM glycine buffer in deionized urea 8 M at 30 °C for 3 h with continuous stirring. After its centrifugation, the protein refolding was initiated by dilution (20 times) of the solution with buffer 50 mM sodium phosphate pH 10.5 in the presence of 10 μM $CuSO_4$.

2.2. QR enzymatic activity determination

The milk clotting activity was measured in 2 mL of Nestle skim milk. Skim milk powder was prepared by dissolving 10 g of skim milk powder in 100 mL of 10 mM $CaCl_2$ solution and was stirred for 30 min. The reconstituted milk was kept at 4 °C and used 1 h after preparation. Before use, the milk was equilibrated at 35 °C for 20 min. The clotting time was determined through a rotation movement until the appearance of the first clot. A control of activity was performed adding QR or QB in a medium of 50 mM pH 6.5 phosphate for 1 h and the activity of the increasing amounts of these solutions was measured through the slope of time clotting vs. 1/volume of added protein solution plot. The inverse of this slope is the U/mL of the solution used. As reference plot, the same experiments were carried out by incubating the same amount of QR in both top and bottom phases for 1 h and the slope of the same plot was compared in order to find the activity values of the unknown samples in all media used. In order to check the stability of the QR different solutions, its activities were compared with the QB calibration curve. A 1.078 mg/mL QB solution was prepared in 50 mM pH 6.5 phosphate buffer; it was used to refer the QR solutions in mg/mL by interpolation in the calibration curve.

2.3. Total proteins concentration determination

Total protein concentration was determined by measuring the absorbance at 230 nm, the bovine albumin was used as standard. Increasing amounts of BSA (10–100 μL) were placed in a cuvette with 2 mL 50 mM pH 7.4 buffer phosphate or the corresponding phase in order to correct the measurement for each phase.

2.4. Preparation of the aqueous biphasic system

The systems PEG/phosphate and PEO/MDX at pH 6.5 were prepared from stock 40% (w/w) PEG solutions of different molecular mass, 30% (w/w), pH 6.5 potassium phosphate (Pi), 50% (w/w) PEO and 40% (w/w) MDX solutions. The systems PEG/Pi were prepared with distilled water according to Ramsch et al. [6]. The system PEO–MDX was prepared in 50 mM pH 6.5 phosphate buffer according to binodial diagram previously obtained by Bolognese et al. [7]. The pH 10.5, 8 M deionized urea PEG/Pi and PEO/Pi systems were prepared with the previous stock solutions but in deionized urea 8 M, pH 10.5 glycin 50 mM buffer. Low-speed centrifugation to speed up phase separation was used after gentle mixing of the system components; then, 1 mL of each phase was mixed to reconstitute the several two-phase system in which the protein partition was assayed.

2.5. Determination of the partition coefficient (K)

Partition coefficient of the proteins between both phases was analyzed by dissolving increasing amount of recombinant QR solution (10–25 μL) in the two-phase preformed system containing 1 mL of each equilibrated phase, the change of the total volume of each phase being negligible. After mixing by inversion for 1 min and leaving it to settle for at least 1 h, the system was centrifuged at low speed for the two-phase separation. Samples were withdrawn from separated phases and after dilution, the protein content in each phase was determined by activity measurements or by the absorption at 230 nm. In this latter case, the blank absorption of each phase in the corresponding amount

was subtracted. The partition coefficient was defined as:

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

where $[P]_{top}$ and $[P]_{bottom}$ are equilibrium concentrations of the partitioned protein in the PEG and phosphate-rich phases, respectively. In the protein concentration range assayed, a plot of $[P]_{top}$ vs. $[P]_{bottom}$ showed a linear behaviour, K being its slope.

In order to evaluate the purification process, the enzyme yield recovery in the top phase ($y\%$) was also calculated according to the given equation:

$$y(\%) = \frac{100}{1 + (1/RK)} \quad (2)$$

$R = V_T/V_B$ and V_T and V_B being the top and bottom volumes, respectively. Absorbance measurements were done in a Carl Zeisse Spekol 1200 spectrophotometer.

2.6. Estimation of the thermodynamics functions associated to the QR partition

The enthalpic change (ΔH°) associated to the protein partition in the ATPS was calculated by applying the known equation:

$$\frac{\partial \ln K}{\partial T} = -\frac{\Delta H^\circ}{R} \quad (3)$$

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

the free energy change ΔG° was calculated from Eq. (4). The entropic change (ΔS°) was calculated from the equation:

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

2.7. Solubilization and refolding of protein from inclusion bodies

The inclusion bodies were dissolved in urea 8 M, and after adequate centrifugation, the oxidative refolding was carried out. A concentration of 20 mg/mL of total soluble protein (prochymosin) was obtained in urea 8 M and after its refolding, 30.00 U/mg (8.14 mg/mL measured as QB) activity was recovered. The same 8 M urea–air oxidation treatment of the inclusion bodies was assayed in PEO–MDX and PEG–phosphate (molecular mass 1500, 3350 and 6000). One gram of inclusion bodies was added into 12 g total ATPS, which were prepared in 8 M urea, 50 mM glycine phosphate buffer at pH 10.5. The solubilization process takes 2 h at 30 °C; after that, the two phases were separated, and the volume and the total protein concentration were measured.

3. Results and discussion

3.1. Partitioning of chymosin in aqueous two-phase PEO–maltodextrin system and the NaCl concentration effect

Fig. 1 shows the partition coefficient of QR in the system PEO–maltodextrin at increasing NaCl concentration. The NaCl concentration was varied between 0 and 8% (w/w), equivalent to 0–1.5 M NaCl, which is a great salt concentration variation.

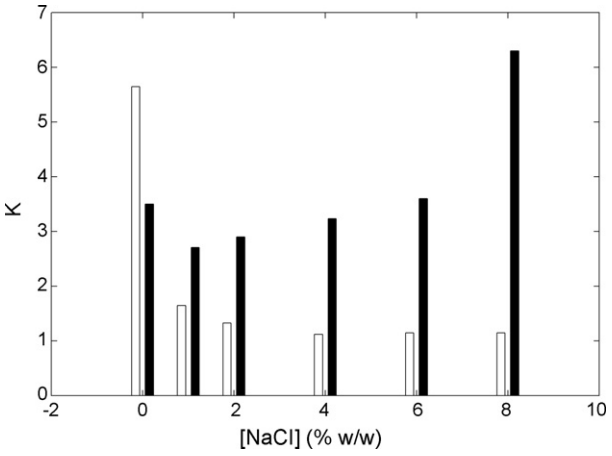

Fig. 1. Effect of NaCl concentration on the partition coefficient of QR in PEO (8.25%, w/w)/maltodextrin (26.1%, w/w) system. Medium 50 mM, pH 6.5, sodium phosphate buffer. Temperature (□) 8 °C and (■) 20 °C.

The effect of the NaCl concentration on the partition was assayed because the salt capacity to induce a modification in the protein partition in favour of one of the phases is well known. NaCl is a non-toxic salt; therefore, it can be used to modify the partition process, without affecting the uses of the protein in any biological process. When NaCl concentration increase (between 0 and 2%) at 8 °C induces a significant decrease of K, remaining it constant at high salt concentration. At 20 °C, the increase salt favoured the QR transfer to the PEO-rich phase.

QR showed high affinity for the PEO-rich phase in the absence of NaCl; when the salt concentration is about 1% the K value decreased, but when the salt concentration is greater than this value an increase in the protein partition in favour of the PEO-rich phase was induced. This dual effect of the salt has been observed for the partition of other proteins [7]. At low salt concentration (between 0 and 0.2 M), the salt modifies the electrical potential ($\Delta\Psi$) between the phases, it depends on the cation and anion affinity to the phases, and K will be modified according to the known state equation for the protein partition:

$$\ln K = \ln K_0 + \frac{\Delta\Psi\, Z_p}{RT} \quad (6)$$

Z_p being the net electrical charge of the protein. Our experimental conditions are salt concentrations greater than 0.3 M such as the ones used by other researchers. The salt induces a significant modification of the structure of the water molecules around the protein hydrophobic area. Flexible chain polymer also interacts with water through hydrogen bond or by formation of structured water around the polymer molecule hydrophobic tail. This ordered water is modified by the presence of water structure making or structure breaking salts. The presence of salt induces loss of this ordered water from the protein and from the flexible chain polymer, thus facilitating the interaction between them. In this way, the PEO–protein interaction will be greater, increasing the partition coefficient in favour of the PEO-rich phase.

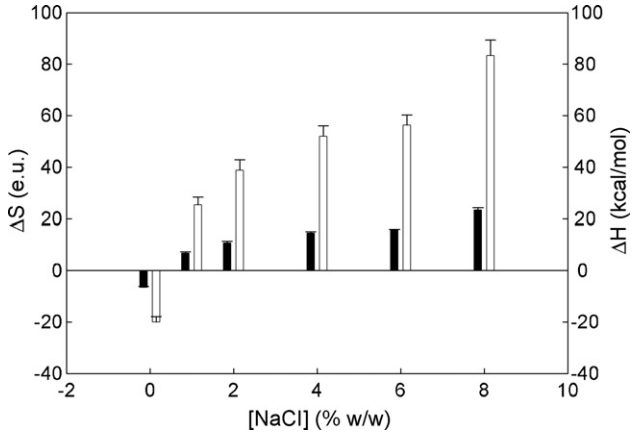

Fig. 2. Dependence of the enthalpic (■) and entropic (□) changes associated to the partition of QR in PEO (8.25%, w/w)/maltodextrin (26.1%, w/w) system at increasing NaCl concentration. Medium 50 mM, pH 6.5, sodium phosphate buffer.

Direct measurements of the heat involved in the protein partition cannot be carried out because of the impossibility of developing this process in a calorimeter; therefore, analysis of the K variation with temperature change may provide thermodynamic data as regards the partitioning process.

Fig. 2 shows the dependence of the enthalpy change (ΔH°) for the QR transfer from MDX phase to PEO phase. In the absence of NaCl, the QR partitioning was exothermic, while the presence of salt produces the opposite effect, being the partition endothermic. Protein partition is, in general, an endothermic process; the inversion of the sign of the energy that participates in the partition is a proof of the PEO–protein interaction sensibly modified by the salt concentration. In the absence of salt, the exothermicity demonstrates the formation of bonds between the PEO and the protein, while the endothermicity observed when NaCl concentration increases suggests a loss of the protein hydration water by PEO interaction. This fact induces an increase of the entropic change (ΔS°) in protein partition as it is shown in Fig. 2. This finding agrees with the good protein–PEO interaction due to the hydrophobic nature of this last interaction. In general, protein partition in PEG/phosphate and PEG/dextran aqueous two-phase systems is associated with positive entropic and enthalpic changes [8]. These values depend on the molecular mass of the polymer; however, in the case of QR–PEO interaction, the negative enthalpic and entropic values observed in the absence of NaCl is suggests a strong polymer–protein interaction. One reason might be the great hydrophobic nature of the PEO molecule compared with the PEG molecule, which is more hydrophilic, favouring in this way the polymer–protein interaction. QR also showed an anomalous and high partition coefficient in comparison with other proteins of similar molecular mass, which suggests the presence of a great hydrophobic character of the QR surface exposure to the solvent.

QR has an isoelectric point of about 4.6; therefore, at pH 6.5 where the partitioning was assayed, the protein has a negative net electrical charge. The chloride anion is hydrophilic so has preference for the more hydrophilic phase (maltodextrin-rich phase). An excess of negative electrical charge is present in the bottom phase. At low salt concentration, the negatively charged protein will be transferred to the top phase [2], resulting in an increase of the partition coefficient. High salt concentrations such as the chloride of monovalent cations of Na^+, Rb^+ and Cs^+ induce a protein partition in favour of the polyethylene glycol-rich phase [9]. Lahore et al. [10] found that the acid protease partition increased towards the PEG-rich phase at increasing NaCl concentration. These authors attributed this effect to a decrease in the water activity in the bottom phase produced by the increase in the salt concentration and thereby hydrophobic protein is excluded from this phase. However, we have demonstrated in a previous report [9] that salts at concentrations above 0.3 M induce a loss of structured water around the PEG molecule; the effect depends on the salt being a structure maker or a water structure breaker. The loss of water structure induces a diminution of the specific volume of the polymer molecule, increasing in this way the volume available of the solution and allowing the protein to go to the top phase.

3.2. QR partitioning in polyethylene glycol–phosphate system at pH 6.5: effect of polymer molecular mass, temperature and NaCl concentration

The pH 6.5 was chosen because it is the QR conservation pH. The systems involved are shown in Table 1. In order to determine the coefficient partition, the enzymatic activity in both phases was measured in all PEG/phosphate systems. The total protein concentration in both phases of all systems was also measured. In Table 2 the QR activity and the specific activity in the top phase of each PEG/phosphate analyzed system can be seen. The values of the specific activity are similar in the three top phases. The activity in the bottom phase was negligible. The possibility that the bottom phase could interfere with the enzymatic activity was not considered because, in those media, the QR standard showed a lineal behaviour of plot: time clotting vs. 1/volume of added protein solution. This fact indicates that the method sensitivity is low for the small protein concentration in the bottom phase.

Fig. 3A–C shows the dependence on the QR partition coefficient (by total protein measured) with the increase of NaCl concentration for systems of different PEG molecular mass. The system compositions are indicated in the corresponding figure legends. It can be seen that in all cases, the partition was slightly affected by the salt concentration, at 8 °C, while at 20 °C in PEG1450 and PEG6000 at low NaCl concentration, a decrease in K was induced and at concentrations higher than 6%, a net increase is produced. In the PEG3350 system, an increase in the partition in favour of the PEG-rich phase was observed for all the NaCl concentration range. Similar results have been found by other proteins partition. Rosa et al. [11] found that the

Table 1
Composition of the employed PEG/Pi SBA

System	PEG (%, w/w)	Pi (%, w/w)	H_2O (%, w/w)
PEG1450/Pi	12.96	12.4	74.64
PEG3350/Pi	10.88	10.1	79.01
PEG6000/Pi	10.11	10.5	79.39

Table 2
QR activity and concentration in the pH 6.5 PEG/Pi system top phases and partition coefficient

Systems	Top phase activity (U/mL)	Top phase specific activity (U/mg)	$[P]_{top}$ (mg/mL)	$[P]_{bottom}$ (mg/mL)	K
PEG1500/Pi	55.01 ± 0.07	4.5 ± 0.1	12.32 ± 0.05	1.77 ± 0.07	6.9 ± 0.1
PEG3350/Pi	48.33 ± 0.07	4.5 ± 0.1	10.66 ± 0.04	1.57 ± 0.06	6.8 ± 0.1
PEG6000/Pi	33.55 ± 0.05	3.7 ± 0.1	9.20 ± 0.05	2.21 ± 0.05	4.2 ± 0.1

partition of hydrophobic proteins such as human immunoglobulin is favoured to the PEG-rich phase by NaCl concentration. Also the addition of NaCl favoured the partition of penicillin acylase [12] to the PEG-rich phase. The presence of NaCl in PEG–phosphate system increases the hydrophobicity difference between the phases and thus inducing the protein partition in favour of the upper phase. Rosa et al. [11] observed a decrease in the volume of the top phase by addition of the NaCl; these authors suggest that an increase in the top phase hydrophobicity is produced due to the loss of water from the environment of ethylene chain residues.

The temperature effect was also assayed, yielding a similar behaviour to that found for the partition of other proteins. The increased temperature induced a significant protein partition increase in ATPS. Fig. 4 shows the dependence on the enthalpic change as a function of the NaCl concentration. It can be seen that the partition endothermicity increased in the same way as the NaCl concentration. PEG1450 induced the greater enthalpic change of the three PEG assayed; its negative value being in the absence of NaCl. The enthalpic change in the pres-

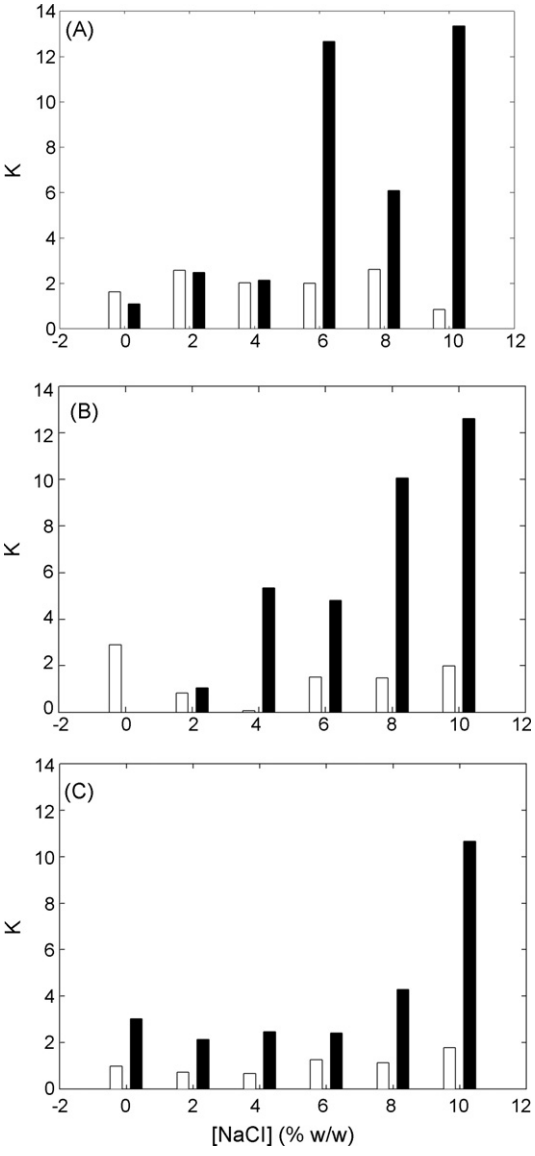

Fig. 3. Effect of NaCl concentration on the partition coefficient of QR in: (A) PEG1450 (12.96%, w/w)/sodium phosphate (12.40%, w/w) systems, (B) PEG3350 (10.88%, w/w)/sodium phosphate (10.10%, w/w), and (C) PEG6000 (10.11%, w/w)/sodium phosphate (10.50%, w/w), pH 6.5. Temperature 8 °C (□) and 20 °C (■).

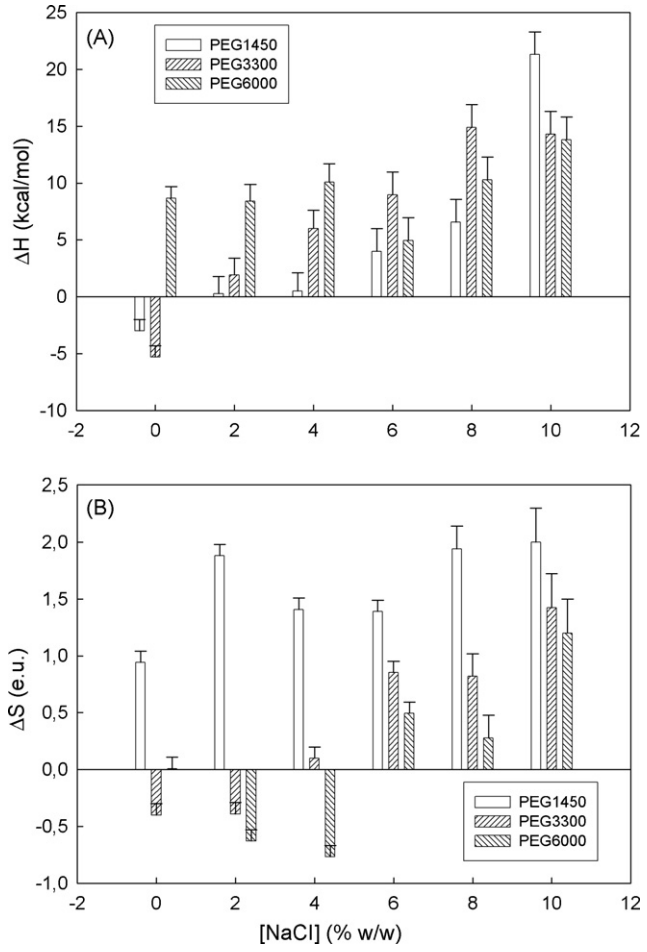

Fig. 4. Dependence of enthalpic (A) and entropic (B) changes associated to the QR partition in PEG/sodium phosphate systems, pH 6.5, at increasing NaCl concentration.

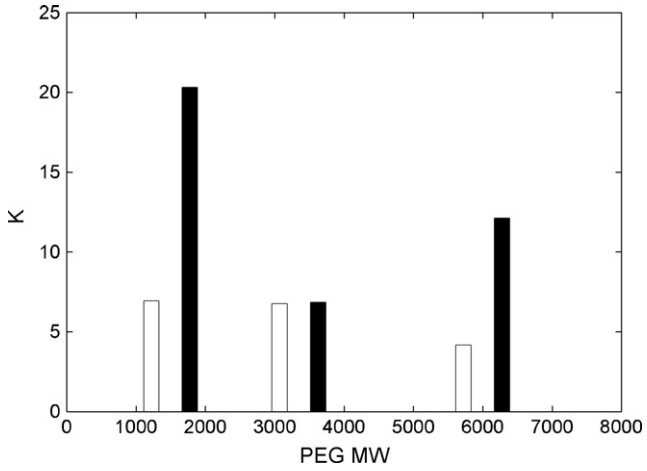

Fig. 5. Partition coefficient of QR vs. the PEG molecular mass, (□) pH 6.5, (■) pH 10.5, urea concentration 8 M. Temperature 30 °C.

ence of PEG3350 was affected in the same way; however, in PEG6000, the effect observed was very poor, showing a relative ΔH° constant. Fig. 4 shows the NaCl effect on the entropic change for the QR partition. It can be seen that ΔS° was always positive; its value increases with the NaCl concentration. The greater variation was induced by PEG1450. This finding agrees with the ΔH° variation effect, and it suggests that when the QR is transferred from the phosphate phase to the PEG-rich phase an increase in the system disorder is produced. Similar results have been found for the partition of other proteins [8] in ATPS.

NaCl is a salt that induces the release of water molecules ordered around the hydrophobic chain (ethylen) of PEG. The loss of the water order around the PEG molecules induces a decrease in the specific volume of the PEG and favoured the interaction between the ethylen chain and the hydrophobic area of the protein exposed to the solvent, increasing in this way the protein transfer to the PEG-rich phase.

When the PEG molecular mass effect was analyzed, a slight decrease in K values is observed, as predicted by the excluded volume theory, due to a decrease in the available free volume as consequence of the PEG molecular mass increase. However, the dependence of K with the molecular mass was less than that reported for other proteins. This finding suggests that the volume available of the solution is not the only factor that affects the tendency shown in Fig. 5, and that other molecular mechanism is also taking place such as a QR–PEG strong interaction.

3.3. QR partitioning in polyethylene glycol–phosphate system at pH 10.5: effect of polymer molecular mass

The QR partitioning was also assayed in pH 10.5, urea 8 M, PEG–phosphate system. The reason for this condition is due to the fact that QR has been expressed in *E. coli* as inclusion bodies, which makes it necessary to solubilize it in urea 8 M previous to an oxidative refolding process at pH 10.5. QR is completely unfolded in urea 8 M, thus is more hydrophobic than the native form, which increases the capacity to interact with the PEG molecule. The PEG of low molecular mass tends to interact with the protein domain because it does not present steric hindrance; therefore, an increase in the partition coefficient can be seen in Fig. 5. PEG of higher molecular mass loses its capacity to interact with the unfolded QR, inducing a decrease of the K, while an increase in the size of the PEG molecule induces an increase in the coefficient partition. This last behaviour could be explained due to the capacity of the PEG molecule to form intra-molecular bonds causing a compact structure that can be accessible to the protein domain [13].

3.4. Solubilization and refolding of protein from inclusion bodies

The general process to purify a protein from inclusion bodies is dissolving them in an unfolding medium such as urea, guanidine or thiocyanate. The solubilized protein may be then refolded decreasing the unfolding agent concentration by dialysis or dilution. During the protein refolding, it is necessary to generate the intra-disulfide bonds so that the protein can acquire its native three-dimensional structure. The condition of the disulfide bond can be carried out by air oxidation. However, QR needs a previous step since there is prochymosin in the inclusion bodies; the refolded prochymosin is carried out at pH 2.0 to facilitate the auto conversion of prochymosin to QR. We have carried out the general procedure for the solubilization in urea and protein refolding in both ATPS processes generated at the same time.

Table 3 shows the total soluble protein concentrations in the top and bottom phases, the protein partition coefficient, the volume relationship and the recovery (y%) in the top phase after the solubilization process. In the PEG–phosphate system, the protein has more affinity for the PEG-rich phase, showing a high partition coefficient, which decreases with the increase of the molecular mass of the PEG.

Both phases underwent the refolding process: first they were diluted 20 times in 50 mM phosphate buffer pH 10.5, with 0.01%

Table 3
Total protein concentrations, partition coefficients, volume relationships (R) and recovery (y%) in top phase after the solubilization process

System	$[P]_{top}$ (mg/mL)	$[P]_{bottom}$ (mg/mL)	K	R	y (%)
PEG1450/Pi	18.00 ± 0.05	0.20 ± 0.06	90.0 ± 0.1	0.54	96
PEG3350/Pi	13.21 ± 0.07	0.24 ± 0.04	55.0 ± 0.1	0.37	95
PEG6000/PI	16.84 ± 0.02	0.56 ± 0.05	30.0 ± 0.1	0.37	98
PEO/MDX	12.16 ± 0.03	0.74 ± 0.03	16.4 ± 0.1	2.60	99
IB		20.00 ± 0.05			

Table 4
Total protein concentrations and activities in the top phase after the refolding process

System	$[P]_{top}$ (mg/mL)	$[P]_{bottom}$ (mg/mL)	Top phase activity (U/mL)	Top phase specific activity (U/mg)	Activity recovery (%)
PEG1450i	15.58 ± 0.02	–	235.0 ± 0.1	15.05 ± 0.04	50
PEG3350i	9.56 ± 0.03	–	–	–	–
PEG6000	12.72 ± 0.05	0.45 ± 0.05	170.40 ± 0.04	17.78 ± 0.09	59
PEO/MDX	8.10 ± 0.03	3.21 ± 0.02	124.20 ± 0.03	9.71 ± 0.06	32
IB	16.43 ± 0.06		491.26 ± 0.03	30.00 ± 0.06	100

(w/v) $CuSO_4$ and then they were kept shaking overnight at 8 °C in an open glass for the oxidation prochymosin process [5]. In order to carry out the autoproteolysis, both solutions were brought to pH 2.5 for 30 min and then to pH 6.5. The total protein concentration was determined in both phases. As a global reference process, the inclusion body underwent the same procedure. Table 2 shows the obtained results. After the refolding and autoproteolysis process, a decrease in the total protein amount in all studied systems was observed. These data are consistent with an observed precipitate when the solutions were taken to pH 2.5. This effect may have been due to the protein aggregation and irreversible precipitation in the acidification process. After protein refolding, the total protein concentration could not be calculated because the sensitivity method was low. The protein concentrations in the bottom phase were practically negligible. The recovery (y%) in all observed cases was very large. These values could be modified with other volume relationships (R) among the phases. In the reference process, the recovery activity was 30 U/mg (8.4 mg/mL measured as QB). This value represents the 42% of QR activity according to Menzella et al. [5]. The enzymatic activity in the studied systems was lower than the reference process although the recovery values of the total protein in the phase were high. The existing difference between the reference process and the studied system is that in the studied systems, the protein is surrounded by PEG or PEO and that these conditions undergo a refolding process. The 1/20 protein dilution in this last process should release the protein from the polymer around. However, the protein refolding should be studied under this condition since the chymosin activity recovery should be improved.

Previous studies have shown the presence of important interactions between PCF and protein [13,14]. When the protein is unfolded, these interactions are important and when the polymer hydrophobicity is high as in this case, they are even more important. PEO is a very hydrophobic molecule; therefore, it interacts with the protein hydrophobic surface area, inducing significant changes in its secondary and tertiary structure, yielding a protein with a loss in the biological activity. The PEG–phosphate systems induce a low protein precipitation, and the proteins are recovered at the top phase (the PEG-rich phase). This finding agrees with the known PEG capacity to avoid the contact between protein and protein molecules, thus avoiding the protein aggregation [14]. PEG significantly reduces the protein aggregation and enhances folding, increasing in this way the thermodynamic stability of a protein. It has been shown that the protein unfolded form is more hydrophobic, having the capacity to interact with PEG molecules, depending on the strength of the interaction of the PEG molecular mass [15].

The presence of urea increases the QR transfer to the top phase, thus increasing the recovery of QR activity.

PEGs interact with the protein domain, avoiding in this way the protein–protein interaction and therefore its irreversible aggregation. PEG of low molecular mass (1450) interacts more strongly with the protein domain, which leads to a loss of the protein tertiary structure, as was demonstrated previously [16]. PEG of high molecular mass induces an increase in the thermodynamical stability of the protein favouring its tertiary structure. This agrees with the experimental result obtained (see Table 4) where PEG1450 yielded a minor QR activity recovery than PEG6000.

4. Conclusion

QR showed high affinity for the PEG-rich phase; this behaviour can be considered as anomalous for the molecular mass of QR, where a partition coefficient around 0.5–1 should be weighted. This finding agrees with a hydrophobic character of QR that suggests the presence of an important enzyme hydrophobic area exposed to the solvent. Because this water is disordered by NaCl, the exposed hydrophobic surface interacts with PEG increasing the protein–polymer interaction.

The recoveries of the specific activity in the top phase are constant, so the PEG medium does not affect the protein activity recovery in these conditions. As in PEO/MDX systems, the interactions involved in the transfer of the protein to the top phase, in NaCl presence, are of hydrophobic and electrostatic nature. The hydrophobic interactions result from non-polar and hydrophobic amino acid remains of the protein. These interactions would cause the water hydrogen bond to break, which is structured around the hydrophobic surfaces, and the system disorder would increase.

The recovery of the chymosin biological activity measured as total protein in the Menzela et al. method [5] was about 44% (using the standard method of urea dissolution and then the enzyme refolding by dilution). For other enzymes this standard method yielded around a 30% of activity [17].

Another advantage of urea 8 M aqueous two-phase system unfolding technique is that it allows us to work with high total protein concentration. In our case, the total protein concentration of about 20 mg/mL, solubilizing approximately an amount of 18 mg/mL of total protein, which increases the handling of the protein several times with respect to the traditional method of solubilization of inclusion bodies.

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