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Features of the acid protease partition in aqueous two-phase systems of polyethylene glycol–phosphate: Chymosin and pepsin

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Abstract

The partitioning of chymosin (from *Aspergilus niger*) and pepsin (from bovine stomach) was carried out in aqueous-two phase systems formed by polyethyleneglycol-potassium phosphate. The effects of polymer concentration, molecular mass and temperature were analysed. The partition was assayed at pH 7.0 in systems of polyethyleneglycol of molecular mass: 1450, 3350, 6000 and 8000. Both proteins showed high affinity for the polyethyleneglycol rich phase. The increase of polyethyleneglycol concentration favoured the protein transfer to the top phase, suggesting an important protein–polymer interaction. Polyethyleneglycol proved to have a stabilizing effect on the chymosin and pepsin, increasing its protein secondary structure. This finding agreed with the enhancement of the milk clotting activity by the polyethyleneglycol. The method appears to be suitable as a first step for the purification of these proteins from their natural sources.
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Keywords: Pepsin; Chymosin; Partition; Acid protease

1. Introduction

The aspartic acid protease family is widely distributed in many organims. Their molecular mass ranges from 32 to 39 kDa with different isoelectrical points due to the presence of isoenzymes or autodegradation products. Chymosin is one of the mostly used acid proteases. It is a neonatal gastric aspartic protease of commercial importance in the cheese industry. Chymosin is more stable at pH values between 5.3 and 6.3; at pH 3–4, the enzyme loses its activity due to autodegradation, while at alkaline pH above 9.8, this loss is due to an irreversible conformation change. Chymosin has a single polypeptidic enzymatic chain of 323 amino acid residues with a low content of basic residues and rich in dicarboxylic acid residues. Its secondary structure consists mainly in a beta sheet with a few small alpha helix segments [1]. Chymosin has the enzymatic capacity to clot milk. This property is used to determine the enzyme activity which is optimal between pH 5.5 and 6.3. At present, other acid proteases with milk clotting activity are used in the cheese industry, such as proteases produced by fungi [2]. We have selected an identical chymosin produced for genetically modified *Aspergilus niger* because it is one of the acid proteases mostly used in the cheese industry.

Other acidic protease widely used in food and pharmaceutic industry is pepsin which has a single polypeptidic enzymatic chain of 324 amino acid residues. It is the principal proteolytic enzyme of vertebrate gastric juice. Its molecular weight is 35,000 and the optimun activity pH and isoelectric point is 1.0 [3].

The traditional methods for the isolation and purification of proteins involve some steps: ammonium sulfate precipitation, ionic and affinity chromatographies, dialysis and final concentration of the product, which require long time and high cost. This induces an increase in the unfolding fraction of the macromolecule, with loss of its biological activity and

Abbreviations: PEG 1450, PEG 3350; PEG 6000 and PEG 8000, polyethylenglycol of average molecular mass 1450, 3350, 6000 and 8000, respectively; CHY, chymosin; PEP, pepsin; MW, molecular weight

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a poor yield of the whole process. The purification process is a great and important 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 purification grade.

As regards this, partitioning in aqueous two-phase systems (ATPS) is a good alternative method to separate and purify mixtures of proteins [4]. ATPS has been used as a first purification step since such systems allow removal contaminants by a simple and economic process because they can be present in a homogenate of a natural or genetically modified product. ATPS have a number of advantages with respect to the conventional methods for the isolation and purification of proteins: the partition equilibrium reaches very fast, it can be applied in scale up and it has the possibility of continuous steady state operation. Besides, its low cost and the materials that form this system are not expensive and can be recycled.

ATPS are formed by mixturing two flexible chain polymers in water or one polymer and a salt (phosphate, citrate, etc.). Proteins are partitioned between two phases with a partition coefficient that can be modified by changing the medium experimental conditions such as pH, salts, ionic strength, etc.

Experimental data on protein partitioning in ATPS are essential for the prediction of the biomolecule distribution coefficient as a function of system variables such as molecular mass and polymer concentration, pH, temperature, etc. [5]. When ATPS are applied to isolate a protein that is present in a complex protein mixture (i.e. an homogenate of a natural product or a protein expressed in a microorganim), the first research step is to determine the partitioning features of the target protein in the pure state and the effect of medium variables on its partition coefficient [6]. Diamond and Hsu [9] found that $\ln K$ decreases with the PEG concentration difference between the phases for different proteins of 12,000–100,000 molecular mass. However, proteins such as chymotrysinogen-A [10] showed a positive slope for the plot $\ln K$ versus Δ[PEG], but this difference in the partition behaviour was not explained by these authors.

Applying the Flory Higgins theory of polymer thermodynamic, Diamond and Hsu [9] arrayed the following simple linear relation to correlate protein partitioning in aqueous two-phase system:

$$\ln K = A[\text{PEG}] + \text{cte} \quad (1)$$

This simplified relationship could adequately describe the partition data of some proteins, A being a constant that includes the polymer and protein molecular mass. Moreover, the constant A involves the protein–medium interaction too and it can be experimentaly calculated from the slope of the $\ln K$ versus Δ[PEG] plot.

Due to the fact that the cheese industry is very important in the area where our laboratory is situated, the requirement of these proteases is essential. Therefore, the goal of this work was to make a preliminary study about the partitioning features of chymosin and pepsin in aqueous two-phase systems of polyethylene glycol-potassium phosphate so that this technique could be applied in the near future as a first step to isolate these enzymes from an animal or microorganism source.

2. Materials and methods

2.1. Chemicals

Chymosin (CHY) was gently donated by Chr. Hansen (Denmark) produced by fermentacion of *Aspergillus niger*. It was previously dialysed against buffer sodium phosphate, 50 mM; pH 7, and concentrated by ultradialysis. Its purification was determined by PAGE electrophoresis, the content of pure enzyme proved to be greater than 96%. Pepsin (PEP), polyethylene glycol of the average molecular weight 1450, 3350, and 8000 (PEG 1450, PEG 3350, PEG 8000) were purchased from Sigma Chem Co. (USA), the PEG 6000 was purchased from Merck, and used without further purifications.

2.2. Binodial curves obtention

The binodial diagram for PEG–potasium phosphaste systems were obtained at pH 7 from the method of turbidimetric titration as it was described in detail in [7,8].

2.3. Preparation of the aqueous biphasic system

To prepare the biphase aqueous systems, stock solutions of the phase components: PEG, 40% (w/w) and 30% (w/w) potassium phosphate solution were mixed according to the binodal partition diagram previously obtained. Low-speed centrifugation was used after gentle mixing of the system components to speed up phase separation, then 2 ml of each phase were mixed to build up several two-phase systems in which the protein partition was assayed.

2.4. Determination of the partition coefficient (K)

The partition coefficient of the proteins between both phases was analyzed by dissolving increasing amounts of protein solution (5–15 μl) in the two phase of pre-formed system containing 2 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 30 min, 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 measuring the native fluorescence emission of the protein at 340 nm, while exciting at 280 nm. The fluorescence values were referred to a protein calibration

curve in each phase. The partition coefficient was defined as:

$$K = \frac{[P]_{\text{top}}}{[P]_{\text{bottom}}} \quad (2)$$

where $[P]_{\text{top}}$ and $[P]_{\text{botton}}$ are equilibrium concentrations of partitioned protein in the PEG and phosphate rich phases, respectively. In the protein concentration range assayed, a plot of $[P]_{\text{top}}$ versus $[P]_{\text{bottom}}$ showed a linear behavior, K being its slope. The enzymatic yield recovery in the top phase (y, %) was also calculated according to the given equation:

$$y\,(\%) = \frac{100}{1 + (1/RK)} \quad (3)$$

where $R = V_T/V_B$, V_B and V_T are the bottom and top phase volumes; and K, the partition coefficient.

The enthalpic change associated to the protein partition in the ATPS was calculated applying the known equation:

$$\frac{\partial \ln K}{\partial T} = -\frac{\Delta H^{\circ}}{T^2} \quad (4)$$

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

$$\Delta G^{\circ} = -RT\ \ln K \quad (5)$$

$$\Delta S^{\circ} = \frac{\Delta H^{\circ} - \Delta G^{\circ}}{T} \quad (6)$$

2.5. Fluorescence spectroscopy measurements

Emission fluorescence spectra were obtained exciting the protein at 280 nm in the top and bottom phases. The spectra were corrected according to a programme given by the instrumental manufacturer. Fluorescence measurements were performed in a S2000 Amicon Bowman with a themostatized cuvette.

2.6. Clotting milk activity measurements

The milk clotting activity was measured in 2 ml of skim milk. Skim milk solution was prepared by dissolving 10 g of a skim milk powder in 100 ml of $CaCl_2$ 10 mM solution and stirring 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. A control of activity was performed adding CHY and PEP in a medium of 50 mM pH 6.5 phosphate during 1 h and the activity of the increasing amounts of these solutions was measured. A 100% of activity was assigned to the slope of time clotting versus 1/volume of added protein solution plot. The same experiments were carried out incubating the same amount of CHY and PEP in both top and bottom phases during 1 h and the slope of the same plot was compared.

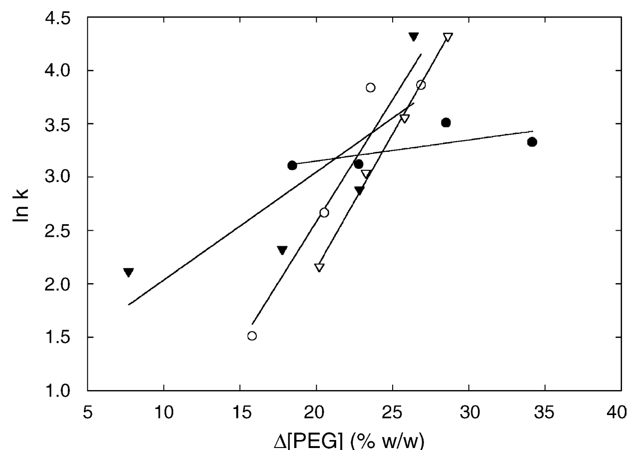

Fig. 1. Dependence of the PEG concentration diference between the top and bottom phases for the CHY partition, PEG 1500 (●), PEG 3350 (○), PEG6000 (▼) and PEG8000 (▽). Medium conditions: pH 7.0. Temperature, 8 °C.

2.7. CD measurements

Circular dichroism spectra (CD) were performed in a Jasco 810 dichrograph, using a thermostatized cuvette of 1 mm of pathlength, a repetitive scanning of four cycles was used.

3. Results and discussion

3.1. PEG concentration and molecular mass effect of the chymosin and pepsin partition

Figs. 1 and 2 show the partition data for CHY and PEP in PEG–phosphate systems at increasing Δ[PEG] values, calculated as the difference between the PEG equilibrium concentration in the top and bottom phases. The general observed trend is a linear relationship between $\ln K$ and Δ[PEG]. This situation has been noticed for the partition of many proteins at

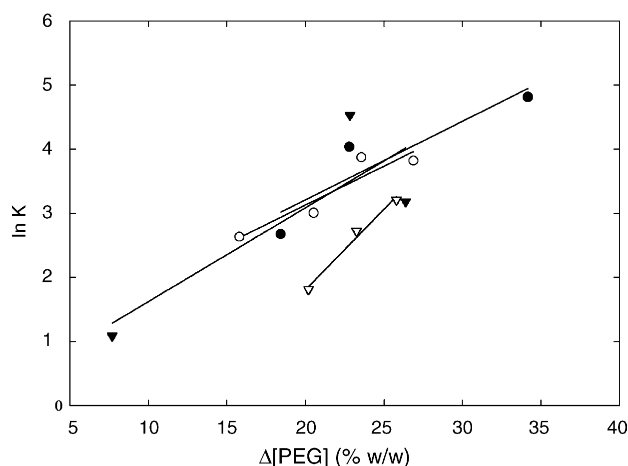

Fig. 2. Dependence of the PEG concentration diference between the top and bottom phases for the CHY partition, PEG 1500 (●), PEG 3350 (○), PEG 6000 (▼) and PEG 8000 (▽). Medium conditions: pH 7.0. Temperature, 8 °C.

Δ[PEG] values up to 25% (w/w), which are our experimental conditions.

The ln K versus Δ[PEG] curves showed to be lineal; moreover, the CHY and PEP partition proved to be in favour of the rich PEG phase. CHY and PEP have shown to have a partition coefficient between 10 and 20, it is seen in the Figs. 1 and 2. A partition coefficient of 2–4 has been reported for chymotrysinogen-A (MW 23,000) [10]. Also, the increase of Δ[PEG] induced a significant increase in the K value, in constrast with previous reports which showed a partitioning decrease at increased Δ[PEG] values [8].

The slope A of Eq. (1) is a function of protein and polymer molecular mass and the interaction of protein with the polymer and water. Our finding agrees with the above equation. in the sense that a net line relationship was obtained. The slope value increases with the PEG mass molar which suggests the presence of a good PEG–protein interaction which is more important than the excluded volume effect. If the PEG excluded volume effect were the only effect that drives the CHY and PEP partition, the slope of Figs. 1 and 2 should have a negative value.

Haghtalab et al. [11] presented experimental results for 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 versus [PEG] curves had both positive or negative values depending on the nature of the protein–PEG–salt interaction coefficient.

Table 1 shows the experimental A values calculated from Figs. 1 and 2 as $M \ln K/M\Delta$[PEG]. It can be seen that the A values of both CHY and PEP proteins were positive for all PEGs. Moreover, the A values increase in the same way that the PEG molecular mass does.

The excluded volume theory shows that the PEG concentration or its increase in the molecular mass induces a diminution of the protein solubility in the phase where the protein is situated. This theory cannot explain the positive slope value for the plots ln K versus Δ[PEG] observed for CHY and PEP under our working conditions.

Previous reports about the PEG–protein interaction [12] have demonstrated the presence of a light interaction between them, but it is not possible to define specific binding sites for the PEG molecule in the protein surface. The affinity constant between a protein and PEG has been measured in an indirect form, such as protein electrophoresis [13] in the presence and absence of PEG. Farruggia and Picó [14], using the optical probe 1-anilino-8-naphthalene sulfonate (ANS) calculated the affinity of albumin by PEG in an indirect form, measuring the displacement of ANS bound to albumin by PEG. They found an affinity in the order of the $10^2\,M^{-1}$ which increases with the PEG molecular mass. This value is very low when it is compared with other measurements for ligands which have a specific interaction with proteins, and suggests that the protein–PEG interaction has an nonspecific nature for a great number of proteins. The authors explained this behaviour taking into account the PEG capacity to form a compact structure with a greater probability to interact with the protein domain, due to the low size.

Table 1
A values of the Eq. (6) calculated as the slope of the curves ln K vs. Δ[PEG] for the CHY and PEP partition at different PEG molecular mass

System	CHY	PEP
PEG 1500	0.020 ± 0.014	0.122 ± 0.053
PEG 3300	0.229 ± 0.047	0.121 ± 0.034
PEG 6000	0.101 ± 0.049	0.146 ± 0.096
PEG 8000	0.252 ± 0.011	0.251 ± 0.030

The data have been taken at a temperature of 8 °C.

Arakawa and Timasheff [15] examined the interaction between PEG and beta lactoglobulin as a function of PEG molecular mass; the preferential exclusion increases with an increase in the PEG size. This result argues in favour of the steric exclusion as the factor to determine the interaction of PEG with proteins. In the case of PEG–betalactoglobumin and PEG–tubulin [16] studied by Lee and Lee [12], the PEG molecular mass increase was found to induce an exclusion of the polymer from the protein domain, increasing in this way the preferential hydration of the protein. However, at high PEG concentration, a decrease of the PEG exclusion for the betalacto–PEG interaction was observed. This opposed behaviour of PEG can be better understood in terms of the PEG solution behavior. It has been demonstrated that PEG, which is a flexible molecule, can acquire compact structure stabilized by intramolecular hydrophobic bonds. The PEG compact structure has a lower interaction with the solvent than the fully extended ones; this allows the PEG molecule to interact with the protein domain. This change in the PEG exclusion grade should be greater for large PEG molecules. In this case, a major interaction PEG–protein was observed.

PEG solution of high concentration passes into the hydration layer of the protein and allows 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 opposed factors: the PEG exclusion and the PEG protein binding through

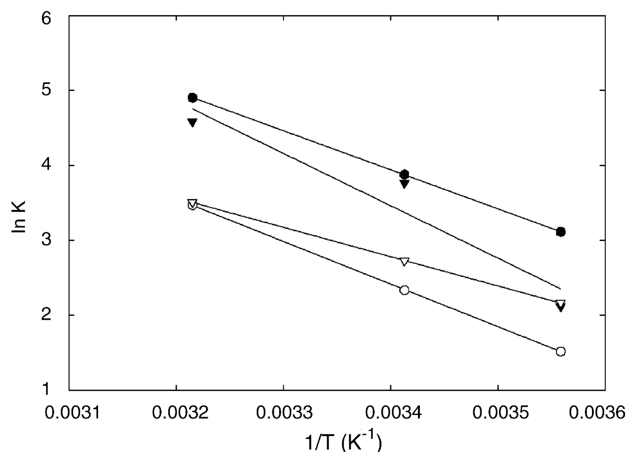

Fig. 3. Vant Hoff plot. Temperature effect of the partition equilibriun of CHY, PEG 1500 (●), PEG 3350 (○), PEG 6000 (▼) and PEG 8000 (▽). Medium conditions: pH 7.0.

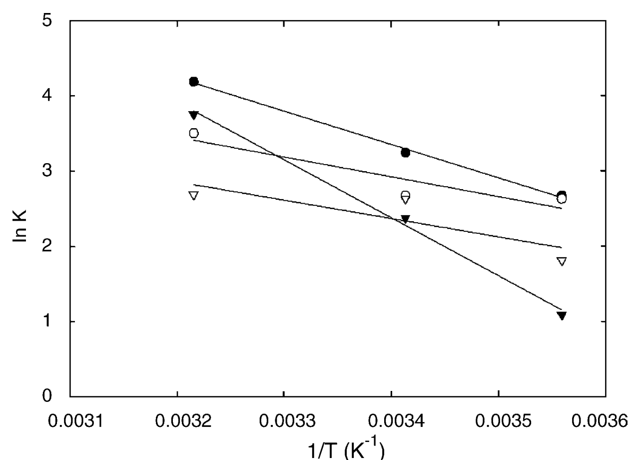

Fig. 4. Vant Hoff plot. Temperature effect of the partition equilibriun of PEP, PEG 1500 (●), PEG 3350 (○), PEG 6000 (▼) and PEG 8000 (▽). Medium conditions: pH 7.0.

the hydrophobic area of the protein exposed to the solvent. This last effect will depend on the chemical structure of the protein. Proteins, with great hydrohobic surface area exposed to solvent, have the possibility of interacting with PEG. This interaction has a high affinity; this factor being the one which drives the protein partition in favour of the PEG-rich phase.

Our finding suggests that the PEG–protein–salt interaction prevails on the excluded volume effect, which allows us to explain why the PEG molecular mass and the PEG concentration increase induced a significant enhancement of the partition in favour of the PEG-rich phase.

3.2. Temperature effect on CHY and PEP partition

Direct measurements of the heat involved in the protein partition cannot be carried out due to the fact that is impossible to develop this process in a calorimeter. Therefore, analysis of the K variation with a temperature change, may provide thermodynamic data as regards the partitioning process. Figs. 3 and 4 show the temperature effect on the partition coefficient expressed as Van't Hoff plots. A linear relation which suggests the presence of only one temperature-dependence process can be seen. In all the cases, an increase in the partition in favour of the PEG-rich phase with an increase in temperature, was observed, in agreement with the endothermic character of the partitioning. This finding is similar to that found for other proteins. [17]. The thermodynamic value functions were calculated as shown Table 2 applying Eqs. (3) and (5).

Table 2
Enthalpic and entropic changes for the CHY and PEP partitioning in aqueous two-phase system of different PEG molecular mass

System	CHY		PEP	
	ΔH (kcal/mol)	ΔS (e.u.)	ΔH (kcal/mol)	ΔS (e.u.)
PEG 1500	10.54 ± 0.025	37.14	8.87 ± 0.24	31.58
PEG 3350	11.40 ± 0.040	40.59	5.26 ± 1.10	18.72
PEG 6000	13.99 ± 2.00	49.80	15.41 ± 0.52	54.87
PEG 8000	7.85 ± 0.042	27.95	4.84 ± 1.48	17.25

It is interesting to note that the entropic change for the two protein partition is positive, and that the entropic change is the factor that drives the protein partition from the thermodynamic point of view. The enthalpic change showed an increase in its endothermicity with the increase in PEG molecular mass, but a decrease at high PEG molecular mass was observed. A similar behavior for the entropic change was observed. The change found in the thermodynamic functions was high for these proteins compared to that obtained for other proteins such as ovoalbumin and serum albumin [15]. For these proteins, the ΔH observed ranged from 1–5 kcal/mol, while the entropic changes were in the order of 0–20 e.u. Since these thermodynamic functions provide information about the molecular mechanism which takes part in the protein transfer from the salt to the PEG-rich phase, the great enthalpic change suggests the breaking of numerous intermolecular bonds (structured water in the protein domain and in the polymer) when this transfer is carried out. This effect could be due to an important PEG–protein interaction which induced a displacement of the water in the protein domain to favour its interaction with PEG.

The important entropic change observed agrees with this mechanism and is associated to an increase of the system disorder by the structure breaking capacity of the PEG in the protein domain.

3.3. Medium effect of the protein structure

When ATPS are used in the isolation and purification of proteins, it is important to determine if the medium affects

Table 3
Effect of PEG on the secondary structure, surface tryptophan and biological activity of the CHY

System	Fluorescence peak (nm)		Specific activity (arbitrary unit)		%Recovery in the top phase	
	CHY	PEP	CHY	PEP	CHY	PEP
None PEG	327.6	341.4	100	100		
PEG 1500	324.6	336.8	100	130	95	93
PEG 3350	324.8	338.8	112	143	81	93.6
PEG 6000	322.8	339.2	150	142	89	74.8
PEG 8000	326	338.8	162	140	89	85.8

Protein native fluorescence and milk clotting activity were measured at a protein concentration of 0.36 mg/ml and 0.082 mg/ml for CHY and PEP, respectively. The enzyme activity was measured at 35 °C.

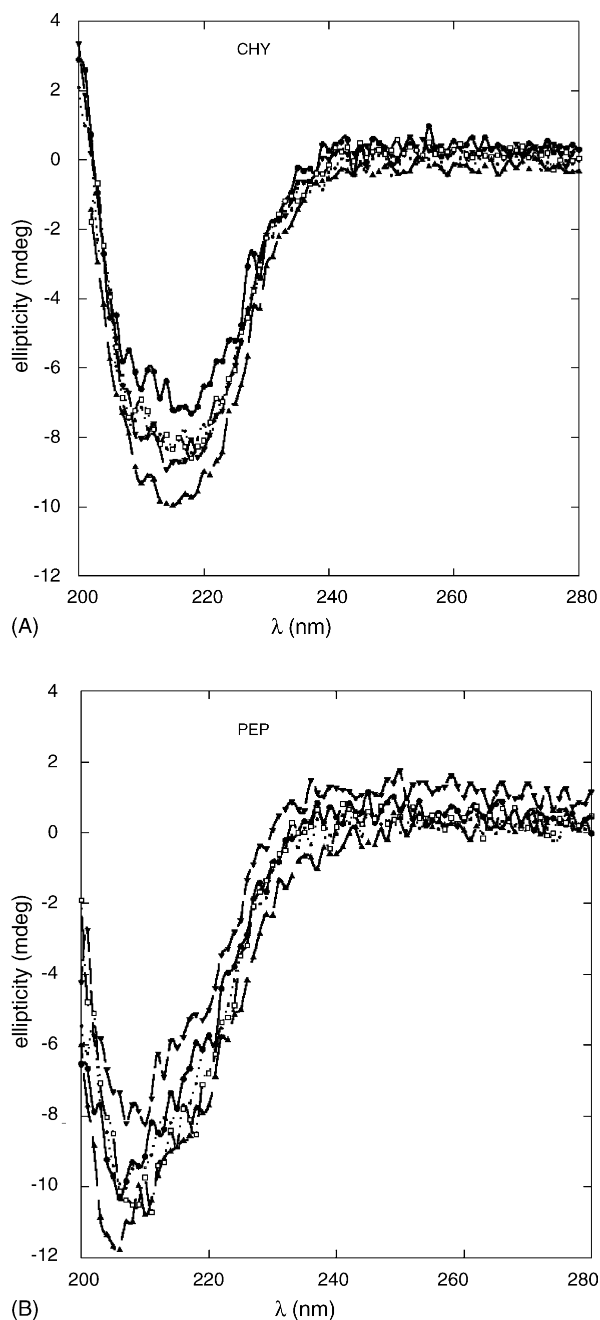

Fig. 5. DC spectra of CHY (A) and PEP (B) in the absence (●) and presence of polyethyleneglycol PEG 1500 (●), PEG 3350 (▼) PEG 6000 (□) and PEG 8000 (▲). Temperature, 20 °C. The PEG media are the top phases of the systems assayed in Fig. 1. The mediun in the absence of PEG was 50 mM pH 7.0 buffer phosphate.

the protein structure significantly as well as its biological activity.

3.3.1. Protein CD spectrum

Circular dichroism is a common tool for the analysis of the protein secondary structure, the CD spectrum in the far UV region (260–190 nm) is related to the alpha helix and the beta sheet content of a protein. Fig. 5 shows the CD spectra of the CHY and PEP in the different top phase media. CHY proved to have a CD spectrum similar to other proteins with a well defined zone between 225 and 210 nm, although the alpha helix content of CHY is low.

The presence of PEG induced a decrease in the CD signal in the 220 nm zone that agrees with a major content of alpha helix which implies a greater protein stability. The protein stability increases according to the PEG molecular mass, the medium of PEG 8000 being the one which induced the main content of alpha helix.

These results are important when the liquid–liquid extraction method is applied to isolate CHY, because it is proof that the PEG-rich phase would stabilize the CHY structure. This finding agrees with the milk clotting capacity of CHY in the top phase as shown in Table 3.

Since PEP has a lower alpha helix content than CHY, it showed a poor CD spectrum. Therefore, it is difficult to obtain good CD spectra in the 210–225 nm zone of this protein in PEG medium as shown in Fig. 5B. Therefore, an effect between the PEG molecular mass and this protein cannot be observed. However, PEG 8000 was shown to induce a significant decrease in the CD signal, in agreement with a major stabilization of the PEP structure in this medium.

3.3.2. Native fluorescence emission spectrum

The native fluorescence spectra of CHY and PEP for the proteins in both top and bottom phases were acquired (data not shown). The peak position is a measure of the accessible tryptophan perturbation of the protein. According to the Lippert equation [18], which allows the study of the interaction between a fluorophore and its environment, the energy of the emitted photon is a direct measurement of the fluorophore–medium interaction. In our case, the position of the peak allows us to have an idea of the strength of the PEG perturbation on the protein surface exposed to the solvent. Table 3 shows the values of the fluorescence emission peak of both proteins in PEG medium of different molecular mass. The experiments were performed at the major Δ[PEG] values assayed in Figs. 1 and 2.

In a medium of 50 mM, pH 7.0 buffer phosphate, the CHY showed a native fluorescence spectum centred at 327.6 nm, which suggests that the tryptophan residue environment has more hydrophobic character than some proteins which have a peak at about 340 nm. This finding agrees with the hydrophobic character of this protein in an aqueous medium. PEP showed a fluorescence peak centred at 341.4 nm which corresponds to the peak position for more hydrophilic proteins. Both proteins showed a significant blue shift in the native fluorescence emission in presence of PEG, the magnitude of the shift depended on the PEG molecular mass. This finding suggests an important PEG protein interaction. It has been reported that bovine serum albumin [19] does not vary its native fluorescence emission in the presence of PEG. A blue shift of 2–3 nm in PEG 600 and 1000 was only found and the effect produced by other PEGs was negligible. It has also been reported that PEG of high molecular mass (8000)

induce a little shift to the red for the albumin fluorescence. The magnitude of the fluorescence shift of 4–6 nm found for CHY and PES in the presence of PEG suggests that: (1) the solvent reaches to the protein tryptophan, (2) an increase in the hydrophobicity on the tryptophan microenvironment is produced in the presence of PEG. This is a proof of the existence of a PEG–protein interaction, a situation which could not be found for other proteins of high molecular weight such as albumin and (3) PEG 8000 induced a minor shift of the fluorescence because, it is excluded from the protein domain to a greater extent.

3.3.3. Milk clotting activity of CHY and PEP in the presence of PEG

The CHY activity increases according to the PEG molecular mass, while PEP activity was not significantly modified with the PEG molecular mass as shown Table 3. This finding agrees with the increase of alpha helix content of CHY induced by PEG. The strong PEG–CHY interaction induces a conformational modification in the catalytic site of the enzyme which increases its milk clotting activity. The increase of the enzyme activity may not be due to the modification of the medium where the measurement was made adding PEG because only a volume of 20–50 ul of the top phase medium containing the enzyme was added to 2 ml of milk; the composition modification of the medium being negligible.

3.4. Recovery of the CHY and PEP by liquid–liquid extraction method

Since one of our goals is to use the liquid–liquid extraction method to isolate CHY and PEP in the near future, the recovery of these proteins (y, %) in the top phase was calculated applying Eq. (2), which predicts that the top/bottom ratio volume determines the recovery capacity of ATPS for a target macromolecule. Table 3 shows the recovery values obtained for the different PEG systems under our experimental conditions (a top/bottom ratio = 1). Both proteins were recovered with a great efficiency in the top phase: CHY, 81–95% and PEP, 74–93%, which could make the liquid–liquid extraction method suitable to be applied as an isolation method for these proteins.

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