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Pepsin extraction from bovine stomach using aqueous two-phase systems: Molecular mechanism and influence of homogenate mass and phase volume ratio

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ABSTRACT

Pepsin partitioning, a gastric acid protease, in aqueous two-phase systems of polyethyleneglycol/potassium phosphate, sodium citrate and ammonium sulphate was assayed using polyethylenglycol of different molecular mass. Pepsin was found to be partitioned towards the polymer-rich phase in all the systems, which suggests an important protein–polymer interaction due to the highly hydrophobic character of the protein surface exposed to the solvent. The pepsin partitioning behavior was explained according to Timasheff's preferential interaction theory. The process was driven entropically with participation of structured water around the polyethyleneglycol ethylenic chains. The best pepsin recovery was observed in the systems polyethyleneglycol molecular mass 600. These systems were chosen in order to assay the bovine stomach homogenate partition and to compare different working conditions such as the top-bottom phase volume ratio and homogenate proportions in the total system. The best purification factors were obtained with PEG600/potassium phosphate with low top-bottom volume ratio using 15% of bovine stomach homogenate in the system total mass.

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1. Introduction

Pepsin (EC 3.4.23.1) is an aspartic protease that acts in food digestion in the mammalian stomach. It has a molecular mass of about 35 kDa. An optimal pH of around 2 allows pepsin to operate in its natural acidic environment [1]. Gastric proteases are traditionally used for the manufacture of most cheese varieties, calf and bovine rennet, the extracts from the abomasum being the most common. Both contain chymosin and pepsin at different levels depending on the age of the animal [2]. Pepsin is also used in leather and pharmaceutical industry. These proteases are all synthesized as zymogens, and they are converted into active enzymes by a limited proteolysis [1], pepsinogen being the pepsin zymogen.

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 a combination of operations such as centrifugation, precipitation, membrane and gel filtrations, ion-exchange and affinity chromatography or dialysis and final concentration of the product. These methods are time-consuming and some expensive reactants contribute to the increase of the cost of the downstream processing.

For this reason, partitioning in aqueous two-phase systems (ATPSs) is a good alternative method to separate and purify mixtures of proteins [3,4]. ATPS are formed by mixing two flexible chain polymers in water or one polymer and a salt (phosphate, citrate, etc.) [3]. Proteins are partitioned between the two phases with a partition coefficient which can be modified by changing the experimental conditions of the medium such as pH, salts, ionic strength, etc.

ATPSs have been used as a first purification step since contaminants can be removed by a simple and economical process and can then be turned into a homogenate of a natural or genetically modified product. ATPSs have a number of advantages over the conventional methods for the isolation and purification of proteins: the partition equilibrium is reached very fast, they can be applied in scale up, they offer the possibility of continuous state operation, the cost is low and the materials are economical and recyclable.

Abbreviations: ATPS, aqueous two-phase system; PEG600, PEG1000 and PEG1450, polyethylenglycol of average molecular mass 600, 1000 and 1450 respectively; Ci, citrate; Pi, phosphate; Su, sulphate; PEP, pepsin; BSH, bovine stomach homogenate; Mm, molecular mass.

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Two steps are necessary in the design of an enzymatic purification method using ATPS: first, it is necessary to know the partition features of the pure target protein, and the effects of the different variables on it. Second, the partitioning of the target protein when it is present in its natural product is measured and the best experimental situation for the separation of this protein should be determined [5].

The goal of this work was to study the shape of the pepsin partition in an aqueous two-phase system formed by PEG/salts with the aim to apply these results to isolate and later purify this protein from bovine stomach. The salts chosen were potassium phosphate, potassium citrate and ammonium sulphate.

2. Materials and methods

2.1. Chemicals

Porcine pepsin (EC 3.4.23.1), (PEP), Bovine Serum Albumin (BSA), polyethyleneglycol of the average molecular weight 600, 1000 and 1450 (PEG600, PEG1000 and PEG1450) were purchased from Sigma Chem Co. (USA) and used without further purification. All the other reagents were of analytical quality.

2.2. PEP concentration determination

Pepsin concentration was determined through enzymatic activity at pH 2.0 and 37 °C with a 2.5% hemoglobin solution as substrate by using the method of Anson and Mirsky [6]. The hydrolysis products were determined by the Lowry method and expressed as mg/mL from the calibration curve which was performed in the same conditions as the unknown sample. PEP calibration curves were carried out in each of the top and bottom phases of all the used systems.

2.3. Total protein concentration determination

The protein concentration in both phases of the systems was determined according to the Lowry method [7] by using bovine serum albumin as standard. Blank systems without proteins were used as reference and no interference from phase components was observed. Absorbance measurements were made on a Jasco V-500 UV/visible spectrophotometer.

2.4. Preparation of bovine stomach homogenate (BSH)

Sections of frozen gastric mucosa of adult bovine were homogenized with about 5 volumes of 50 mM sodium phosphate buffer, pH 7.0. Then the homogenate was filtered, fractioned and frozen. Before using it, the fat was withdrawn and the homogenate centrifuged at 15,000 × *g* for 30 min. Total protein concentration and enzymatic activity was carried out in the supernatant [8].

2.5. Pepsinogen activation

The procedure for pepsinogen activation was made at 20 °C by slow addition of HCl concentrate to the homogenate solution in order to diminish the pH until the solution of homogenate reached a value of 2.5. It was allowed to rest for 30 min. A slight turbidity was observed in all the cases due to a minimal precipitation of proteins [9]. Then it was taken to pH 6.4 with concentrate NaOH (3 M). After that, the solution was centrifuged at 1000 × *g* during 5 min.

Table 1
Total compositions of systems tested and the PEG concentration difference among the two phases in % (w/w)

System	[PEG] (%, w/w)	[salt] (%, w/w)	Δ[PEG] $[PEG]_T - [PEG]_B$ % (w/w)
PEG600/Pi	17.00	16.90	29.22
PEG1000/Pi	15.00	15.70	27.94
PEG1450/Pi	13.88	15.74	28.61
PEG600/Ci	18.55	15.26	27.94
PEG1000/Ci	15.92	13.97	23.84
PEG1450/Ci	16.27	12.09	24.68
PEG600/Su	24.07	18.80	49.48
PEG1000/Su	21.00	20.00	53.50
PEG1450/Su	18.50	16.00	41.20

2.6. Preparation of the aqueous two-phase systems

The PEG molecular masses chosen in this work were PEG600, PEG1000 and PEG1450. The PEG/salts systems were prepared from stock 40% (w/w) PEG solutions of different molecular mass and 28% (w/w) pH 7.0 potassium phosphate (Pi), 25% (w/w) pH 5.3 potassium citrate (Ci) and solid ammonium sulphate. In this latter system, the pH is determined by the salt. The system compositions, which are indicated in Table 1, were chosen according to the binodial diagrams previously obtained by Lei et al. [11], Tubío et al. [12] and Albertsson et al. [5]. In the PEG/Pi and PEG/Ci systems, the tie lines of each system were considered with similar variation of the PEG concentration (Δ[PEG]) between both phases. The PEG/Su systems were chosen with the Δ[PEG] corresponding to the lower possible concentration that produces a better phase separation at the two temperatures assayed. Low-speed centrifugation to speed up phase separation was used after gentle mixing of the system components, and then 1 mL of each phase was mixed to reconstitute the different two-phase systems in which the protein partition was assayed.

2.7. Determination of the total protein (K_p) and PEP coefficients (K_e)

Partition constant of the proteins between both phases was analyzed by dissolving an increasing amount of PEP solution (10–25 μL) in the two-phase pre-formed system containing 1 mL of each equilibrated phase, the change of the total volume of each phase being negligible. After mixing it 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 total protein concentration measurements.

$$K_p = \frac{[PEP]_{TOP}}{[PEP]_{BOTTOM}} \quad (1)$$

where $[PEP]_{TOP}$ and $[PEP]_{BOTTOM}$ are equilibrium concentrations of the partitioned PEP in the PEG and salt-rich phases, respectively. In the protein concentration range assayed, a plot of $[PEP]_{TOP}$ vs $[PEP]_{BOTTOM}$ showed a linear behavior, the partition constant (K_p) being its slope.

When the bovine stomach homogenate was partitioned, samples of top and bottom phases were taken for the enzyme assay and total protein, K_e and K_p, were defined as the ratio of enzyme activity or protein concentration in the top and bottom phases, respectively.

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

$$y(\%) = \frac{100}{1 + (1/K_{p \text{ or } e} R)} \quad (2)$$

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Table 2
Values of R and BSH percentage in the PEG600/Pi total system

Systems (%, w/w)	R	BSH mass (g) per 100 g of total system
PEG600/Pi (23.4/12.0)	1.9	7.5 15.0 25.0
PEG600/Pi (17.0/16.9)	0.8	7.5 15.0 25.0
PEG600/Pi (10.9/22.0)	0.37	7.5 15.0 25.0

where $R = V_T/V_B$ and V_T and V_B were the top and bottom volume, respectively. The yield recovery will be expressed as yield recovery of enzyme or yield recovery of protein according to whether K_p or K_e is used in this equation.

The purification factor was calculated by the equation:

$$PF = \frac{A_{TOP}/[P_{TOTAL}]_{TOP}}{A_{BSH}/[P_{TOTAL}]_{BSH}} \quad (3)$$

where A_{TOP}, $[P_{TOTAL}]_{TOP}$ and A_{BSH}, $[P_{TOTAL}]_{BSH}$ are the enzymatic activities and the total protein concentration in top phase and the homogenate, respectively.

2.8. Estimation of the thermodynamic functions associated with PEP partition

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

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

$$\frac{\partial \ln K}{\partial T} = -\frac{\Delta H^\circ}{T^2} \quad (5)$$

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

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

3. Results and discussion

3.1. The effect of PEG molecular mass on PEP partition in ATPs

In the second stage of this work, in order to analyze the effect of the top-bottom volume ratio (R) and the mass of the homogenate added to each studied system, 10 g of PEG600/Pi systems with three different R and also different amounts of BSH were prepared as can be seen in Table 2. The systems with different R were chosen from the Lei et al. binodial diagrams [11], on the same tie line of the previous experience and calculating their compositions from the binodial graphic. All the systems were prepared by adding the components in the same order: firstly, the solid PEG, secondly, BSH and buffer Pi and, finally, the necessary amount of water for each case.

Fig. 1 shows the influence of the PEG molecular mass on the pepsin partitioning in systems of PEG-salts at one of the two working temperatures, 8 and 30 °C. For all the systems, the partition coefficients were higher for the PEG/Pi than in the other ones (Fig. 1). The values of logarithm K vs logarithm PEG molecular mass were plotted (Fig. not shown). A straight line was obtained for all the systems and the values of the slope were calculated and shown in Fig. 2. All the systems yielded negative slope values, consistent with the increase of the PEG excluded volume in such a way that its

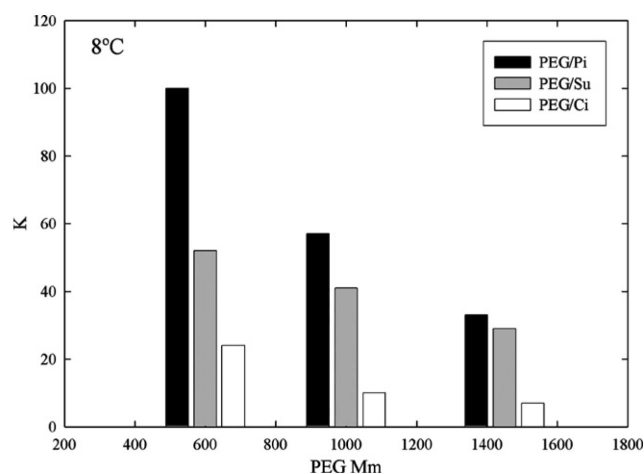

Fig. 1. Pepsin partition constant as a function of the PEG molecular mass in ATPSs of different salts. Temperature 8 °C. Medium PEG/Pi pH 7, PEG/Ci pH 5.3 and PEG/Su pH 5.0.

molecular mass increased because this induced a transfer of protein to the salt-rich phase.

According to Wyman's theory, if two reactions, such as protein partition and the interaction of a cosolute (PEG) with the protein, take place simultaneously, the direction in which the cosolute presence displaces the partition equilibrium may be expressed thermodynamically in terms of Wyman's linkage relation [13]. Thimasheff's theory [14] defines the preferential binding parameter as: $\partial m_3/\partial m_2$, where m_2 and m_3 are the molar concentration of protein and the cosolute (in this case, PEG), respectively. It is the expression of the amount of cosolute that would have to be added to (or removed from) the system to restore thermodynamic equilibrium when the protein is added.

The combination of the preferential binding parameter definition and the Wyman equation gives the following equation [15]:

$$\frac{\partial \ln K}{\partial \ln a_3} = \left(\frac{\partial m_3}{\partial m_2}\right)_{TOP} - \left(\frac{\partial m_3}{\partial m_2}\right)_{BOTTOM} = \nu_3^{TOP} - \nu_3^{BOTTOM} \quad (7)$$

where K is the partition coefficient, a_3 is the cosolute activity (PEG), and the $(\partial m_3/\partial m_2)_{TOP} - (\partial m_3/\partial m_2)_{BOTTOM}$ is the change in preferential binding parameter of the protein and PEG in the top and bottom phases. The preferential binding parameter, with a small

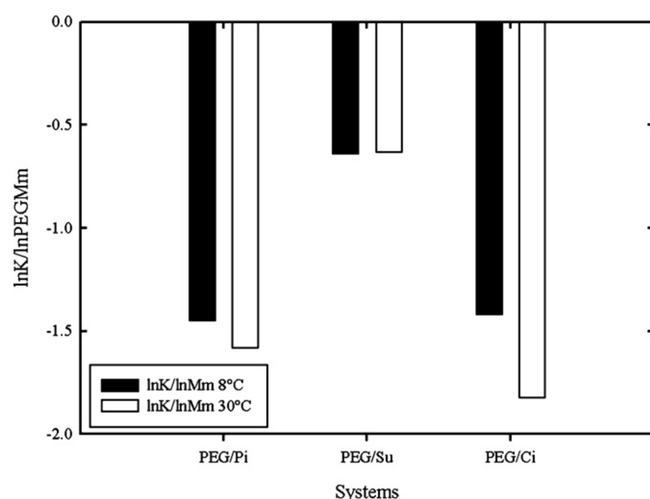

Fig. 2. Slopes of the ln K/PEG mM plots for the systems studied in Fig. 1, at 8 and 30 °C.

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approximation, is similar to ν_3, the binding parameter of Scatchard notation (ν_3 = moles of bound ligands per mole of protein) [14].

The parameter $\partial \ln K/\partial \text{PEG}_{\text{Mm}}$ has the opposite sign to the parameter $\partial \ln K/\partial \ln a_3$, because the PEG molar concentration decreases when the PEG molecular mass increases, consistent with the system concentration chosen. As found in other proteins, the increase of PEG molecular mass induced a pepsin transfer towards the salt-rich phase. Albertsson et al. (1987) [5] reported a similar behavior for some common proteins and based their discussion on the Flory-Huggins theory for polymers in solutions. They expressed the partition coefficient of a protein as a function of the molecular mass of the flexible polymer and the Flory coefficient of interaction between the protein and the polymer.

Positive values of the preferential binding parameter suggest a good interaction of the cosolute with the protein surface. Moreover, Abbott et al. [16] postulated that the attractive interaction between polymers and proteins could cause the collapse of polymer coils onto the protein surface, forming a polymer-protein complex. From inspection of Table 1, it is possible to note that for each type of systems, since the PEG concentrations are similar, the cosolute activity (a_3) decreases as the PEG molecular mass increases, therefore, the term $\partial \ln K/\partial \ln a_3$ is positive in all cases and so are the other two members of the Eq. (7). We have assumed that all the terms of the equation are positive because PEP is a hydrophobic protein that has an important interaction with PEG molecules. The central member of the Eq. (7), or the term $\nu_3^{\text{TOP}} - \nu_3^{\text{BOTTOM}}$, can be considered as the difference in moles of bound cosolute (PEG) per mole of protein between the top and bottom phase, which is higher than the moles of bound ligands per mole of protein in the top-bottom phases. The $\ln K$ vs $\ln a_3$ plot could not be plotted but the $\ln K$ vs PEG molar concentration plot (not shown) indicated that $\partial \ln K/\partial \ln a_3$ was not linear. The plots increased as the PEG concentration increased, which suggested that the preferential interaction parameter decreased with the increase in PEG molecular mass.

This fact agrees with the increase in the excluded volume of the PEG molecule with its molecular mass. The increase in temperature results in an increase in the slope value (see Fig. 2), suggesting that the temperature is another factor of protein exclusion from the polymer phase or that the temperature produces an increase in the electrostatic attraction forces between the salt and the protein.

In all cases, the major partition coefficients were observed in PEG600 systems, so they could be chosen in order to separate PEP from its natural source.

3.2. Temperature and salt effect on PEP partitioning

PEP partitioning was assayed at two different temperatures for the three ATPSs studied. By applying Eqs. (4)–(6), the enthalpic and entropic changes were calculated as shown in Fig. 3A and B. PEG-phosphate systems yielded negative enthalpic changes which decreased when PEG molecular mass increased. PEG/Su and PEG/Ci systems showed the opposite effect (i.e. the partition was endothermic). This effect decreased when the PEG molecular mass increased. This last effect has also been found for the partition of other proteins in ATPSs [17]. The positive and negative signs of ΔH° associated with the PEP partitioning in different salt media, suggest different molecular mechanisms for the partition of both kinds of systems. Whereas PEP transfer from salts to PEG phases is enthalpically favoured in PEG/Pi systems, PEP transfer in PEG/Ci and PEG/Su is driven entropically. The magnitude of this interaction decreased when the PEG molecular mass increased due to the fact that the preferential binding parameter decreased in the top phase with an increase in the PEG molecular mass (see Section 3.1).

When the entropic and enthalpic changes data were plotted one vs the other (Fig. not shown), a straight line was obtained consistent

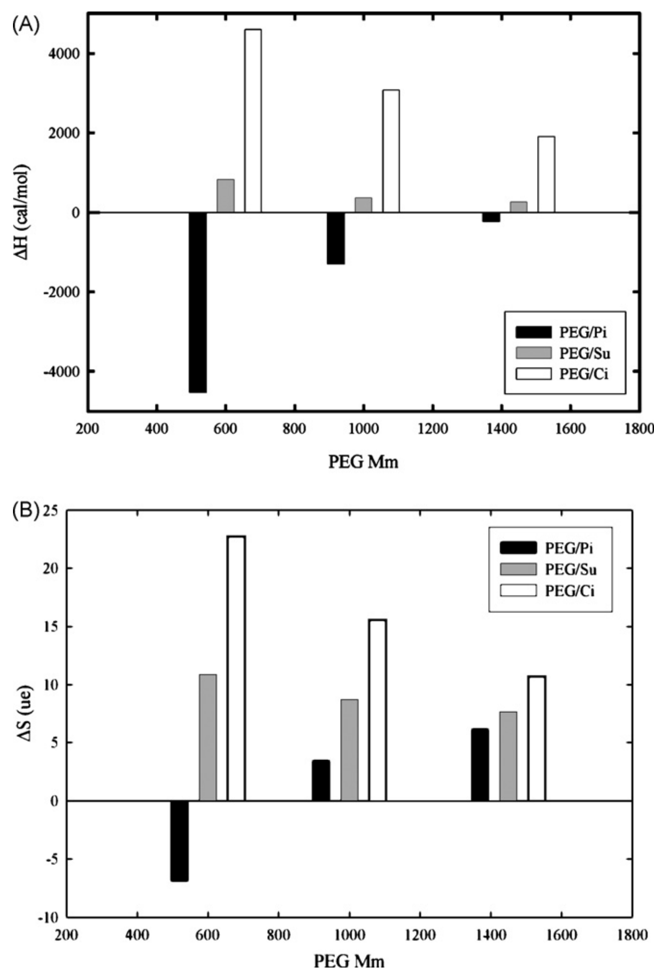

Fig. 3. Enthalpic (A) and entropic (B) changes as a function of PEG molecular mass. The experimental conditions are the same of Fig. 1.

with an entropic–enthalpic compensation process. This compensation suggests an order which is characteristic of the process, clearly due, to the interaction between the hydrophobic groups and the water solvent [18]. In this case, this could be associated with the structured water around the hydrophobic ethylene chain of the PEG molecule. When a protein is transferred from a salt-rich phase to a PEG-rich phase, a protein–PEG interaction takes place with the release of this ordered water, which explains the positive enthalpic and entropic changes.

3.3. PEP liquid–liquid extraction from a bovine stomach homogenate

In agreement with the results obtained, the systems of PEG600/salts were chosen to partition PEP from the bovine stomach homogenate at 20 °C because they presented the major partition constant at both temperatures. These results agree with a previous report from our laboratory [19] which indicates that the best systems to separate the PEP are those having low PEG molecular mass. In the chosen systems, some water necessary to form them was replaced by 1.5 g of the adequately prepared homogenate (15% BSH in the total system). After mixing, the systems were centrifuged at 3000 rpm for 3 min and left 2 h at a bath at 20 °C. Samples of top and bottom phases were taken in order to quantify the enzyme activity and total protein concentration. Besides, top and bottom phase volumes were measured in order to calculate the R parameter.

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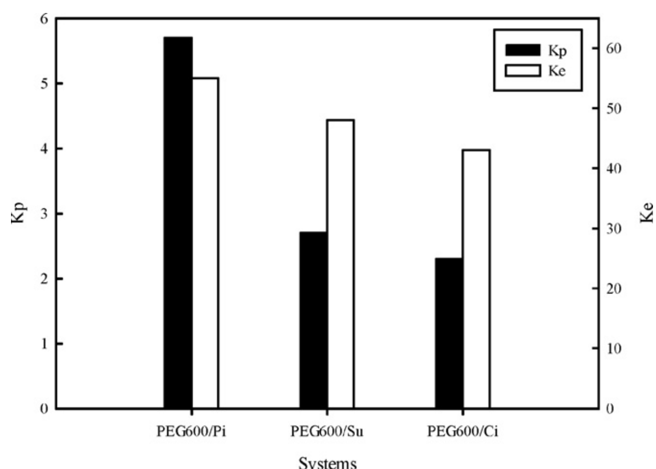

Fig. 4. Total protein and PEP partition coefficients (K_p, K_e) for the BSH partition, in the systems PEG600/Pi, R 1.14, PEG600/Su, R 0.94 and PEG600/Ci, R 0.89. The relation BSH/total system was 15%.

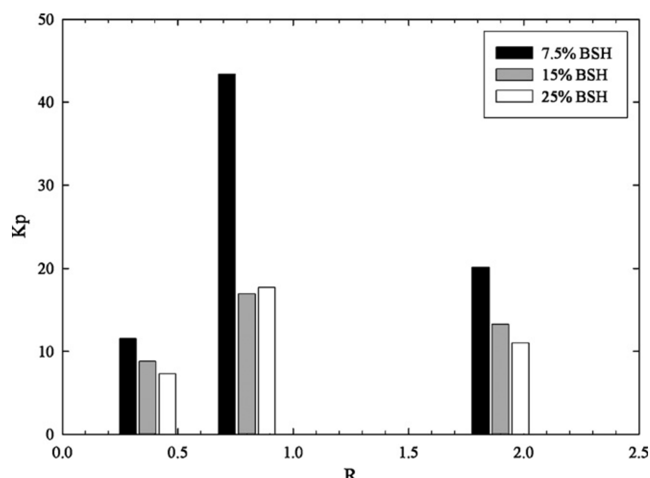

Fig. 6. Partition coefficients as a function of R for the PEG/salts systems with different relations of BSH.

Fig. 4 shows the partitioning coefficients of enzyme and total protein, K_e and K_p, for the PEG600/Pi (R = 1.14), PEG600/Su (R = 0.94) and PEG600/Cit (R = 0.89) systems. As can be seen, the K_p and K_e have an important difference between them, which suggests a high recovery of the enzymatic activity in the top-phase relation with the total protein partition. In Fig. 5 the purification factor indicates that the PEG600/Pi has the best value although all systems show a better activity recovery than protein recovery.

3.4. Analysis of PEP recovery from PEG600/Pi systems with variable R and percentage of BSH added

Three systems with different R and also different quantity of BSH added were studied. Fig. 6 shows the K_p value for different systems with variable R and total BSH percentage. The minor value of K_p was observed for the system with low volume ratio (R = 0.37), which indicates that the total proteins were partitioned towards the bottom phase as regards other systems. Fig. 7 shows the PEP purification factor (PF) for the different systems studied with different R values. The PF can be seen to increase at low R values and with medium or high quantities of BSH in the system.

The difference between the partition coefficients of PEP alone and in a bovine stomach homogenate may be due to the presence of the protein–protein interaction. It has been well demonstrated that the K_p value for a pure protein differs from that corresponding to the target protein in a natural product [14] owing to several causes. Usually, a natural product contains several hydrophobic proteins, cell debris, some electrolytes and peptides. Generally, the hydrophobic proteins have high molecular mass and precipitate in an irreversible manner when the homogenate is added to the biphasic system by interaction with its components.

Another point to be taken into account is that the binodial diagram of an ATPS may change when a natural product is partitioned, modifying the PEG and salt concentration at dealt with equilibrium, thus modifying the protein partition coefficient. Rito-Palomares et al. [20] have addressed this question, suggesting that the partition coefficient of a target protein should be determined from the natural product where it is present and not from a solution of a pure target protein.

Fig. 7 clearly shows that the variation in the homogenate mass influences significantly on the PF of PEP. When a mass of 7.5% of a total mass was added, the PF was low and remained independent of the top-bottom volumen ratio. However, when 15 and 25% were used, the PF increased only for a R value of 0.3, while at a greater R, the PF was low and similar between them. This last finding is very important when a design of a separation method using ATPs is carried out. At present, there is no state equation that allows us to

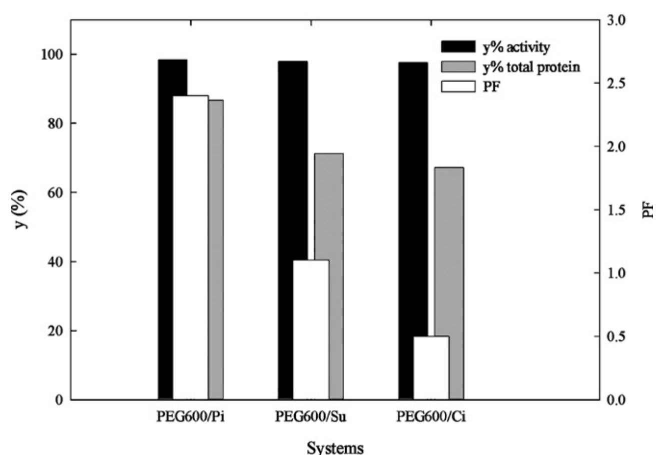

Fig. 5. Total protein and activity recovery (y (%)) and purification factor in the same systems of Fig. 4.

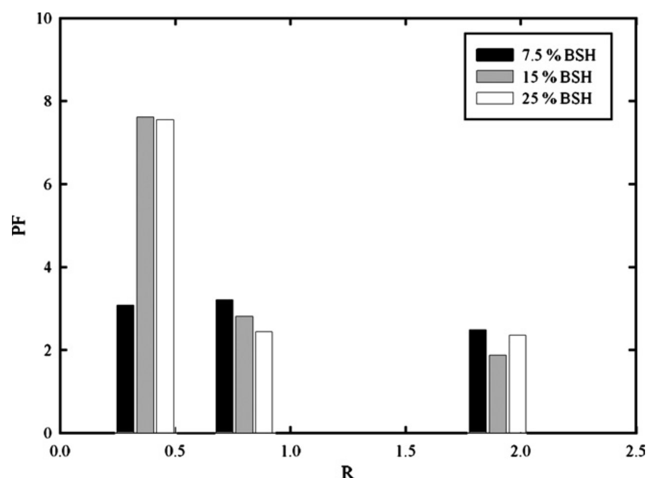

Fig. 7. Purification factor as a function of R for the PEG/salts systems with different relations of BSH.

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predict the behavior of the PF in function of the homogenate mass and the top-bottom ratio. One reason may be that each homogenate has a different behavior because the components are very different in quality and quantity, the interaction between protein and cellular debris are is dramatic and depend on the method of obtaining the homogenate. In same cases, obtaining a tissue from an animal or vegetal source is very complex and the different final homogenates obtained in different days using a standardized method produce different yields of the enzyme.

4. Conclusions

In all systems the pepsin is partitioned towards the top phase with a high partition coefficient which indicates an important interaction between the protein and PEG. This fact suggests a high solvent exposure of the hydrophobic character of the protein surface. The process is entropically conducted and could be due to the participation of structured water in the ethylenic chains around PEG molecule. The preferential interaction parameter decreases with the increase in PEG molecular mass, which agrees with the increase in the excluded volume increase of the PEG molecule with its molecular mass. The temperature is another factor of protein exclusion from the polymer phase and produces an increase of the electrostatic forces between the salt and the protein.

The pepsin recovery is more important in PEG600/salt systems and, when the BSH was partitioned in these systems, the partition of total proteins, though higher than one in all cases, is very much lower than the enzymatic activity partition.

From these results, several systems were tested with different volume relations chosen and the best recovery of enzymatic activity and the major purification factor were found in systems PEG/Pi.

On the PEG/Pi systems chosen, an analysis of variables such as volume ratio among the phases and homogenate quantity added to the total system was performed. The results shown that, with a low *R*, it is possible to concentrate the protein in the top phase and to isolate the PEP from the other contaminant protein. The best quantity of homogenate added to the total system was a 15%.

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