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Bromelain partitioning in two-phase aqueous systems containing PEO–PPO–PEO block copolymers

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Abstract

Bromelain is an enzymatic complex obtained from pineapple (*Ananas comosus*) fruits and stem. Thermoseparation of bromelain by poly(ethylene oxide) (PEO)– poly(propylene oxide) (PPO)– poly(ethylene oxide) (PEO) block copolymers aqueous solutions was studied. Triblock copolymers with different EO percentages and different molecular mass were evaluated. Copolymer solutions at different pH values, buffer concentrations and copolymer concentrations were investigated. It was found that cloud point temperature increases as a function of %EO and decreases with copolymer molecular mass, copolymer concentration and buffer concentration. The results showed that all the studied factors influenced enzyme partition. The best conditions were copolymer with 10% EO and molecular mass of 2000 g/mol, temperature of 25 °C, copolymer concentration of 5% (w/w), pH 6.0 and salt concentration of 15 mM. Enzyme activity recovery around 79.5%, purification factor around 1.25 and activity partition coefficient around 1.4 were obtained.

Keywords: Partitioning; Aqueous two-phase systems; Enzyme purification; Thermoseparation; Bromelain

1. Introduction

Aqueous two-phase systems are widely used for separation and purification of biomolecules [1,2]. These systems are suitable for purification of biological material as the phases contain 70–90% water, thus reducing the denaturation of labile molecules [3]. The advantages of aqueous two-phase extraction compared to other purification methods lie in volume reduction, high capacity and short processing times.

Recently, thermoseparating copolymers properties and their application in aqueous two-phase systems have been investigated, due to the possibility of utilization of these systems for solubilizing labile biological molecules, such as proteins [4–7]. When thermoseparating polymers are heated above a critical temperature, known as cloud point, the solubility of the polymer will decrease and a system composed of two phases (water and polymer phases) is formed. The cloud point is characteristic of each polymer and can be determined by the solution turbidity. The temperature induced phase separation makes it possible to partition a protein between the phases and consequently easily recycle the polymers in aqueous two-phase systems. Examples of thermoseparating polymers are random, diblock and triblock copolymers of hydrophobic poly(propylene oxide) (PPO) and hydrophilic poly(ethylene oxide) (PEO). Polymer hydrophobicity increases with increasing content of PO. The polymers behavior in solution depends on the percentage of PEO and PPO polymers, molecular mass, PEO and PPO block sizes. PEO and PPO content can be expressed in mass percentage, molar percentage or polymers block size. The dissolution rate decreases as the copolymer molecular mass increases for copolymer groups with the same PPO/PEO composition ratio. This is probably a result of the degree of hydrogen bonding between the copolymer molecules, and is also reflected in the physical form of the copolymers (liquid for low molecular mass, low PEO content; solid for high molecular mass, high PEO content copolymers) [8].

PEO–PPO–PEO are commercially available non-ionic polymeric surfactants. These polymers are dissolved as unimers at low temperatures, where water is a good solvent for both PEO and PPO. Water becomes a poor solvent for the PPO block at higher temperatures, and

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micelles are formed with a core of PPO chains and a water-swollen mantle of PEO chains [9]. At higher temperatures, polymers aggregate and form aqueous two-phase systems.

By using the thermoseparating polymers it has been possible to combine partition in aqueous two-phase systems with temperature-induced phase separations. Some examples of systems used are PEO–PPO–PEO/dextran and PEO–PPO–PEO/hydroxyopropyl starch, where in both cases the top phase polymer was PEO–PPO–PEO copolymer. Target proteins can be partitioned to the copolymer top phase. A water/PEO–PPO–PEO two-phase system is formed when the temperature is increased above the cloud point of the copolymer. Proteins have been found to be partitioned to the water-rich phase. Another possibility is the cloud point extraction, that consists of dissolving a thermoseparating copolymer in protein extract and heating the solution above the copolymer cloud point temperature; when this aqueous solution separates into two phases, a top water-rich phase and a bottom copolymer-rich phase are formed. The protein is partitioned to one of them, usually the top phase.

Many authors presented good results for biomolecules extraction using aqueous two-phase systems formed by thermoseparating copolymers [3,4,10–13]. These works show an easy copolymer recovery and phase separation, plus good results in partition of biomolecules. The influence of many variables, such as copolymer molecular mass, copolymer concentration, PEO percentage in copolymer and presence of cosolutes, like salts and surfactants, on biomolecules partition was studied.

The target enzyme in this work was fruit bromelain. Bromelain is obtained from various species of *Bromeliaceae*, and is found in the fruit and stem of *Ananas comosus* (L.) *Merr*, known as pineapple. Stem bromelain (EC 3.4.22.4) presents isoelectric point at 9.5 [14] and fruit bromelain (EC 3.4.22.5) presents isoelectric point at 4.6 [15]. Thermal denaturation of stem bromelain was studied using circular dichroism and differential scanning calorimetry [16]. The authors showed that the denaturation process is irreversible and seems to follow a simple two state mechanism. They also reported that native enzyme fraction is zero in 50 min at 46.1 °C at pH 3.4.

The aim of this work is to study the bromelain partitioning and purification using aqueous two-phase systems formed by thermoseparating copolymers (cloud point extraction). The influences of thermoseparation temperature, copolymer molecular mass, copolymer concentration, pH and salt concentration on fruit bromelain partition were determined. In the evaluation of enzyme partition, the following parameters were analyzed: partition coefficient of enzyme activity, purification factor and percentage of enzyme activity recovery. In order to select a suitable system for enzyme partition, the cloud point temperatures for different PEO–PPO–PEO block copolymers were determined as well as their dependence on various factors.

Table 1
PEO–PPO–PEO block copolymers evaluated

%EO	Molecular mass (g/mol)	Physical form
10	1100	Liquid
10	2000	Liquid
10	2800	Liquid
30	4400	Liquid
30	5800	Solid (paste)
40	2900	Liquid
50	1900	Liquid
80	8400	Solid

2. Materials and methods

2.1. Chemicals

PEO–PPO–PEO block copolymers (Table 1) were purchased from Aldrich (Milwaukee, WI, USA) and all other chemicals of analytical grade were obtained from Synth (Diadema, SP, Brazil). Distilled water was used to prepare the solutions. No chemicals were further purified before being used.

In this work, mass percentage of poly(ethylene oxide) in PEO–PPO–PEO copolymer is represented by %EO or %PEO.

2.2. Enzyme

Fruit bromelain (EC 3.4.22.5) were obtained from fruit extract of pineapple, species Perola.

2.3. Cloud point determination

2.3.1. Sample preparation

All copolymer concentrations were calculated as % (m/m). Copolymer solutions were weighed out and mixed with buffer of varying pH and salt concentration. The total mass of the systems was 2 g. The systems were carefully mixed and kept under controlled temperature for 2 h in the water bath (Nova Etica, Model 521 D, Vargem Grande Paulista, SP, Brazil).

Systems of different pH values (6.0, 7.0 and 8.0), copolymer concentrations (5, 10, 15, 20, 25 and 30%, m/m) and salt concentrations (15, 45 and 100 mM) were evaluated.

2.3.2. Cloud point measurements

The cloud points of copolymer solutions were determined at different pH values, concentration of the copolymer (%, m/m), in the presence of varying salt concentrations (mM) and pH values by gently heating solutions in thin glass tubes immersed in a thermostated water bath and the temperature was raised slowly, 0.5 °C/min, until turbidity was noted in the tube. The temperature at which turbidity was observed first was taken as the cloud point.

When measuring the cloud point of copolymers at different pH values, the pH was measured in the solution at room temperature before the samples were placed in the thermostated water bath.

All cloud point measurements were performed three times.

2.4. Measurements of fruit bromelain partition in two-phase aqueous systems

Polymer solutions and enzyme extract were weighed out and mixed with buffer of varying pH values (6.0, 7.0 and 8.0) and salt concentrations (15, 45 and 100 mM). The total mass of the system was 2 g, including the enzyme extract that represented 20% (m/m) of total mass. All partition experiments were performed three times. The systems were carefully mixed and the separation took place at temperatures 5 and 10 °C above the cloud point temperature of the copolymer. The thermoseparation was performed during 2 h. This resulted in formation of a two-phase system consisting of an upper water-rich phase and a lower copolymer-rich phase.

Partition of total proteins and enzymes between the phases was determined by removing appropriate amounts of each phase and assaying for total protein and enzyme activity.

For enzymes, the activity partition coefficient, K_a, is defined as

$$K_a = \frac{A_t}{A_b} \quad (1)$$

where A_t and A_b are the enzyme activity in units/l in the top and bottom phases, respectively.

Purification factor (PF) for the top phase is defined as

$$\mathrm{PF} = \frac{A_t/C_t}{A_i/C_i} \quad (2)$$

where A_t and A_i are the enzyme activity in units per liter in the top phase and in the initial extract (before partition), respectively and C_t and C_i are total protein concentration in grams per liter of the top phase and the initial extract (before partition), respectively.

Percentage of activity recovery after partition ($\%R_a$) is defined as

$$\%R_a = \left(\frac{A}{A_i}\right) \times 100 \quad (3)$$

where A and A_i are the enzyme activity in units/l in both phases after extraction and initial (before extraction), respectively.

2.5. Total protein assay

Protein was determined using Coomassie Brilliant Blue G and measured at 595 nm with bovine serum albumin as standard [17].

2.6. Bromelain sample preparation

Pineapple fruit was triturated and filtered. The filtrate, named as extract or pineapple juice, contained the enzyme bromelain. Samples containing 10 g of pineapple juice were frozen [18].

2.7. Enzyme activity assay

Enzyme activity was determined by casein method, measuring the absorbance at 280 nm with tirosine as standard [15,19]. Spectrophotometer UV-Vis GBC Model 911 A (Victoria, Australia) was used for total protein and enzyme activity determination.

3. Results and discussion

3.1. Cloud point determination

For a fixed copolymer concentration, the increase in temperature results in thermoseparation of copolymer solutions in a water-rich phase and a copolymer-rich phase. It is possible to manipulate the cloud point of a copolymer solution by addition of salt, by changing the copolymer molecular mass, by changing the ratio between ethylene oxide to propylene oxide or by changing copolymer concentration.

Cloud point temperatures measured have an accuracy of 0.5 °C in all experiments, except those of copolymer with 50% EO (%, m/m) and molecular mass of 1900 g/mol, when the accuracy was 1.0 °C.

The gellification was observed in some conditions, usually in copolymers with high EO content and high copolymer concentration. The gellification tendency increases with increasing in EO (%, m/m) of copolymer, increasing in copolymer molecular mass and in copolymer concentration [20]. In this work, gellification was observed in solutions formed by copolymers containing 50% EO and 80% EO (m/m).

Cloud point temperatures of copolymer with 30% EO (m/m) and molecular mass of 5800 g/mol and copolymer with 80% EO (m/m) and molecular mass of 8400 g/mol were higher than 85 °C, that was the highest temperature reached in experiments.

3.1.1. Influence of copolymer concentration on cloud point temperature

As can be seen in Fig. 1, the increase in copolymer concentration resulted in lower cloud point temperatures. For example, when the copolymer concentration varied from 5 to 30% (m/m), the cloud point temperature changed from 32 to 18 °C, respectively, for solutions containing copolymer with 10% EO and molecular mass of 1100 g/mol. The high number of copolymer molecules increases the interactions, and easily aggregate, resulting in phase separation at lower temperatures.

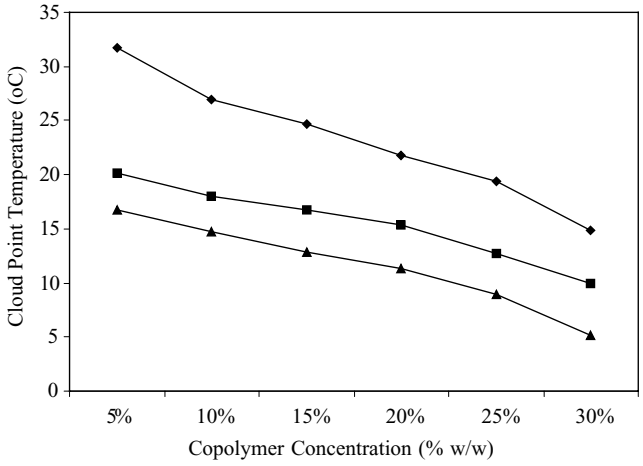

Fig. 1. Influence of copolymer concentration, and copolymer molecular mass on cloud point temperature. Results were obtained at pH 6, salt concentration 15 mM and copolymers with 10% EO (m/m) and molecular mass of 1100 g/mol (◆), molecular mass of 2000 g/mol (■) and molecular mass of 2800 g/mol (▲).

For polymers that should be used in aqueous two-phase systems for the purification of biomolecules, it is important to observe that the cloud point temperature cannot be too high as this could lead to protein denaturation.

As can be seen in Fig. 2, the cloud point temperature of the solution containing copolymer with 50% EO was not influenced significantly by the copolymer concentration, unlike what was observed in the solution containing copolymer with 10% EO. The solutions with 50% EO have presented a cloud point temperature of 65 °C. For solutions containing copolymer with 10% EO and molecular mass of 2000 g/mol, the cloud point temperatures varied from 18 to 5 °C, as copolymer concentration changed from 5 to 30% (m/m), respectively. This is probably a result of the degree of hydrogen bonding between copolymer molecules. As

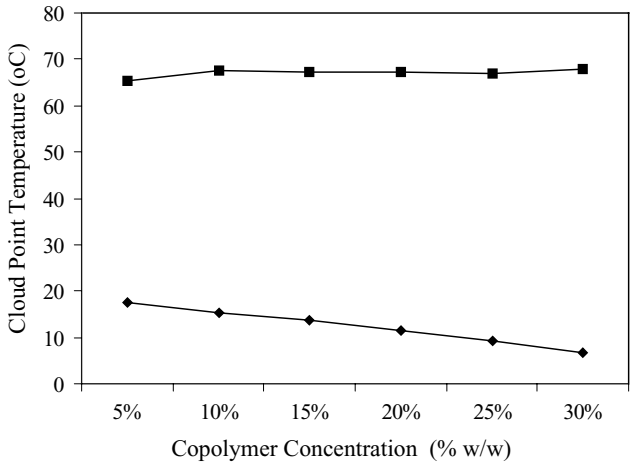

Fig. 2. Influence of %EO of copolymer on cloud point temperature. Results obtained at pH 7.0, salt concentration of 100 mM, copolymer with 10% EO and molecular mass of 2000 g/mol (◆) and copolymer with 50% EO and molecular mass of 1900 g/mol (■).

PEO molecules are the hydrophilic or polar blocks in the copolymer, they are important for hydrogen bonding and the influence of copolymer concentration is not significant.

3.1.2. Influence of copolymer molecular mass on cloud point temperature

The influence of copolymer molecular mass on cloud point temperature can be seen in Fig. 1. It was observed that the cloud point temperature of copolymers with molecular mass of 2800 g/mol is lower than the cloud point of copolymers with molecular masses of 2000 and 1100 g/mol and the same %EO. For example, at copolymer concentration of 5% (m/m), cloud point temperatures for solutions containing copolymer of 10% EO and molecular mass of 1100, 2000 and 2800 g/mol were 32, 21 and 17 °C. All other solution conditions are the same. The higher the copolymer molecular mass the more hydrophobic the copolymer, because they aggregate easily at lower temperatures. As these copolymers are large, the number of hydrophilic and hydrophobic interactions is higher.

3.1.3. Influence of copolymer PEO content on cloud point temperature

As can be seen in Fig. 2, the cloud point temperature of solutions containing copolymer with 50% EO is higher than for solutions containing copolymer with 10% EO and similar molecular mass. In Fig. 2, it can be seen that at the copolymer concentration of 5% (m/m), the cloud point temperature for solutions containing copolymer with 50% EO and molecular mass of 1900 g/mol was 65 °C and for solutions containing copolymer with 10% EO and molecular mass of 2000 g/mol, it was 18 °C. The copolymer PEO–PPO–PEO is formed by PEO and PPO blocks. PPO blocks are hydrophobic and PEO blocks are hydrophilic, therefore copolymer with higher PEO content is more hydrophilic, thus dissolving easily in water. This is probably a result of the degree of hydrogen bonding between the copolymer molecules. At higher temperatures, water is a worse solvent for copolymers than at low temperatures. Therefore, thermoseparation is observed at higher temperatures.

The same behavior was observed when comparing cloud point of solutions containing copolymer formed by 10% EO (m/m) with molecular mass of 2800 g/mol and solutions of copolymer formed by 40% EO and molecular mass of 2900 g/mol.

3.1.4. Influence of salt concentration on cloud point temperature

As can be seen in Fig. 3, the cloud point temperature is dependent on salt concentration. The cloud point temperature of solutions containing 100 mM of phosphate salts is lower than those of solutions containing 45 and 15 mM of phosphate salts. In conditions presented in Fig. 3, cloud point temperatures in solutions containing copolymer with 30% EO (m/m), molecular mass of 4400 g/mol and copolymer concentration of 5% (m/m) were 12, 10

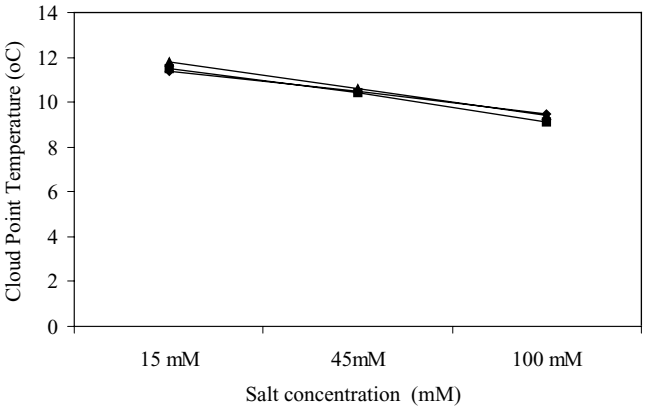

Fig. 3. Influence of pH and salt concentration on cloud point temperature. Results obtained with solutions containing 5% (m/m) of copolymer 30% EO (m/m) with molecular mass of 4400 g/mol and pH 6.0 (◆), pH 7.0 (■) and pH 8.0 (▲).

and 9 °C at phosphate salt concentrations of 15, 45 and 100 mM, respectively. This behavior can be a reflection of the "salting out" effect of phosphate salts. However, in the range of phosphate salt concentration evaluated (15, 45 and 100 mM), a small variation on cloud point temperature was observed.

The addition of electrolytes, having cations and anions of different sizes and polarizabilities, resulted in an increase or a decrease in cloud point [8]. The effect of salts was discussed in terms of "salting in" and "salting out" and followed the Hofmeister series. Phosphate salts are hydrophilic and present a "salting out" effect.

The results had been corroborated by Cunha et al. [13]. The authors found that the variation in salt concentration (0–50 mM) had not caused any significant change in cloud point temperature, however at phosphate salt concentrations above 50 mM, the cloud point temperature decreased with the increase in salt concentration.

3.1.5. *Influence of pH on cloud point temperature*

As PEO–PPO–PEO copolymers are non-ionic molecules, the cloud point temperature is not dependent on pH, as can be seen in Fig. 3, for the pH values evaluated here (pH = 6.0, 7.0, and 8.0).

3.2. *Fruit bromelain partitioning*

In this work, the target enzyme was fruit bromelain. Some partition experiments using bromelain obtained from stem were carried out but enzyme activity recovery was very low, 15% at most. The authors concluded that conditions used caused the inhibition of enzyme activity or denaturation of enzymes obtained from stem.

All results presented are refereed to fruit bromelain because this enzyme presented a better activity recovery than stem bromelain. Enzyme activity recovery was around 79.5% for fruit bromelain under the best conditions.

The aqueous systems for bromelain partitioning were chosen according to the results obtained in experiments of cloud point determination. It is important to evaluate the viscosity, and volume of the phases as well as the cloud point temperature, which should not be high to avoid enzyme denaturation.

The following systems were used for enzyme partition studies: copolymer with 10% EO (m/m) and molecular mass of 2000 and 2800 g/mol in concentrations of 5, 20 and 30% (m/m), different pH values (6.0, 7.0 and 8.0) and different salt concentrations (15, 45 and 100 mM). Aqueous systems of other copolymers studied were not used because the phases viscosities and time for phase separation were too high, e.g. phase separation time longer than three hours.

The results presented here are the average taken of three experiments where the maximum error admitted was 20%.

3.2.1. *Activity enzyme (bromelain) recovery (%R_a)*

All the factors evaluated influenced the activity enzyme recovery (%R_a), although the most important were copolymer concentration, thermoseparation temperature and pH, at the conditions studied. %R_a is low at higher copolymer concentration, as can be seen in Fig. 4. For example, solutions containing 15 mM of phosphate salts and copolymer concentration of 5% (m/m) presented %R_a = 39%, whereas solutions with copolymer concentration of 20 and 30% presented %R_a = 18% in the conditions described in Fig. 4. High copolymer concentration seems to cause the inactivation of fruit bromelain activity as a consequence of the greater number of interactions between copolymer and enzyme, at higher copolymer concentration. Inactivation of this enzyme in concentrated and hydrophobic phases is possible. In phases virtually free of copolymer, enzyme activity increases greatly resulting in a high yield [21]. Temperature also influenced %R_a as observed in Table 2. At temperature 30 °C for 2 h, that is the thermoseparation time, the enzyme activity recovery is lower than at 25 °C. At fixed conditions and varying only the temperature, %R_a was 79.5 and 40.0% at 25 and 30 °C, respectively. Therefore, %R_a was higher at the lower thermoseparation temperature. This behavior

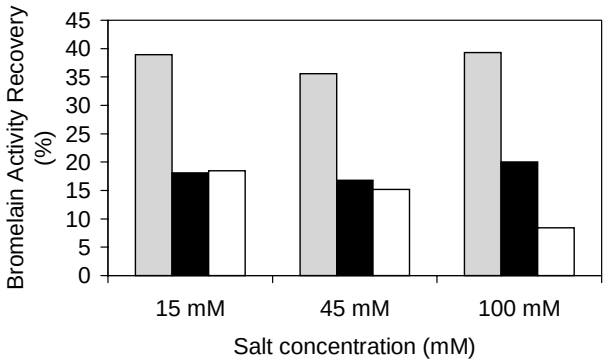

Fig. 4. Influence of copolymer, and salt concentration in solution on percentage of activity recovery in solutions containing copolymer 10% EO with molecular mass 2000 g/mol at pH 8 and copolymer concentrations (%, m/m) of 5% (▨), 20% (■) and 30% (□).

Table 2
Influence of Temperature and pH on enzyme partition

pH	Temperature (°C)	K_a	PF	$\%R_a$
6	25	1.40 ± 0.18	1.25 ± 0.20	79.5 ± 4.5
	30	0.85 ± 0.11	1.01 ± 0.20	45.3 ± 3.0
8	25	1.27 ± 0.18	0.57 ± 0.10	50.6 ± 5.0
	30	0.94 ± 0.10	0.67 ± 0.10	38.8 ± 5.0

Results were obtained using copolymer formed by 10% EO (m/m) and molecular mass of 2000 g/mol, copolymer concentration of 5% (mm) and salt concentration of 15 mM.

showed that fruit bromelain is sensitive to temperature. In Table 2, it can also be seen that $\%R_a$ is reduced as pH increases from 6.0 to 8.0. At 25 °C, $\%R_a$ was 79.5% (pH 6.0) and 45.0% (pH 8.0). Therefore, to obtain a high percentage of bromelain activity recovery, it is adequate to operate at low copolymer concentration (5%, m/m), low temperatures (25 °C) and pH 6.0, according to the conditions evaluated.

3.2.2. Bromelain activity partitioning

Analogously to the behavior observed for the $\%R_a$, all factors studied have influenced bromelain (enzyme) activity partitioning coefficient (K_a), however, only the most important are commented below. Aqueous two-phase systems formed by copolymer with 10% EO (m/m) and molecular mass of 2000 and 2800 g/mol were used to evaluate the influence of copolymer molecular mass on enzyme partition. In Fig. 6, it can be seen that K_a increases as the copolymer molecular mass increases. For example, at fixed solution conditions, K_a was 0.8 in an aqueous two-phase systems formed by copolymer with 10% EO and molecular mass of 2000 g/mol and 1.25 in aqueous two-phase systems formed by copolymer with molecular 2800 g/mol. The difference in the partition coefficient values observed reflects the "volume exclusion" effect, as molecules with molecular mass of 2800 g/mol are larger than those with molecular mass of 2000 g/mol. Therefore, enzyme molecules are "pushed" from the bottom phase, the copolymer-rich phase, to the top phase, rich in water. Although aqueous systems containing copolymers with higher molecular mass presented a higher K_a, there was a pronounced increase in phase viscosity and the time required for phase separation. As a consequence, aqueous systems formed by copolymers with high molecular mass are worse for enzyme partition than others with lower molecular mass and consequently, lower phase viscosity and phase separation time.

As can be seen in Table 2, K_a is high at 25 °C, and was the lowest thermoseparation temperature studied. In the conditions shown in Table 2, at 25 °C $K_a = 1.4$ and at 30 °C $K_a = 0.75$ in solutions of pH 6.0. This behavior is also related to higher enzyme activity recovery obtained at 25 °C. According to Fig. 5, K_a is higher at high salt concentrations, although this effect was less significant for lower values of K_a, in the conditions studied. In the conditions presented on Fig. 5 and for solutions containing copolymer with 10% EO

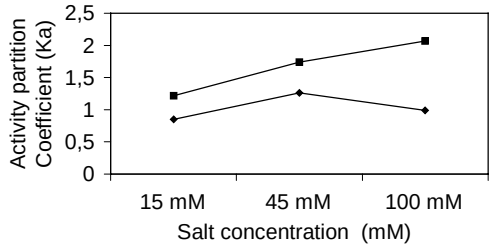

Fig. 5. Influence of copolymer molecular mass, and salt concentration on K_a. Results obtained with copolymer concentration of 5% (m/m), temperature 10 °C above the cloud point temperature, pH 6.0 and copolymer 10% EO (m/m) with molecular mass of 2000 g/mol (◆) and with molecular mass of 2800 g/mol (■).

and molecular mass of 2800 g/mol, K_a was 1.25 at phosphate salt concentration of 15 mM and $K_a = 2.25$ at phosphate salt concentration of 100 mM. Solutions containing copolymer with 10% EO and molecular mass of 2000 g/mol showed a K_a value of 0.8 at phosphate salt concentration of 15 mM and 1.0 at phosphate salt concentration of 100 mM.

This behavior can be explained by the "salting out" effect of phosphate salts, thus raising K_a. In addition, the greater number of ions in solutions with high salt concentration attract to the top phase more electrically charged protein molecules, owing to the pH of solution.

K_a dependence on pH was of little significance, under the conditions studied, as can be seen in Table 2. The values of studied pH were 6.0, 7.0 and 8.0, and fruit bromelain p*I* is 4.6. Therefore, enzyme molecules were electrically charged and this influenced the enzyme partition. However, the effect of the electrical charges in enzyme molecules due to pH variation is not clear in the conditions analyzed here.

3.2.3. Purification factor (PF)

This analysis was done only for the purification factor in the top phase (PF), as the bottom phase PF was very low. In Fig. 6, it is observed that PF decreased as phosphate salt and copolymer concentration are increased. Probably, copolymer molecules interact with enzyme molecules, mainly the hydrophobic interactions, causing the inactivation of the enzyme. Of the copolymers studied, the ones used in the

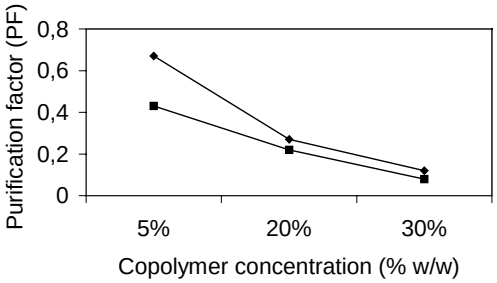

Fig. 6. Influence of copolymer, and salt concentration on purification factor (PF) in solutions containing copolymer 10% EO (m/m) with molecular mass of 2000 g/mol at pH 8.0 and salt concentration of 15 mM (◆) and 45 mM (■).

Table 3
Influence of studied conditions on bromelain partitioning and purification

	Experimental conditions									
	Copolymer concentration		Copolymer molecular mass (g/mol)		Phosphate salt concentration (mM)		Temperature (°C)		pH	
	5% (m/m)	30% (m/m)	2000	2800[b]	15	100	25	30	6.0	8.0
$\%R_a$	High	Low	[a]	[a]	[a]	[a]	High	Low	High	Low
K_a	[a]	[a]	Low	High	Low	High	High	Low	[a]	[a]
PF	High	Low	Low	High	High	Low	High	Low	High	Low

[a] Influence is not significant.
[b] Solutions containing this copolymer presented high viscosity and phase separation times.

partition experiments were the most hydrophobic among copolymers studied. They were chosen due to their low cloud point temperature and phase viscosities. Therefore, lower copolymer concentration (5%, m/m) is more suitable to enzyme partition and purification because PF and $\%R_a$ were higher in this condition. Purification Factor in the top phase decreased from 0.7 to 0.4 as phosphate salt concentration changed from 15 to 45 mM at copolymer concentration of 5% (m/m), according to Fig. 5. This can be explained by the "salting out" effect, which causes the increase of protein concentration in the top phase. PF reduction indicates that proteins other than fruit bromelain were attracted to the top phase. Consequently, K_a increased and PF decreased. At higher copolymer concentration PF was lower and the salt concentration influence was not significant. The reduction of the Purification Factor in the top phase can be explained by the "volume exclusion" effect, at high copolymer concentration. There many copolymer molecules in the bottom phase, at high copolymer concentration, so copolymer molecules "expel" protein molecules from the bottom to the top phase. Therefore, despite the increase in bromelain (enzyme) concentration in the top phase, PF was lower as total protein concentration also increased. Copolymer molecular mass also influences PF. In Fig. 7, it is seen that PF was higher in solutions containing copolymer formed by 10% EO and molecular mass of 2800 g/mol compared to solutions containing copolymer formed by 10% EO and molecular mass of 2000 g/mol. For example, PF was 2.8 and 1.0 in solutions of phosphate salt concentration of 15 mM, containing copolymer of 2800 and 2000 g/mol, respectively. This behavior can also be explained by the "volume exclusion" effect, as it was described earlier.

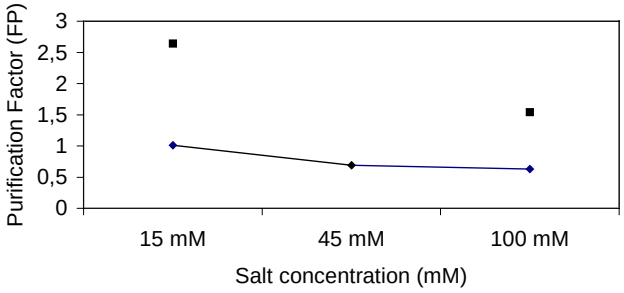

Fig. 7. Influence of copolymer molecular mass, and salt concentration in top phase purification factor. Results obtained with copolymer concentration of 5% (m/m), temperature 10 °C above the cloud point temperature, pH 6.0 and copolymer 10% EO (m/m) with molecular mass of 2000 g/mol (◆) and with molecular mass of 2800 g/mol (■).

In Table 2, the PF behavior in relation to thermoseparation temperature can be observed. The PF in the top phase was higher at 25 °C (1.25, at pH 6.0) than at 30 °C (0.9, at pH 6.0). This behavior can be an evidence that in the aqueous systems studied, enzyme inactivation is reduced at lower temperatures, as was the case for $T = 25$ °C.

The PF in the top phase was higher at pH 6.0 than at pH 8.0, according to Table 2. At 25 °C, PF was 1.25 at pH 6.0 and 0.5 at pH 8.0. Therefore, pH 6.0 was more suitable to bromelain purification in the aqueous two-phase systems studied than pH 8.0.

3.2.4. Influence of experimental conditions on bromelain partitioning

The influence of the factors studied on bromelain partitioning and purification using aqueous two-phase systems formed by thermoseparating copolymers is summarized in Table 3. As can be seen, the best conditions for bromelain partitioning and purification were: Copolymer concentration: 5% (m/m), copolymer molecular mass: 2000 g/mol, phosphate salt concentration: 15 mM, thermoseparation temperature: 25 °C and pH 6.0. In these conditions, the following results were obtained: $\%R_a$, 79.5%; K_a: 1.4 and PF, 1.25.

4. Conclusions

The composition and concentration of block copolymer PEO–PPO–PEO and all the experimental conditions evaluated, except the pH, influence significantly the cloud point temperature. At the conditions defined here, the cloud point changed from temperatures of approximately 3.0 °C to values around 85.0 °C. Therefore, thermoseparating copolymers PEO–PPO–PEO can be used for biomolecules extraction and partitioning since suitable temperatures can be reached using appropriate copolymers and conditions. Cloud point temperature decreases as copolymer molecular mass, copolymer concentration, percentage of PPO blocks

and phosphate salt concentration increase. In the latter case, the block copolymers can easily form aggregates, owing to salting out effect caused by phosphate salts.

The fruit bromelain partition in aqueous two-phase systems formed by copolymers with 10% EO (m/m) and molecular mass of 2000 g/mol and 2800 g/mol was studied. A significant bromelain inactivation was observed for some conditions, namely high copolymer concentration (20 and 30%, m/m) and higher temperatures (30 °C). Low copolymer concentration and temperature avoid denaturation. High molecular mass copolymers form rather viscous solutions, in some conditions. The best results achieved in this work were percentage of enzyme activity recovery of 79.5%, top phase purification factor of 1.25 and enzyme activity partition coefficient of 1.4. These results were obtained using the conditions: copolymer with 10% EO (m/m) and molecular mass of 2000 g/mol, copolymer concentration of 5% (m/m) and temperature 5 °C above the cloud point, that is 25 °C in this case, pH 6.0 and salt concentration of 15 mM. Better results were obtained in systems containing copolymer with 10% EO and molecular mass of 2800 g/mol, but the viscosity and time separation of the phases were too high.

The aqueous two-phase systems studied proved to be simple and consequently promising as an enzyme purification method.

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References

[1] D.E. Brooks, H. Walter, D. Fisher, Partitioning in Aqueous Two Phase Systems, Academic Press, Orlando, 1985.
[2] P.A. Albertsson, Partition of Cell Particles and Macromolecules, third ed., Wiley/Interscience, New York, 1986, 323 pp.
[3] H. Walter, G. Johansson, Methods in Enzymology, vol. 228, Academic Press, Londres, 1994, pp. 600–608.
[4] P.A. Alred, F. Tjerneld, A. Koslowski, J.M. Harris, Bioseparation 2 (1992) 363.
[5] T.T. Franco, Y.U. Galaev, R. Hatti-Kaul, N. Holberg, L. Bülow, B. Mattiasson, Biotechnol. Tech. 11 (1997) 231.
[6] J. Persson, H.-O. Johansson, F. Tjerneld, J. Chromatogr. A 864 (1999) 31.
[7] J. Persson, L. Nystrom, H. Ageland, F. Tjerneld, J. Chem. Technol. Biotechnol. 74 (1999) 238.
[8] P. Alexandridis, T.A. Hatton, Colloids Surf. A: Physicochem. Eng. Aspects 96 (1995) 1.
[9] S. Hvidt, Colloids Surf. A: Physicochem. Eng. Aspects 112 (1995) 201.
[10] J. Persson, A. Kaul, F. Tjerneld, J. Chromatogr. B 743 (2000) 115.
[11] K. Berggren, H.-O. Johansson, F. Tjerneld, J. Chromatogr. A 718 (1995) 67.
[12] T. Saitoh, H. Tani, T. Kamidate, H. Watanabe, Trends Anal. Chem. 14 (1995) 213.
[13] M.T. Cunha, F. Tjerneld, J.M.S. Cabral, M.R. Aires-Barros, J. Chromatogr. B 711 (1998) 53.
[14] G. Feinstein, J.R. Whitaker, Biochemistry 3 (1964) 1050.
[15] T. Murachi, Bromelain enzymes, in: L. Lorand (Ed.), Methods in Enzymology, vol. XLV, Academic Press, New York, 1976, pp. 475–485.
[16] A. Arroyo-Reyna, A. Hernández-Arana, Biochim. Biophys. Acta 1248 (1995) 123.
[17] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[18] A.C.W. Cesar, R. Silva, A.C. Lucarini, RIC, São Carlos, SP, Brazil 01 (1999) 47–54.
[19] V.L.S. Baldini, M. Iaderoza, E.A.H. Ferreira, A.M. Sales, I.S. Draetta, E.J. Giacomelli, Colet. ITAL, Campinas 23 (1993) 44.
[20] G. Karlstron, J. Phys. Chem. 89 (1985) 4962.
[21] P.A. Alred, A. Koslowski, J.M. Harris, F. Tjerneld, J. Chromatogr. A 659 (1994) 289.