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The Effect of pH on Bromelain Partition from *Ananas comosus* by PEG4000/Phosphate ATPS

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ABSTRACT

In this work, the thermodynamic equilibrium and applying of PEG4000/Phosphate ATPS on the purification of bromelain extracted from pineapple was studied. A rigorous study of the equilibrium curves and tie-line length from PEG4000/phosphate ATPS were done for the pH 6-11 at 25°C. Results showed that there was augment in the PEG and salt contents with the high pH value from PEG4000/Phosphate ATPS and two-phase formation needed only increasing the PEG content. Two tie-line length at pH 11 from PEG4000/Phosphate ATPS were optimal condition for bromelain purification, one on composition of 14% PEG and 13% salt and other at 12.6% PEG and 12.2% salt, while a 25-62 folds of enzyme was found. SDS-PAGE electrophoreses had one band only, which showed that bromelain was purified. Optimum conditions of bromelain use were found at pH 7 and between 30-40°C.

Key words: bromelain, aqueous two-phase systems, partitioning, characterization, pineapple

INTRODUCTION

Bromelain from pineapple steam

Bromelain is the name of a group of powerful protein-digesting, or proteolytic, enzymes that are found in the pineapple plant (*Ananas comosus*). Discovered in 1957, and widely studied since then, bromelain is particularly useful for reducing the muscle and tissue inflammation and as a digestive aid. Besides the pharmacological effects, bromelain is also employed in food industries, such as breweries and meat processing. The optimum pH and temperature of bromelian are 6.5-7.5 and 37°C, respectively (Fisher et al., 2007; Lopes et al., 2007 and 2009; Silveira et al., 2009).

These enzymes are a mixture of proteinases derived from pineapple stem, which is sold as a nutritional supplement to "promote digestive health" and as an anti-inflammatory medication in some developed countries (Hale et al., 2005a and 2005b; Wen et al., 2006). Bromelain is clinically used from the pineapple extract and the natural product. Literatures have reported anti-inflammatory and immunomodulatory activities (Sercor et al., 2005). It has been applied in the anticancer activity (Harrach et al., 1994), in the immunization of influenza virus (Veroniková et al. 1995), in the treatment of allergic airway disease (Sercor et al., 2005) and is also used in cosmetic compositions (Chatsworth, 1996). Adverse results had been reported on an allergic activity of bromelain caused by the inhalations of beer or wheat snack (Antón et al., 2005).

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However, bromelain is instable and frequently is deactivated spontaneously, due the action of agents of meddle. Concentrated bromelain solutions are more resistant to spontaneous inactivation of their proteolytic activity than are dilute solutions. The relative stability of concentrated versus dilute bromelain solutions to inactivation under physiologically relevant conditions suggests that the delivery of bromelain as a concentrated bolus would be the preferred method to maximize its proteolytic activity in vivo (Hale et al., 2005b; Arroyo-Reyna and Hernández-Arana, 1995). Other problem in vivo studies using bromelain, is the limitation by the lack of assays to control for potential differences in the composition and proteolytic activity of this naturally derived proteinase mixture (Arroyo-Reyna and Hernández-Arana, 1995). Thus, the concentration of pineapple juice is needed to retain the bromelain activity and to standardize the composition and proteolytic activity.

The bromelian, according to Sigma (2006), had a price about U$ 1,075.40 for kilogram of purified material, at 30% of protein contents. Thus, the recovery these enzymes from pineapple steam will add value this agricultural product. **So**, this study aimed to characterize and to recover the bromelain from pineapple steam and skin, by a PEG 4000/ phosphate aqueous two-phase systems liquid-liquid extraction. Batch assays were performed aiming the enzyme extraction and recovery, using the partition coefficient as an indicator of the enzyme purification.

Aqueous two-phase system partitioning

In many biotechnological industries, including food and pharmaceutical ones, the selective separation of a protein out of fermentation broths or vegetable sources has been a primary research interest for downstream processing operations. It is difficult and expensive to selectively recover a targeted protein from a broth due to the low protein concentration and the similarity of the physical properties between the proteins present in the same solution. Aqueous two-phase systems (ATPS), expanded bed adsorption (EBA) and membrane separation process are more recent downstream processes used in the purification of biomolecules (Biazus et al., 2006, 2007 and 2010; Curvelo-Santana et al., 2008; Fisher et al., 2007; Lopes et al., 2007 and 2009; Padilha et al., 2009; Severo Jr. et al., 2007 and 2009; Silveira et al., 2009; Toledo et al., 2007).

The liquid-liquid extraction process consists of transferring a substance from a liquid mixture to another immiscible (or partially miscible) liquid phase by putting them in contact. This process is widely used in chemical and pharmaceutical industries, such as in the recovery of antibiotics or organic acids from fermentation broths. Nevertheless, the application in the purification of proteins is still limited mainly due to the possibility of protein denaturation if in contact with organic solvents, yielding a useless product (Aires-Barros et al., 1994; Albertsson, 1986; Matiasson and Kaul, 1986; Zaslasvsky, 1995).

The partitioning of a solute (e.g., a protein) between the phases is described by the partition coefficient, ***K***, defined as the ratio of the concentrations of solute in the upper phase to that in the lower phase, given t equation 1 (Albertsson, 1986; Zaslasvsky, 1995).

$$K = \frac{C_{top}}{C_{bottom}} \quad (1)$$

The bromelain from pineapple juice (*Ananas comosus*) has been purified by batch and continuous extraction by reversed micelles ATPS. On optimal condition of batch purification a 3-folds of enzyme recovery was obtained and, a purification factor of 6-folds was found for the continuous ATPS operating at 0.5 for top/total flow and 2 s for the time interval between the pulses (Fileti et al., 2009 and 2010; Fisher et al., 2007). A study of bromelain partitioning in two-phase aqueous systems containing PEO-PPO-PEO block copolymers was made an value 1.25 was the maximum purification factor found for the bromelain recovery from pineapple fruit via two-phase aqueous extraction (Rabelo et al., 2004). Lopes et al. (2009) purified a bromelain from pineapple juice in a continuous system of the membrane separation process. The best operation condition to bromelain concentration using the plain membrane was at pH 7.5 and transmembrane pressure of 0.05 bar, while 85% of bromelain activity was recovered.

EXPERIMENTAL

Material

Polyethylene glycol with molecular weight of 4000 was obtained from Sigma (Switzerland).

Milli-Q-quality distilled water was used. An UV-VIS spectrophotometer VIS was used to determine the protein concentration. Acetone (PA), acetic acid, sodium acetate and potassium sodium tartrate were obtained from Vetec (São Paulo, Brazil). Potassium hydrogen phosphate, comassie brilliant blue G, di-sodium hydrogen phosphate, agar-agar, sodium hydroxide, yeast extract, peptone, TRIS and chlorine acid were obtained from Merck (Darmstadt, Germany). Pineapple fruits were acquired from a supermarket in Campinas (Brazil).

Methods

Enzyme assays

Fruit bromelain (EC 3.4.22.5) was obtained from the fruit extract of the Perola pineapple species. Pineapple pulp was triturated and filtered. The filtrate contained the bromelain enzyme. Samples were frozen at -5^{o}C (Fileti et al., 2009 and 2010; Fisher et al., 2007; Silveira et al., 2009). Bromelain activity was measured by hydrolysis of a 2% (w/w) casein solution at pH 7.5 and 37^{o}C for 10 min. Tricloroacetic acid (TCA) was used in the precipitation of the non-hydrolyzed product. The amount of soluble peptides in TCA was determined by measuring the absorbance at 280 nm. The method defines one unity of enzyme activity as the amount of enzymes that modifies by 1.0 the absorbance at 280 nm (Baldini et al., 1993; Fileti et al., 2009 and 2010; Fisher et al., 2007; Lopes et al., 2007 and 2009; Murachi, 1976).

Preparation of aqueous two-phase systems

Solutions of PEG 4000 (50% w/w) and Phosphate buffer (22.5%, w/w) of known concentrations were prepared at pH value of 6 to 11. A given volume of PEG solution was added to the buffer solution until it became turbid, indicating the beginning of the two-phase system. The phase contents were determined to obtain the binodal curve compositions. PEG and phosphate buffer solutions were mixed together until equal volumes of the phases. The phase contents were determined to obtain the tie-line length compositions (Albertsson, 1986; Zaslasvsky, 1995).

Bromelain partitions

Pineapple juice was added to different ATPS compositions. Total protein and bromelain activity in samples of bottom and top phases were determined by the Bradford method (Bradford, 1976) and bromelain activity method (Baldini et al., 1993; Fileti et al., 2009 and 2010; Fisher et al., 2007; Murachi, 1976). The partition coefficient was obtained with Equation 1.

RESULTS AND DISCUSSION

Figure 1 showed the experimental binodal curves of PEG4000/phosphate ATPS obtained at pH value of 6 to 11. The equilibrium curves of PEG 4000/ phosphate ATPS had a behavior random with the pH of systems, that is, this behavior cannot be described. This has also been observed by Diamond and Hsu (1982), Zaslasvsky (1995) and Johansson et al. (1995).

Experimental data of tie line lengths composition from PEG4000/phosphate aqueous two-phase systems are shown in Tables 1 to 6, for the pH value of 6 to 11, respectively. Three tie lines showed in some tables, it was denoted the number 1 for the high PEG composition, until number 3 for the low PEG composition. It was noted that PEG and salt contents for phase formation increased to pH value.

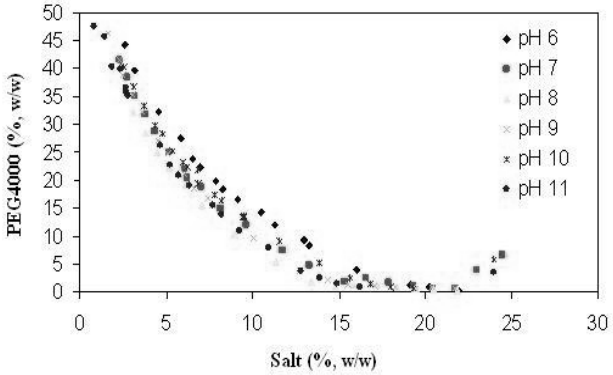

Figure 1 - Binodal curves of PEG/phosphate aqueous two-phase systems, at 25 ± 2°C.

Table 1 - Phase compositions from PEG 4000/ Phosphate Salt Aqueous two-phase Systems, at 25 ± 2ºC and pH 6.

Tie Line	System (%w/w)			Top phase (%w/w)			Bottom phase (%w/w)		
	Salt	PEG	H_20	Salt	PEG	H_20	Salt	PEG	H_20
1	13.31	16.7	69.99	20.0	0.9	79.1	4.9	31.5	63.6
2	13.82	11.42	74.76	20.0	0.9	79.1	7.5	20.5	72.0
3	14.02	8.02	77.96	16.1	3.3	80.6	11.0	12.0	77.0

Table 2 - Phase compositions from PEG 4000/ Phosphate Salt Aqueous two-phase Systems, at 25 ± 2ºC and pH 7.

Tie Line	System (%w/w)			Top phase (%w/w)			Bottom phase (%w/w)		
	Salt	PEG	H_20	Salt	PEG	H_20	Salt	PEG	H_20
1	13.5	16.23	70.27	4.30	30.0	65.70	21.8	2.5	75.7
2	13.4	12.40	74.20	20.41	1.0	78.59	6.4	22.0	71.6
3	13.5	8.51	77.99	18.00	0.5	81.50	8.1	15.9	76.0

Table 3 - Phase compositions from PEG 4000/ Phosphate Salt Aqueous two-phase Systems, at 25 ± 2ºC and pH 8.

Tie Line	System (%w/w)			Top phase (%w/w)			Bottom phase (%w/w)		
	Salt	PEG	H_20	Salt	PEG	H_20	Salt	PEG	H_20
1	11.68	16.95	71.37	20.5	0.5	79	3.12	32.3	64.58
2	11.47	14.88	73.73	20.5	0.5	79	3.10	30.0	66.90
3	11.00	12.00	77.00	5.0	24.0	71	16.5	0.3	83.20

Table 4 - Phase compositions from PEG 4000/ Phosphate Salt Aqueous two-phase Systems, at 25 ± 2ºC and pH 9.

Tie Line	System (%w/w)			Top phase (%w/w)			Bottom phase (%w/w)		
	Salt	PEG	H_20	Salt	PEG	H_20	Salt	PEG	H_20
1	13.5	19.80	66.70	23.5	9.0	67.5	4.00	30.00	66.00
2	12.2	18.50	69.30	3.0	35.2	61.8	20.80	1.40	77.80
3	11.2	16.03	72.77	4.0	30.0	66.0	18.24	0.72	81.04

Table 5 - Phase compositions from PEG 4000/ Phosphate Salt Aqueous two-phase Systems, at 25 ± 2ºC and pH 10.

Tie Line	System (%w/w)			Top phase (%w/w)			Bottom phase (%w/w)		
	Salt	PEG	H_20	Salt	PEG	H_20	Salt	PEG	H_20
1	14.18	19.54	66.32	24.0	5.7	70.3	3.7	33.17	63.13
2	12.84	17.89	69.36	4.0	31.5	64.5	21.0	2.00	77.00
3	11.10	15.39	73.51	5.1	28.0	66.9	16.9	1.70	81.40

Table 6 - Phase compositions from PEG 4000/ Phosphate Salt Aqueous two-phase Systems, at 25 ± 2ºC and pH 11.

Tie Line	System (%w/w)			Top phase (%w/w)			Bottom phase (%w/w)		
	Salt	PEG	H_20	Salt	PEG	H_20	Salt	PEG	H_20
1	14.53	18.37	67.1	4.2	30	65.8	25	7.5	67.5
2	13.03	14.03	72.94	21.5	0.5	78	4.85	26	69.15
3	12.2	12.64	75.16	18	0.3	81.7	5.5	25	69.5

Results of protein partition from pineapple juice by PEG4000/phosphate aqueous two-phase systems are shown in Figure 2. It was observed that on the tie lines two (14% PEG/13% salt) and three (12.6% PEG/12.2% salt) at pH 11, the total protein from pineapple juice was concentrated in top phase by 25-62 folds. These values were more than partition coefficients obtained by Fileti et al. (2009 and 2010), Fischer et al. (2007), Lopes et al. (2009) and Rabelo et al. (2004).

After the batch ATPS process, the top phase sample of the best system was analyzed by SDS–PAGE for the determination of molecular weight and degree of purification of the enzyme (Fig. 3).

The analysis showed that the extracted enzyme was pure and the molecular weight was 24.5 kDa. This molecular weight was approximated to the 31 kDa of the bromelain reported by Martins et al. (1992).

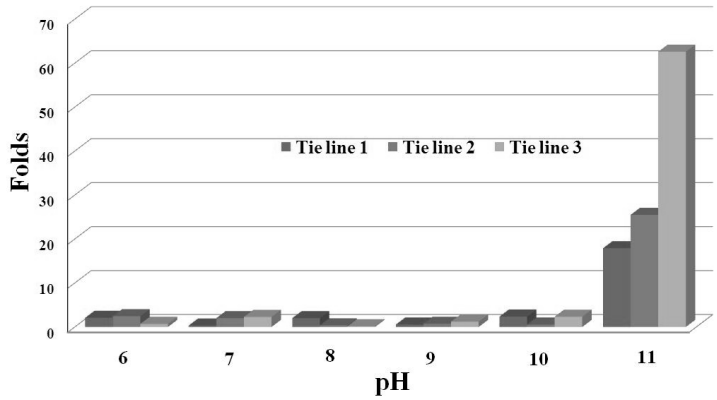

Figure 2 - Results of bromelain partitioning, in top phase from PEG4000/ Phosphate ATPS.

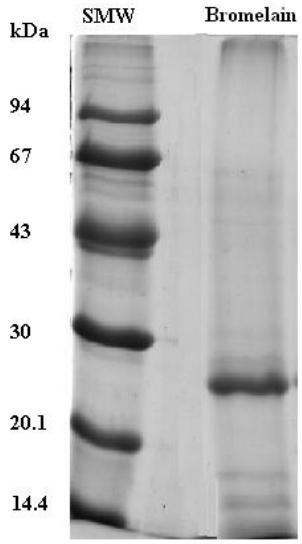

Figure 3 - Molecular weight determination by SDS-PAGE electrophoreses. SMW is standard of molecular weight, it is compound of following proteins: phosphorylase b (94kDa), bovine serum albumin (67kDa), ovalbumin (43kDa), carbonic anhydrase (30kDa), trypsin inhibitor (20.1kDa) and α-lactoalbumin (14.4kDa). Bromelain is the enzyme purified by PEG4000/phosphate ATPS.

Figures 4 and 5 showed biochemical characterization of bromelain purified from pineapple juice. The optimum pH was about 7, the optimum temperature was among 30-40 °C and the K_M *and* V_{max} values were 330.4 mg/L and 2.539 mg BSA/L.min, respectively. The optimal pH and temperature were accordance with the reported values (Dixon and Webb, 1976; Forgaty and Kelly, 1987). The minor peaks showed in Figure 4.a from pH effect showed that the enzyme had activity in the neighborhood of pH 4.5 and 8.5, as perceived for the amylases from maize malt by Biazus et al. (2009).

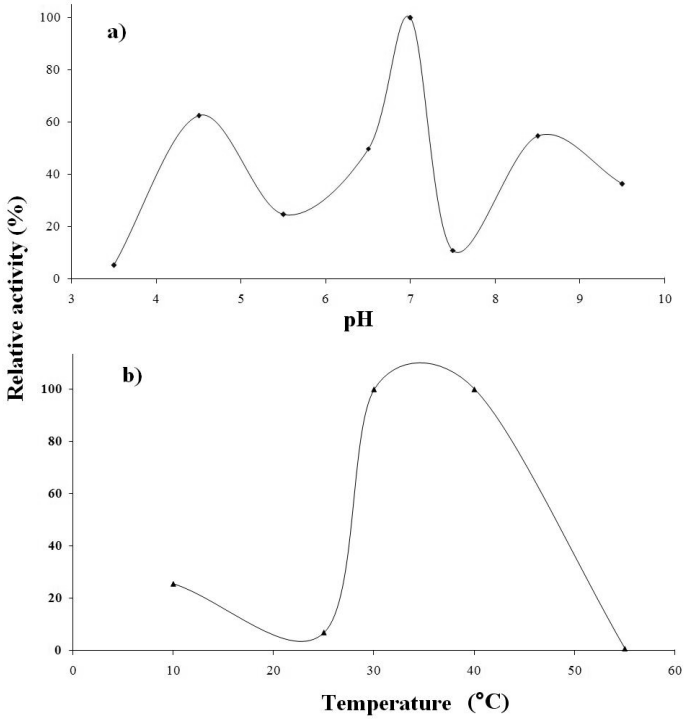

Figure 4 - Effects of (a) pH and (b) temperature on the bromelain purified from pineapple juice by PEG4000/ Phosphate ATPS.

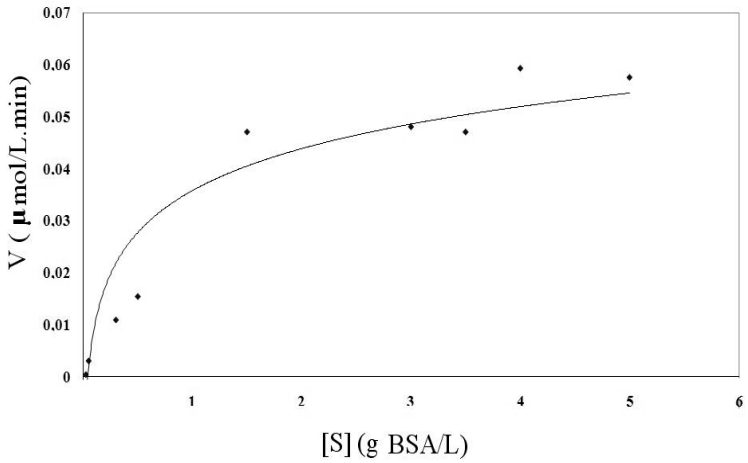

Figure 5 - Kinetic of protein hydrolysis by bromelain purified from pineapple juice by PEG4000/ Phosphate ATPS.

CONCLUSIONS

It was observed that the equilibrium curves of PEG 4000/ phosphate had a behavior random with the pH of systems for the formation of aqueous two phases. On the tie lines two (14% PEG/13% salt) and three (12.6% PEG/12.2% salt) at pH 11, the enzyme was concentrated in top phase by 25-62 folds.

The enzyme characterization showed that the optimum pH was about 7, the optimum temperature was between 30-40 °C and the K_M *and* V_{max} values were 330.4 mg/L and *2.53*9 mg BSA/L.min respectively. The SDS-page analysis showed that this enzyme had a molecular weight of 24.5 kDa.

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