

Purification of Bromelain from *Ananas comosus* by PEG/Phosphate ATPS

Juliana Ferrari Ferreira*, Iara Rocha P. Bresolin, Edgar Silveira, Elias Basile Tambourgi

Department of Engineering of Chemical Systems, School of Chemical Engineering,
State University of Campinas (UNICAMP)
University Campus Zeferino Vaz, Av. Albert Einstein, 500, Campinas, Brazil.
jferrarif@hotmail.com

The bromelains are proteases from vegetable origin, obtained from several species of Bromeliaceae family, which are found in pineapple's stem and fruit. The bromelains uses are based on its proteolytic activity on food industries and mostly at Brazilian pharmaceutical industry. The bromelains has been documented by its therapeutic effects at all inflammatory conditions, in addition to its attested efficiency of cure of various health problems, such as angina, indigestion and respiratory problems.

Various bromelains' purification methods have been studied, particularly bromelain of pineapple stem. Aqueous two-phase system has been applied for several years as a good laboratorial separation technique and it can be used as a pre-purification step. This system has been tested with large success, it is formed by two aqueous phases immiscible or partially miscible within themselves, obtained by the addition of hydrophilic polymers or one hydrophilic polymer and a salt, such as the poly(ethylene glycol) – PEG – and potassium phosphate salt systems.

This work studied the enzyme characterization and recovery presented in the pineapple's stem and peel, by aqueous two-phase systems (ATPS) liquid-liquid extraction. Batch assays were performed aiming the enzyme extraction and recuperation, using the partition coefficient as indicator.

The phase diagrams from PEG and potassium phosphate salts aqueous two-phase systems (ATPS) were obtained for PEG with molecular weight of 4000 at pH 6.0 to 11.0, at 25 °C. Three different tie-lines were also studied. The pH and tie-line length influence on the enzyme partition coefficient were analyzed.

1. Introduction

Bromelain is the name of a group of powerful protein-digesting, or proteolytic, enzymes that are found in the pineapple plant (*Ananas comosus*). 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). The bromelian, according to Sigma (2006), had a price about US\$ 1,075.40 per kilogram of purified

material, at 30% of protein contents. Thus, the recovery these enzymes from pineapple stem will add value to this agricultural product. 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; Zaslavsky, 1995).

So, this study aimed to characterize and to recover the bromelain from pineapple stem and skin, by a PEG 4000/ phosphate aqueous two-phase systems (ATPs) liquid-liquid extraction. Batch assays were performed aiming the enzyme extraction and recovery, using the partition coefficient as an indicator of the enzyme purification.

2. Materials and Methods

2.1 Reagents

Polyethylene glycol (PEG) 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. Potassium hydrogen phosphate, comassie brilliant blue G, di-sodium hydrogen phosphate, agar-agar, sodium hydroxide were obtained from Synth (São Paulo, Brazil). Pineapple fruits were acquired from a supermarket in Campinas (Brazil).

2.2 Enzyme assays

Fruit bromelain (EC 3.4.22.5) was obtained from the fruit extract of the pineapple's Perola cultivar. Pineapple pulp was triturated and filtered with nylon filter. The filtrate contained the bromelain enzyme. Samples were frozen at -5°C to avoid the denaturation (Fileti et al., 2009; Fisher et al., 2007; Silveira et al., 2009).

2.3 Preparation of aqueous two-phase systems (ATPs)

Solutions of PEG 4000 (50% w/w) and Phosphate buffer (22.5%, w/w) were prepared at pH value of 6 to 11 and temperature of 25°C . A known volume of PEG solution was added to the buffer solution until it became turbid, indicating the beginning of the two-phase system for determination binodal curves.

2.4 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; Fisher et al., 2007; Murachi, 1976).

3. Results and Discussion

The Figure 1 showed the experimental binodal curves of PEG4000/ phosphate ATPS obtained at pH value of 6 to 11. This interval of pH was chosen because the enzyme

shows the maximum of activity. The equilibrium curves of PEG 4000/ phosphate ATPS had a random behavior with the pH of systems, that is, this behavior cannot be described. This has also been observed by Diamond and Hsu (1992), Zaslavsky (1995) and Johansson et al. (1995).

Experimental data of tie line lengths composition from PEG4000/phosphate aqueous two-phase systems (ATPs) 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.

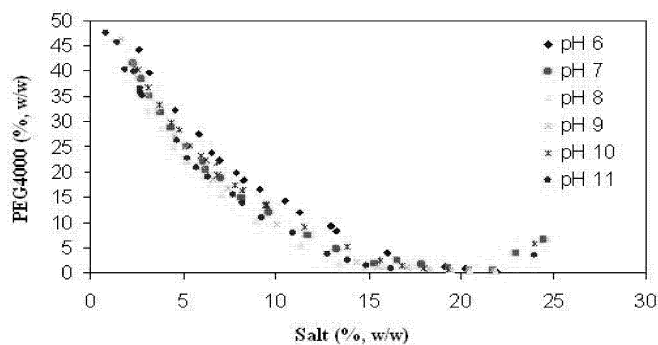


Figure 1. Binodal curves of PEG/phosphate aqueous two-phase systems (ATPs), at $25 \pm 2^\circ\text{C}$.

Table 1. Phase compositions from PEG 4000/ Phosphate Salt Aqueous two-phase systems (ATPs), at $25 \pm 2^\circ\text{C}$ and pH 6.

Tie Line	System (%w/w)			Top phase (%w/w)			Bottom phase (%w/w)		
	Salt	PEG	H ₂ O	Salt	PEG	H ₂ O	Salt	PEG	H ₂ O
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 (ATPs), at $25 \pm 2^\circ\text{C}$ and pH 7

Tie Line	System (%w/w)			Top phase (%w/w)			Bottom phase (%w/w)		
	Salt	PEG	H ₂ O	Salt	PEG	H ₂ O	Salt	PEG	H ₂ O
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 (ATPs), at $25 \pm 2^\circ\text{C}$ and pH 8.

Tie Line	System			Top phase			Bottom phase		
	(%w/w)			(%w/w)			(%w/w)		
	Salt	PEG	H ₂ O	Salt	PEG	H ₂ O	Salt	PEG	H ₂ O
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 (ATPs), at $25 \pm 2^\circ\text{C}$ and pH 9.

Tie Line	System			Top phase			Bottom phase		
	(%w/w)			(%w/w)			(%w/w)		
	Salt	PEG	H ₂ O	Salt	PEG	H ₂ O	Salt	PEG	H ₂ O
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 (ATPs), at $25 \pm 2^\circ\text{C}$ and pH 10.

Tie Line	System			Top phase			Bottom phase		
	(%w/w)			(%w/w)			(%w/w)		
	Salt	PEG	H ₂ O	Salt	PEG	H ₂ O	Salt	PEG	H ₂ O
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 (ATPs), at $25 \pm 2^\circ\text{C}$ and pH 11.

Tie Line	System			Top phase			Bottom phase		
	(%w/w)			(%w/w)			(%w/w)		
	Salt	PEG	H ₂ O	Salt	PEG	H ₂ O	Salt	PEG	H ₂ O
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

It was observed that on the conditions of tie lines two (14% PEG/13% salt) and three (12.6% PEG/12.2% salt) at pH 11, the enzyme presented the best phase separation and the maximum activity. These values were more than partition coefficients obtained by Fileti et al. (2009), Fischer et al. (2007), Lopes et al. (2009) and Rabelo et al. (2004).

4. Conclusions

It was observed that the equilibrium curves of PEG 4000/ phosphate had a random behavior with the systems of pH 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. So, the ATPs is a good promising system for bromelain enzyme extraction, being the enzyme preferentially partitioned on the top phase.

References

- Aires-Barros, M. R.; Taipa M. A.; Cabral, J. M. S. (1994). Isolation and purification of lipases. In: *Lipases: their structure, biochemistry and application*. Great Britain: Cambridge University Press, Eds. Wooley P, Petersen SB. pp. 234-270.
- Albertsson, P.-Å. (1986). *Partition of cell particles and macromolecules*. 3^a ed., John Willey, New York.
- Baldini, V. L. S. et al. (1993). Ocorrência da bromelina e cultivares de abacaxizeiro. *Colet. ITAL*, 23(1), 44-55.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein. Utilizing the principle of protein-dye binding. *Anal. Biochem.* 72 248-254.
- Diamond, A. D. and Hsu, J. T. (1992). Aqueous two-phase systems (ATPs) for biomolecule separation. *Advances in Biochemistry Engineering*. 47 89-135.
- Fileti, A. M. F.; Fischer, G. A.; Santana, J. C. C. and Tambourgi, E. B. (2009). Batch and Continuous Extraction of Bromelain Enzyme by Reversed Micelles. *Braz. Arch. Biol. Technol.* v.52 n.5: pp. 1225-1234.
- Fischer, G. A.; Ferrari, J. F.; Santana, J. C. C.; Fileti, A. M. F.; Tambourgi, E. B. (2007). Aqueous two-phase systems (ATPs) chromatography and biochemistry characterization of a enzyme from *Ananas comosus*. *Trend in Chromatography* 3, 85-95.
- Hale, L. P.; Greer, P. K.; Trinh, C. T.; Gottfried, M. R. (2005a). Treatment with oral bromelain decreases colonic inflammation in the IL-10-deficient murine model of inflammatory bowel disease. *Clinical Immunology* 116(2):135-142.
- Hale, L. P.; Greer, P. K.; Trinh, C. T.; James, C. L. (2005b). Proteinase activity and stability of natural bromelain preparations. *International Immunopharmacology* 5(4):783-793.
- Johansson, H.-O.; Karlström, G. Mattiasson, B. & Tjerneld, F. (1995). Effects of hidrofobicity and counter ions on the partition of amino acids in termosering Ucon-water two-phase systems. *Bioseparation* 5: 269 – 279.

- Lopes, F. L. G.; Severo Júnior, J. B.; Souza, R. R.; Ehrhardt, D. D.; Santana, J. C. C.; and Tambourgi, E. B. (2009). Concentration by Membrane Separation Processes of a Medicinal Product Obtained from Pineapple Pulp. *Braz. Arch. Biol. Technol.* v.52 n.2: pp. 457-464.
- Matiasson, B. and Kaul, R. (1986). Use of aqueous two-phase systems (ATPs) for recovery and purification in biotechnology. In: *Separation, Recovery, and Purification and Biotechnology*. American chemical Society, pp.79- 92.
- Murachi T. (1976). Bromelain Enzymes. In: Lorand L. *Methods in Enzymology*. New York: Academic Press, v.XLV, pp. 475-485.
- Rabelo, A. P. B.; Tambourgi, E. B.; Pessoa Jr., A. (2004). Bromelain partitioning in two-phase aqueous systems containing PEO-PPO-PEO block copolymers. *J. Chrom. B*, 807, p.61-68.
- Sercor, Jr., E. R.; Carson, W. F.; Cloutier, M. M.; Guernsey, L. A.; Schramm, C. M.; Wu, C. A.; Thrall, R. S. (2005). Bromelain exerts anti-inflammatory effects in an ovalbumin-induced murine model of allergic airway disease. *Cellular Immunology* 237(1):68-75.
- Sigma Chemical Co, Catalogue. (2006). Biochemical and chemical reagents, pp.116.
- Silveira, E.; Souza Jr.; M. E.; Santana, J. C. C.; Chaves, A. C.; Porto, A. L. F.; Tambourgi, E. B. (2009). Expanded bed adsorption of bromelain (EC 3.4.22.33) from *Ananas comosus* crude extract. *Braz. J. Chem. Eng.*, v.26, n.1, pp.149-157.
- Wen, S.; Huang, T. H. W.; Li, G. Q.; Yamahara, J.; Roufogalis, B. D.; Li, Y. (2006). Bromelain improves decrease in defecation in postoperative rats: Modulation of colonic gene expression of inducible nitric oxide synthase. *Life Sciences* 78(9), 995-1002.
- Zaslavsky, B. Y. *Aqueous two-phase partitioning - Physical Chemistry and Bioanalytical Applications*, MerceL Dekker, Inc. New York (1995).