Studies on Bromelain Precipitation by Ethanol, Poly (Ethylene Glycol) and Ammonium Sulphate

Paulo Soares[1,4], Diego Coelho[2], Priscila Mazzola[3], Edgar Silveira[1], Maria das Graças Carneiro-da-Cunha[4], Adalberto Pessoa Jr.[1], Elias Tambourgi[2]

[1]FCF – Universidade de São Paulo
Av. Lineu Prestes, 580, 05508-000, São Paulo -SP, Brasil
[2]FEQ – Universidade Estadual de Campinas
P.O. Box 6066, 13083-970, Campinas-SP, Brasil
[3]FCF – Universidade Estadual de Campinas
Rua Tessália Vieira de Camargo, 126, 13087-887, Campinas-SP, Brasil
[4]DBIOq – Universidade Federal de Pernambuco
Av. Prof. Moraes Rego, s/n, 50670-420, Recife-PE, Brasil

Brazil produces over than 58.000 hectares of pineapple, beeing the world leader in pineapple production. Aiming the recovery of agriculture residues (stalk and leaves), and fruit processing residues (stem and bark), several researches have been carried out for the recovery of pineapple's enzymes, such as the bromelain. These enzymes performs important role in proteolytic modulation at cellular matrix, in numerous physiologic process, including tissue morphogenesis, tissue repair, angiogenesis and tissue modulation, decreasing bruises, swelling, pain and healing time. This work aims the recovery on bromelain from pineapple stem, bark and leaves. Aqueous extract of bromelain was prepared through stem, bark and leaves processing in a common blender. Precipitation studies were performed under refrigeration (4 °C). Poly(ethylene glycol) precipitations were carried out in a single step, in various concentrations. Ethanol and ammonium sulphate precipitations were carried out stepwise, where several concentrations of ethanol and ammonium sulphate were added to perform a fractional precipitation. Results showed that bromelain was not precipitated by poly(ethylene glycol) at all. However, bromelain was recovered with 30-70% ethanol fraction, in which were achieved a purification factor of 2.07 fold, and yielded over than 98% of enzymatic activity. Precipitation with ammonium sulphate showed that bromelain was recovered at 20-40 saturation fractions, yielding up to 44% of protein recovery, and a purification factor of 4.4 fold. Although the ethanol precipitation had achieved a lower purification factor, it seems to be more suitable for bromelain recovery, based on process time and local ethanol price, since Brazil is the largest ethanol producer worldwide.

1. Introduction

The pineapple (*Ananas comosus* L.) is cultivated extensively in Hawaii, Philippines, Caribbean, Malaysia, Australia, Mexico, South Africa and Brazil. Brazil is the second producer worldwide with more than 58,000 hectares planted. The main producer areas are the rain forest and country's northeast, although it is cultivated all around the country (Silveira et al., 2009).

The stem bromelain (formerly E.C. 3.4.22.4) is extracted from *A. comosus*, considered by many to be a whole fruit, it is in fact an infructescence: many independent fruits are bound together in one common body around a fibrous stem. The stem bromelain (E.C. 3.4.22.32) is the most abundant cystein endopeptidase in pineapple's stem, leaves and skin. It exhibits a broad specificity for protein cleavage, having a strong preference for Arg-Arg-|-NHMec amongst other substrates (Haq et al., 2005, Harrach et al., 1998).
Bromelain belongs to a group of proteolytic enzymes, which are used as drugs for oral systemic treatment of inflammatory, blood-coagulation-related and malignant diseases. These enzymes offer a wide spectrum of therapeutic efficacies: they demonstrate, *in vitro* and *in vivo*, antiedemateous, anti-inflammatory, antithrombotic and fibrinolytic activities (Maurer, 2001).
Bromelain is a crude extract constituting an unusually complex mixture of different thiol-endopeptidases and other not yet completely characterized components such as phosphatases, glucosidases, peroxidases, cellulases, glycoproteins and carbohydrates, among others (Rowan et al., 1990). Nowadays, bromelain is prepared from cooled pineapple juice by centrifugation, ultrafiltration and lyophilisation. This process yields a yellowish power with 40% of protein content (Harrach et al., 1998).
This work describe the studies on bromelain precipitation with ethanol, ammonium sulphate and poly(ethylene glycol) as an alternative process for the production of commercial bromelain.

2. Materials and Methods

1 Enzyme Extract

The enzymatic extract, referred to as crude extract for now on, was obtained from *Ananas comosus*'s stem, bark and leaves. The vegetable tissue was processed in a common blender and than filtered on cheesecloth and centrifuged at 2000g for 20 minutes at 4˚C for insoluble particles removal.

2 Protein and Enzyme Activity Assay

The protein concentration was determined by the method described by Bradford (1976). The Enzymatic Activity Assay was performed by the azocasein method, as described by Oliveira et al. (2006). Azocasein 1.0% (w/v) (Sigma) was solubilised in ethanol 4% (v/v) and 0.1 M phosphate buffer, pH 7.0, and used as substrate. One activity unit was defined as the amount of bromelain needed to produce 1 μmol tyrosine per minute at 37°C.

3 Poly(Ethylene Glycol) Precipitation

The poly(ethylene glycol) precipitation was carried out according to the methodology described by Walker (2002). A solution of PEG 1500, 4000 and 6000, each in several concenrations (10 to 50% w/w) and cooled to 4˚C, was slowly added to the crude extract in a jacked Becker at 4˚C and homogenized by 20 minutes. The resultant solution was centrifuged at 2000g for 20 minutes at 4˚C, and the resulting pellet was resolubilised in 0.02 M Phosphate Buffer, pH 7.0.

4 Ammonium Sulphate Precipitation

Ammonium Sulphate precipitation was performed by the method described by Englard and Seifter (1990), where solid $NH_4(SO_4)_2$ was added to 0˚C cooled crude extract until the desired saturation (20%, 40%, 60%, 80% and 100%) was reached. After each step,

the solution was centrifuged at 2000g for 20 minutes at 4˚C, and the resulting pellet was resolubilised in 0.02 M Phosphate Buffer, pH 7.0.

5 Ethanol Precipitation

The ethanol precipitation was performed according to de methodology described by Englard and Seifter (1990). Ethanol was cooled to 0˚C and added dropwise in the crude extract until the desired concentration (10 to 90% v/v) was achieved. The solution was than centrifuged at 2000g for 20 minutes at 4˚C, and the resulting pellet was resolubilised in 0.02 M Phosphate Buffer, pH 7.0.

3. Results and Discussion

It was observed that PEG would not precipitate bromelain, nor any other *A. comosus*'s proteins. The PEG precipitation of proteins is well documented in literature (Fontes et al., 2005, Kumar et al., 2009); however, it emphasizes the solubility of serum proteins. Kumar et al. (2009) described that PEG would decrease the solubility of BSA from 0.6 mg/ml to less than 0.1 mg/ml after addition of 18% (w/v) of PEG 400 or 25% (w/v) of PEG 1450. Fontes et al. (2005) reported the precipitation of whole virus, in which had an initial protein content of 0.82 mg/ml. The lack of precipitation at the *Ananas comosus*'s proteins over PEG addition should be a result of the little concentration of total proteins at the crude extract, less than 0.3 mg/ml.

Table 1 shows the effects of Ammonium Sulphate saturation on bromelain precipitation. The crude extract produced five pools of proteolytic activity successively separated by ammonium sulphate precipitation. It was observed that more than 44% of total activity was collected in the 20-40%'s saturation fraction. This activity is probably due to bromelain itself, since it is the most abundant endopeptidase in the crude extract and should be soluble in solutions with $(NH_4)_2SO_4$ saturation less than 20%. Narayan et al. (2008) reported that increasing $(NH_4)_2SO_4$ concentration, the purification degree decreased significantly. As in conventional salting out, the extent of protein precipitation is a function of $(NH_4)_2SO_4$ concentration (Roy and Gupta, 2000).

Table 1: Effects of ammonium sulphate saturation on bromelain precipitation

	Protein Concentration ($mg.mL^{-1}$)	Bromelain Activity ($U.ml^{-1}$)	Specific Activity ($U.mg^{-1}$)	Y (%)	Purification Factor
Crude Extract	0.201	16.25	80.85	--	--
0-20%	0.005	0.13	24.69	2.62	0.31
20-40%	0.089	31.97	359.28	44.27	4.44
40-60%	0.054	3.06	56.93	26.74	0.70
60-80%	0.036	2.00	55.59	17.90	0.69
80-100%	0.006	0.21	34.03	3.07	0.42

Devakate et al. (2009) described the precipitation of fruit bromelain (E.C. 3.4.22.5) with ammonium sulphate, where it achieved 2.97 fold in the 40-70% saturation range.

However, the fruit bromelain is a completely different enzyme, as in biochemical characteristics as in physiological functions, from stem bromelain, which should explain this discrepancy.
Table 2 shows the effects of ethanol concentration in bromelain precipitation. Whenever ethanol concentrations on crude extract were over than 60% (v/v), the enzymatic activity recovery was near its initial value. It was also observed that with 30% of ethanol (v/v) there was no bromelain precipitation. However, under this conditions more than 26% of proteins presented in crude extract were precipitated. Ethanol precipitation was then performed in two steps, 0-30 and 30-70%, and so it was possible to extract bromelain without any loss in its activity and with an increase on purification factor from 1.13 to 2.34.

Table 2: Effects of ethanol concentration on bromelain precipitation

	Protein Concentration ($mg.mL^{-1}$)	Bromelain Activity ($U.mL^{-1}$)	Specific Activity ($U.mg^{-1}$)	Y (%)	Purification Factor
Crude Extract	0.201	16.25	80.85	N.A.	N.A.
0-30%	0.052	0.00	0.00	26.01	0.00
30-70%	0.084	15.96	189.5	41.91	2.34

Cesar (1999) achieved, in a single step, total bromelain recovery from *A. comosus* with 80% of ethanol (v/v), and increased the specific activity 5 times. Rabelo (2004) described bromelain recovery using thermoseparating aqueous two-phase systems, in which was obtained a purification of 1.25 fold. Umesh Hebbar et al. (2008) achieved 106% of bromelain activity recovery and purification factor of 5.2 fold from *A. comosus* wastes when using reversed micelles systems.
The main purpose of using a precipitation method, such as ammonium sulphate or ethanol, is to concentrate the target molecule. Concentration is a necessary step to reduce the volume making it easier to handle for further purification, specially when it is integrated with a purification step. Thus considering the economics, it is always advisable to include a concentration step, even though if there is a compromise in the yield. Therefore, even doing a simple precipitation step, such as ethanol or ammonium sulphate fractionation, it is possible to purify considerably the enzyme presented in *A. comosus*'s wastes. It also represents a cost saving procedure, since it would reduce the overall steps number in biomolecule purification.

4. Conclusions

The bromelain was precipitated by ammonium sulphate mainly at the 20-40% saturation fraction yielding a purification factor of 4.44. Ethanol precipitation, on other hand, yielded 2.07. Bromelain was not precipitated by poly(ethylene glycol) whatsoever. Although ethanol precipitation had achieved a lower purification factor, it seems to be more suitable for bromelain recovery, based on process time, low ionic strength (which

is desirable for further purification steps) and the local ethanol prices, since Brazil is the largest ethanol producer worldwide.

References

Bradford, M.M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry. 72, 248-254.

Cesar, A.C., Silva, R. and Lucarini, A.C., 1999, Recuperação das enzimas presentes na casca e no talo do abacaxi, Revista de Iniciação Científica - São Carlos/SP. 1, 47-53.

Devakate, R.V., Patil, V.V., Waje, S.S. and Thorat, B.N., 2009, Purification and drying of bromelain, Separation and Purification Technology. 64, 259-264.

Englard, S. and Seifter, S., 1990, Precipitation techniques. in: Guide to protein purification, Eds. Deutscher, M.P., Academic Press, San Diego, USA.

Fontes, L.V.Q., Campos, G.S., Beck, P.A., Brand„o, C.F.L. and Sardi, S.I., 2005, Precipitation of bovine rotavirus by polyethylen glycol (PEG) and its application to produce polyclonal and monoclonal antibodies, Journal of Virological Methods. 123, 147-153.

Haq, S.K., Rasheedi, S., Sharma, P., Ahmad, B. and Khan, R.H., 2005, Influence of salts and alcohols on the conformation of partially folded intermediate of stem bromelain at low pH, The International Journal of Biochemistry & Cell Biology. 37, 361-374.

Harrach, T., Eckert, K., Maurer, H.R., Machleidt, I., Machleidt, W. and Nuck, R., 1998, Isolation and characterization of two forms of an acidic bromelain stem proteinase, Journal of Protein Chemistry. 17, 351-361.

Kumar, V., Sharma, V.K. and Kalonia, D.S., 2009, Effect of polyols on polyethylene glycol (PEG)-induced precipitation of proteins: Impact on solubility, stability and conformation, International Journal of Pharmaceutics. 366, 38-43.

Maurer, H.R., 2001, Bromelain: biochemistry, pharmacology and medical use, Cellular and Molecular Life Sciences (CMLS). 58, 1234-1245.

Narayan, A., Madhusudhan, M. and Raghavarao, K., 2008, Extraction and Purification of *Ipomoea* Peroxidase Employing Three-phase Partitioning, Applied Biochemistry and Biotechnology. 151, 263-272.

Oliveira, L.A., Porto, A.L.F. and Tambourgi, E.B., 2006, Production of xylanase and protease by Penicillium janthinellum CRC 87M-115 from different agricultural wastes, Bioresource Technology. 97, 862-867.

Rabelo, A.P.B., Tambourgi, E.B. and Pessoa, A., 2004, Bromelain partitioning in two-phase aqueous systems containing PEO-PPO-PEO block copolymers, Journal of Chromatography B. 807, 61-68.

Rowan, A.D., Buttle, D.J. and Barrett, A.J., 1990, The cysteine proteinases of the pineapple plant, Biochem. J. 266, 869-875.

Roy, I. and Gupta, M.N., 2000, Current trends in affinity-based separations of proteins/enzymes, Current Science. 78, 587-591.

Silveira, E., Souza-Jr, M.E., Santana, J.C.C., Chaves, A.C., Porto, A.L.F. and Tambourgi, E.B., 2009, Expanded bed adsorption of bromelain (E.C. 3.4.22.33) from *Ananas comosus* crude extract, Brazilian Journal of Chemical Engineering. 26, 149-157.

Umesh Hebbar, H., Sumana, B. and Raghavarao, K.S.M.S., 2008, Use of reverse micellar systems for the extraction and purification of bromelain from pineapple wastes, Bioresource Technology. 99, 4896-4902.

Walker, J.M., 2002, The Protein Protocols Handbook. Humana Press, Totowa, New Jersey.