ASIAN JOURNAL
ISSN: 0976-3376

Available Online at http://www.journalajst.com

ASIAN JOURNAL OF SCIENCE AND TECHNOLOGY

Asian Journal of Science and Technology
Vol. 2, Issue, 1, pp.016-022, January, 2011

RESEARCH ARTICLE

A COMPARATIVE STUDY ON DETERMINING THE EFFICACY OF SALT PRECIPITATION AND BIPHASIC SYSTEM IN THE EXTRACTION OF BROMELAIN FROM *Ananas comosus*

Sankaran, *K[1]., Vadanasundari, V[1]., Hemavathy, R.V[1] and Thirunavukkarasu, A[2]

[1]Department of Biotechnology, P.R.Engineering College, Thanjavur, Tamil Nadu
[2]Department of Biotechnology, P.S.G. College of Technology, Coimbatore, Tamil Nadu

Received 19th October, 2010; Received in revised from; 15th December, 2010; Accepted 27th December, 2010; Published online 19th January, 2011

Purification of bromelain from wet *Ananus comosus* latex by extraction in aqueous two-phase system was studied and compared with the traditional procedure involving a two-step salt precipitation. The bromelain obtained by the latter method was usually contaminated with other proteins, and its purity was dependent on the initial protein concentration in the material used for processing. Highly pure bromelain was obtained in a much shorter processing time directly from the stem than the fruit portion with the use of an aqueous two-phase system consisting of 8% (w/w) polyethylene glycol and 15 % (w/w) ammonium sulfate. The purified product obtained from stem and fruit were further characterized by SDS-PAGE and their molecular weights were determined as 23 KDa (Biphasic) and 19 KDa (Salt Precipitation) for the fruit sample. Similarly, 28 KDa (Biphasic) and 20 KDa (Salt precipitation) for the Stem sample. From this investigation, it is confirmed that the Bromelain, molecular size is found to be high in the Biphasic system.

Key words: *Ananus comosus* – Aqueous two phase system – Salt precipitation - SDS-PAGE

INTRODUCTION

Bromelain, (EC 3.4.22.33) derived from the pineapple fruit, is one of the groups of proteolytic enzymes. Proteases are the single class of enzymes which occupy a pivotal position with respect to their application in both physiological and commercial fields. Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes. Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals is a relatively new development and has conferred added biotechnological importance.

***Corresponding author:** *sivamsanthosh@gmail.com*

The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications. Bromelain is an anti-inflammatory agent and for this reason, it is helpful in healing minor injuries, particularly sprains and strains, muscle injuries, and the pain, swelling, and tenderness that accompany sports injuries. Topically applied bromelain in the form of a cream may be beneficial for frostbite, possibly enhancing the rate of healing and for cleaning debris from burns. Also it is a natural blood thinner because it prevents excessive blood platelet stickiness (Monograph: Bromelain). In addition, bromelain reduces the thickness of mucus, which may benefit patients with asthma or chronic bronchitis (Tattersall *et al.,* 1983).

Bromelain is a collective name for proteolytic enzymes or proteases found in tissues including stem, fruit and leaves of the pineapple plant family Bromeliaceae. The isolation of enzymes from pineapple fruit and its study have been investigated since 1894. The enzymes occurring in the stem and the fruit of *Ananas comosus* (Smouth cayenne) are the most studied. Unlike crude stem bromelain, which is used widely in industry, fruit bromelain is not commercially available despite the large quantities of waste pineapple fruit portions at pineapple canneries. The continued interest in bromelain, for its numerous applications in the food industry as well as in medicine and pharmacology make this enzyme one of the best vegetal proteases. The potential therapeutic value of bromelain is due to its biochemical and pharmacological properties and hence, it is desired to obtain bromelain in its highest purified form. The preparation in pure form of a proteolytic enzyme has always proved difficult, and the bromelains appear to be no exception. The crude commercial bromelain used in the manufacture of pharmaceuticals is not chemically homogeneous. The main ingredient in crude bromelain is a proteolytic enzyme named glycoprotein, besides substances such as insoluble materials, e.g. colored pigments, organic acids, minerals, protease inhibitors, organic solvents and excipient used for enzyme recovery have been reported. The present study examines the extract of fresh pineapple fruit for the presence of cysteine proteinases, besides protein degradation kinetics.

Purification of bromelain from pineapple juice has traditionally been achieved by precipitation methods; however, the purified enzyme still remains contaminated with other proteases. An alternative purification strategy has involved the use of chromatographic techniques like size exclusion chromatography. Aqueous two-phase systems (ATPS), made up of two polymers or one polymer and a salt in water, have shown interesting potential for downstream processing of proteins, especially in view of providing integrated clarification, concentration and purification of the target product in one unit operation. They have been successfully applied for large-scale enzyme separation and purification. A desired partition of proteins in such systems can be obtained by manipulating a variety of system parameters. The polymer– salt systems have the advantage of low cost and low viscosity compared to polymer–polymer systems (David *et al.,* 1988). Aqueous two-phase systems are widely used for separation and purification of biomolecules. These systems are suitable for purification of biological material as the phases contain 70–90% water, thus reducing the denaturation of labile molecules. The advantages of aqueous two-phase extraction compared to other purification methods lie in volume reduction, high capacity and short processing times. The most frequently used among the former systems has been the polyethylene glycol (PEG)–phosphate however other salts with multivalent anions have also been used (Haiyan Yue *et al.,* 2007). Use of PEG–phosphate system has earlier been reported for separation and purification of bromelain from pineapple juice. This paper reports the use of ATPS composed of PEG and ammonium sulfate for purification of bromelain from *A.comosus* fruit juice and compares it with the conventional method based on precipitation.

Purification of enzymes has traditionally been achieved by precipitation methods however; the purified enzyme still remains contaminated with other proteases. An alternative purification strategy has involved the use of biphasic system, made up of two polymers or one polymer and a salt in water have shown interesting potential for downstream processing of proteins, especially in view of providing integrated clarification, concentration and purification of the target product in one unit operation. It has been successfully applied for large-scale enzyme separation and purification. A desired partition of proteins in such systems can be obtained by manipulating a variety of system parameters. The polymer–salt–water systems have the advantage of low cost and low viscosity compared to polymer–polymer–water systems. The most frequently used among the former systems has been the polyethylene glycol (PEG)–phosphate (Veide *et al.,* 1983 and Hustedt *et al.,* 1985). Use of PEG–phosphate system has earlier been reported for separation and purification of papain from papaya latex (Kuboi *et al.,* 1990). This study reports the use of ATPS composed of PEG and potassium phosphate for purification of

bromelain from stem and fruit portion of *Ananus Comosus* and compares it with the conventional method based on precipitation. The fruit and stem of *A.Comosus* (Pine apple) is a rich source of the cysteine endopeptidases, including bromelain, glycyl endopeptidase, chymobromelain and caricain, which constitute more than 80% of the whole enzyme fraction (Azarkan *et al.,* 2003). Bromelain (EC 3.4.22.2) is a minor constituent (5–8%) among the pineapple endopeptidases (Barrett *et al.,* 1998). The enzyme is used widely as meat tenderizer, and has also several other applications, like defibrinating wounds, treatment of edemas, shrink proofing of wool.

MATERIALS AND METHODS

The study focuses on finding out the extraction efficiency of aqueous two phase system (PEG 4000 –Potassium Phosphate system) with the conventional salt precipitation (Ammonium sulphate) for the enzyme Bromelain extracted from the fruit and stem portion of pineapple (*Ananas comosus).*

Sample preparation from Ananus comosus Fruit Bromelain

The pineapple fruit (*A. comosus*) used in this study was obtained from the local market. The fruits were washed and peeled. After removal of core, the fruit was cut into small pieces and stored at 4^{o}C for further use. The frozen pieces of pineapple were homogenized in cold extraction buffer (0.01M sodium phosphate buffer, pH-7) and homogenate was filtered. The filtrate was centrifuged at 10,000 rpm (4 ^{o}C, 20 min) and the supernatant (crude enzyme extract) was used for further experiments.

Stem Bromelain

Fresh extract from the stem portion of *A.comosus* was collected. The same methodology has been followed for the enzyme extraction.

Salt precipitation (Ammonium sulphate) of bromelain from fruit and stem extract of *A.comosus*

The precipitation of bromelain from clarified pineapple fruit and stem extract (20 ml) was carried out by slow addition of ammonium sulfate $(NH_4)_2SO_4$ at 4 ^{o}C under constant stirring. Initially, 6 g of $(NH_4)_2SO_4$ was added to get 30% saturation. The stirring was continued for 30 min after the complete addition of salt to allow equilibration between the dissolved and aggregated proteins. The salt-enriched solution was centrifuged at 10,000 rpm for 15min and the precipitate was collected. The collected precipitate was then analyzed for total protein (Bradford assay) and Bromelain (gelatin digestion test).

Aqueous Biphasic extraction (PEG 4000-Potassium Phosphate) of bromelain from fruit and stem extract of *A.comosus*

A biphasic system was allowed to form with Polyethylene glycol 4000 and Potassium Phosphate, thus we tried to achieve a polymer-salt phase system. The two phases were constructed with 8% PEG 4000 and 15% Potassium Phosphate in such a way that maximum partitioning of our desired protein in the salt phase has been achieved. Accurately weighed quantities of phase forming solutes were allowed to stand for 15 min to have a biphasic system. The crude enzyme extract from the fruit and stem portion were added to make the total weight of the system 100% (w/w). During the experiment the weight percentage of crude enzyme extract was maintained at 20% (w/w). The contents were mixed thoroughly using a magnetic stirrer for equilibration and were allowed to separate for about 5 h in a separating funnel. After clear separation of two phases, the volumes of top and bottom phases of the system were noted and analyzed for total protein (Bradford assay) and bromelain (gelatin digestion test) All partition experiments were conducted in triplicates at 25±1 ◦C.

Gelatin digestion test for the confirmation of Bromelain in the crude extract

The presence of Bromelain in the precipitated protein is confirmed by Gelatin digestion test, by viscometric analysis. 3ml of crude protein was added to 12ml of the substrate, gelatin and incubate the mixture at 45ºC for an hour and observe their viscosities.

Purification of Bromelain by dialysis

The precipitated protein from the salt precipitation contains our desired enzyme bromelain and other proteins along with the salts used for their precipitation. The salts in the samples were removed by dialysis using ammonium phosphate buffer (50mM, pH 7.0) as a process for buffer exchange. Dialysis membrane (Hi-Media Laboratory, Mumbai, India) having molecular weight cut-off of 20kDa was used for dialysis. The specific enzyme activity units were determined by GDU - Gelatin Digestion Unit assay framed by Enzyme Development Corporation, (EDC), and New York**.** One Gelatin Digestion Unit is the amount of enzyme which will liberate 1 mg of amino nitrogen from a standard gelatin solution at pH 4.5 or pH 5.5 after 20 minutes of digestion at 45°C.

GDU/g = ((T-B) x 14 x N x 50))/ Wt(g)
Where:
T = Test titer (ml 0.1 N NaOH)
B = Blank titer (ml 0.1 N NaOH)
N = Normality of standardized NaOH (i.e. 0.100)
Wt (g) = Initial weight of enzyme (50mg)

Similar purification was performed for the salt phase of the aqueous biphasic system. The GDU assay has been done for finding the specific enzyme activity units and the comparison has been made with the salt precipitation.

Confirmation of the enzyme Bromelain through SDS electrophoresis

The presence of Bromelain in the extract has been confirmed with the relative positioning of the sample loaded in the 12% SDS-PAGE gel with the unstained protein molecular marker UPM, which ranges from 14.4kDa to 116kDa. The molecular weight of is 28kDa and 23kDa for stem and fruit bromelain respectively (Hames *et al.,*1984) and it has been validated for the loaded sample (Figure 1a & 1b).

RESULTS AND DISCUSSION

In Salt precipitation method, the fresh extract contains 33±2 µg protein and 2966±28 units

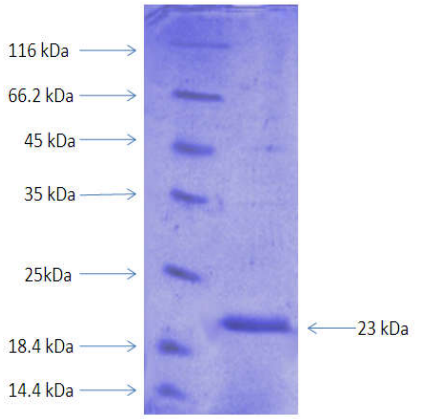

Fig. 1a. Confirmation of Enzyme Bromelain (STEM)

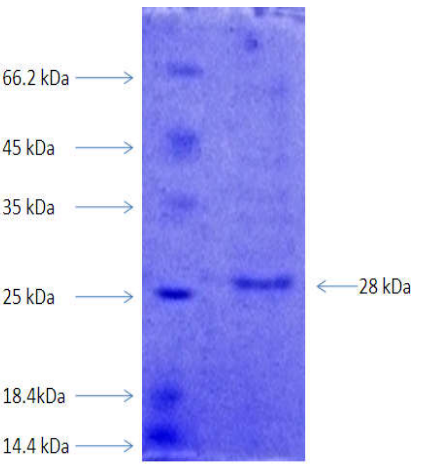

Fig. 1b. Confirmation of Enzyme Bromelain (Fruit)

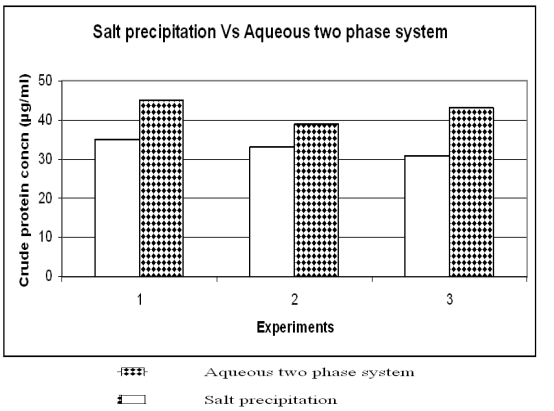

Fig. 2a. Stem extract

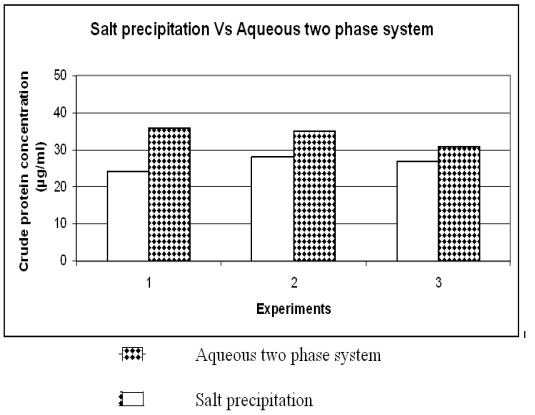

Fig. 2b. Fruit extract

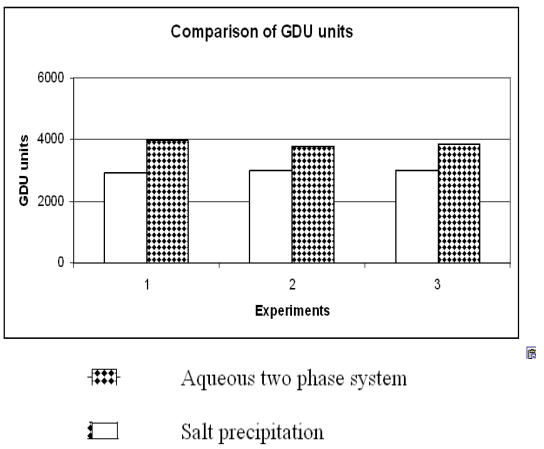

Fig. 3a. Stem extract

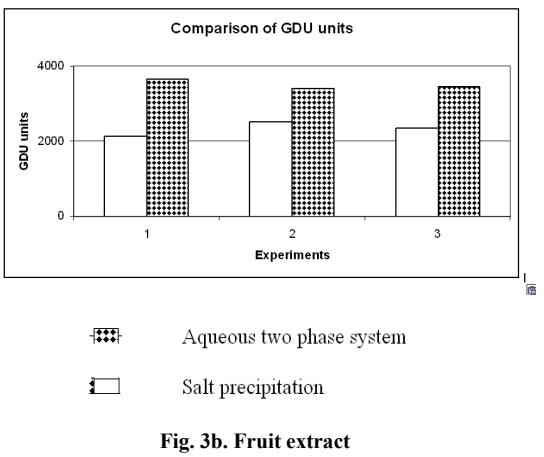

Fig. 3b. Fruit extract

Table 1. Two step salt precipitation

Expt.	Steps		Crude protein concentration (µg/ml)			Specific GDU assay (Units)	
			Stem extract	Fruit juice		Stem extract	Fruit juice
A	I step	PRECIPITATION	35	24	DIALYSIS	2925	2124
	II step		12	8		1221	758
B	I step		33	28		2987	2528
	II step		14	6		741	256
C	I step		31	27		2986	2357
	II step		8	9		885	579

Table 2. Biphasic extraction

Expt.	PHASE	Crude protein concentration (µg/ml)			Specific GDU assay (Units)	
		Stem extract	Fruit juice		Stem extract	Fruit juice
A	SEPARATION	45	36	DIALYSIS	3958	3652
B		39	35		3785	3412
C		43	31		3857	3458

protease activity from stem, 26±2 µg protein and 2336.3±202 units protease activity from fruit. In biphasic system, the fresh extract contains 41±2 µg protein and 3844±28 units protease activity from stem, 32±2 µg protein and 3529 ±104 units protease activity from fruit**.**

As bromelain is a protease of broad specificity and no specific synthetic substrate is available, gelatin was used as a substrate to determine the total protease activity present in the extract while the purity of bromelain was determined by chromatography. On SDS page gel electrophoresis, the proteins in the stem extract were identified as bromelain according to its mobility that was locating nearer to the 23 kDa of the unstained protein marker. Similarly the proteins in the fruit extract were identified as bromelain according to its mobility that was locating nearer to the 28 kDa of the unstained protein marker.

Purification of bromelain by two-step salt precipitation

A modification of a two-step precipitation method proposed by Baines and Brocklehurst was used for purification of bromelain from the fruit and stem extract. The precipitation was performed in two stage using ammonium sulfate. As shown in Table 1, increase in protein concentration used during each precipitation step resulted in an increase in the amount of protease activity (including bromelain) recovered in the precipitate.

Purification of bromelain by aqueous two-phase extraction

As ammonium sulfate is commonly used for separation of cysteine proteases, a two-phase system composed of PEG–Potassium phosphate was evaluated for partitioning of bromelain. According to the phase diagram of PEG–K_2PO_4 system by Salabat, the system comprising 12% (w/w) PEG 6000–15% (w/w) K_2PO_4 provides two separated phases. These were the maximum concentrations of the phase components that could be used for purification of bromelain from extract. The system containing more than 12% (w/w) of PEG resulted in a highly viscous mixture, whereas the system consisting of more than 15% ammonium sulfate provoked the precipitation of protein from the extract. Studies on partitioning of total protease activity of bromelain showed that extremely low levels of total protease activity were recovered in the PEG rich upper phase, which contained the major fraction of bromelain present in the extract (Table 2). This may reflect a higher salting out effect of potassium phosphate on bromelain owing to its higher surface hydrophobicity as compared to the other protease.

Consequently, bromelain is preferentially partitioned to the salt phase. This is also in accordance with the relatively higher bromelain recovery during potassium phosphate precipitation reported in Table 1, Fig 2 a & b. Increase in potassium phosphate concentration used to make the two-phase system increased only the recovery of bromelain in the salt phase (Table 2, Fig 3 a & b). Partitioning at protein concentrations ranging between 10 and 40 mg protein/ml in 12% PEG–15% K_2PO_4 revealed a notable variation in protease partition to the salt phase at their maximum concentration. Significant decrease in partition was indeed observed at higher protein concentrations (60 mg/ml and above) probably due to the limiting solvating effect of PEG. Highest recovery of protease (including bromelain) activity was achieved at pH 5.

This study illustrates that the bromelain obtained using biphasic system was found to be more pure when compared to the ammonium sulphate precipitation method .This is due to the high resolving power of aqueous two-phase system for separating enzymes. This technique provided an easy means of obtaining pure bromelain that was free from contaminating protease activities. High selectivity in separation was easily achieved by a suitable choice of phase components and partitioning conditions. The quickness factor, simplexes and easy scalability of the two-phase systems are the important features that favor the large-scale use of this technique for the purification of bromelain.

Acknowledgement

We convey our deep felt gratitude to the faculty members of PRGI (Ponnaiyah Ramajayam Group of Institutions) who have enlightened our life with Quality and Discipline along with academic focus and financial support.

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