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Comparative studies of bromelain extraction- conventional vs ionic liquid based aqueous two phase extraction

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ABSTRACT

Bromelain is a major proteinase, isolated from pineapple (Ananas comosus). In the plant, bromelain is accumulated in the entire plant to different extent and properties depending on its source. The objective of present study was to compare the extraction of bromelain present in stem juice of the plant using Ionic liquid (IL) based aqueous two phase (ATP) and Reverse micellar(RM) system. Separation and purification of high value bio-molecules using IL based ATP extraction is an emerging green separation process. Here the Phosponium based IL ATP system was used and it is the best alternative for the conventional organic solvent extraction and polymer based ATP systems. The current study is to compare the partition coefficient of bromelian from the stem juice of *Ananas comosus* and the same was compared with reverse micellar system. The resulting protein was purified on gel filtration chromatography column and it is confirmed with SDS page analysis. From the results it was found that IL based ATP system is highly selective and yield higher than the reverse micellar system.

KEYWORDS

Bromelian;
Ionic liquid;
ATP;
Protease;
Purity.

INTRODUCTION

Aqueous two phase extraction system is employed for the isolation and extraction of target enzyme in a restricted single step[1]. The aqueous two phases are formed by polymer/ polymer, polymer/salt, salt/salt combinations[2]. Polyethylene Glycol (PEG) is one of the phase forming component involved in the Aquoeus two phase formation[3]. It is selected because of its biodegradability, less melting points, maximum solubility in aqueous medium, economic,etc. These qualities made PEG usage in different processes, especially in Aqueous two phase systems. When compared to polymer-polymer ATP system, polymer/salt ATP system is more advantageous as it exhibit better biocompatibility and phase separations, fast process and profitable which satisfies the expectations of downstream processing[4]. The limitations of PEG in ATP system is in the extraction of hydrophobic molecules, because its hydrophilic nature restricts the protein extraction[5]. This can be overcome by applying Ionic liquids as phase forming component instead of PEG in ATP system[6]. This ionic liquid based ATP system provides high chemical stability and possess negligible vapour pressure as it is con-

sidered as the green solvent[7].

Bromelain (EC 3.4.22.5) is noticed in the tissue of the Bromiliaceae family of which pineapple (*Ananas comosus)* is the well identified source of the proteolytic enzyme[8]. The pineapple (*Ananas comosus*) is found in tropic region having ripe fruit consisting of combined berries. Bromelain has been widely used in food, medical, pharmaceutical and cosmetic industries[9]. In the present study, Bromelain from pineapple stem juice was extracted by different bioseparation methods and compared with high selective bio extraction method using ionic liquids[10].

Assessment of purity tools develops the specific activity and functional usage of bromelain enzyme. It also requires a precise separation means with cost effective operations. This ionic liquid based ATP system proves not only for high selectivity but also economic as well[11]. From the purity factor, the ionic liquid based ATP system shows not only high resolving power for bromelain enzyme separation and it is also a much simpler single step purification process in bioseparation in comparison to other methods[12,13].

The design of an ionic liquid based aqueous two phase extraction for the enzyme purification from its crude source is the main objective of this work. The resulting fractions undergo gel filtration chromatography technique for purity range. This study moreover investigates the specific activity of the aqueous two phase extraction of bromelain enzyme, conventional vs ionic liquid based ATP system.

MATERIALS

Ripened pineapple fruits were purchased from a local supermarket. Ionic liquids- 1-Butyl phosphonium bromide, KH_2Po_4, AOT, Iso octane, casein and bovine serum albumin (BSA), trichloroacetic acid (TCA) were purchased from Sigma Aldrich. All chemicals used in this work were of AR grade.

METHODOLOGY

Crude extract preparation

The juice was collected by peeling,slicing and crushing the pineapple and stored in a clean tube. The collected latex was centrifuged at 15,000 rpm for 10 minutes at 4°C. The supernatant finally collected was referred as crude extract.

Purification of Bromelain by ammonium sulphate precipitation

Englard and Seifter (1990) described the performance of Ammonium sulphate precipitation method, where the $NH_4(SO_4)_2$ solution was added drop by drop with the crude extract maintained at 0°C until the desired saturation was reached. The saturation range was ffrom 30 to 80%. The precipitation was found to occur at 40% of the salt solution. After reaching the saturation point, the solution was centrifuged at 2000 rpm for 10 minutes at 4°C. Then the resulted precipitation was dissolved in a buffer maintained at pH 7.2.

Purification of Bromelain by extraction in reverse micellar system

The stationary phase of the reverse micellar system was formed by suspending the surfactant in organic solvent, and the movable phase was the sodium phosphate buffer maintained at suitable pH values where the Kcl adjusts the ionic strength. For equilibration, the mobile phase used was 0.150 mM of Kcl. The preparation of a Reverse micellar system was by dissolving 60mM AOT in Iso-octane mixture.. The injection of transportable phase into the Reverse micellar phase gives equilibration and the constant and clear reverse micellar phase were obtained.

Purification of bromelain by ATP system

PEG-4000 polymer and $NH_4(SO_4)_2$ were taken to form the Aqueous Two Phase system. Crude extract was added to the polymer salt mixture and mixed gently and centrifuged at 6,000 rpm for 30 minutes at 4°C. Two phases formed were separated carefully. The top phase separated was added with 150mM salt and centrifuged at 4000 rpm and the bottom phase was carefully separated to carry out further estimations.

Purification of bromelain by ionic liquid based ATP system

An ionic liquid based ATP system was formed by applying three different ionic liquid as one of the phase forming components with the KH_2PO_4 salt. Ionic liquids of preferred volume mixed with KH_2PO_4 salt was added to the equal amount of diluted sample solution.

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In order to attain equilibrium, the mixture was refrigerated at 25°C for 20 minutes. Addition of 0.5M Nacl alters the ionic strength of the ATP and the mixture was centrifuged for phase separation. The upper and bottom phases were gathered separately for further analysis.

Determination of total protein assay, Enzyme assay and partition coefficient

Total protein was determined based on Lowry protein assay method[14] with BSA as standard. Enzyme assay activity was resolved by enzymatic hydrolysis of 2%(w/v) casein at pH 7.5, with temperature of 37°C.

Partition coefficient is defined as the ratio of concentration of protein in extract phase to the concentration of protein in the bottom phase.

$K = Y/X$

Where Y= concentration of protein in extract phase

X= concentration of protein in bottom phase.

Gel filtration chromatography

The gel filtration chromatography was carried out for the conventional and ionic liquid based ATP system. The purity analysis for the bromelain enzyme was performed on Sephadex G 100 (Akta prime plus, GE life sciences, Sweden). The column was equilibrated using a buffer maintained at pH 7 and the resulted bromelain extracts from the conventional and ionic liquid based systems were introduced into the column. The resulted fractions were considered for total protein and specific activity.

RESULT AND DISCUSSION

The extraction of bromelain from crude sample was carried out through conventional methods like ammonium sulphate precipitation, reverse micellar extraction and aqueous two phase system and compared with the ionic liquid based aqueous two phase system. The results show that the maximum extraction was found at ionic liquid based ATP system. The parameters like pH, temperature and ionic liquid concentration were analysed against specific activity.

Conventional method vs ionic liquid extraction for extraction of bromelain:

The specific activity was found to be 520 U/mg in Ammonium sulphate precipitation method where aqueous two phase extraction gives 548 U/mg and reverse micellar extraction shows 960 U/mg were shown in TABLE 1, 2 and 3. Of all the conventional methods, a maximum yield of 32% was found in reverse micellar extraction method. The ionic liquid based ATP system gives maximum specific activity and yield. Three ionic liquids were proposed for bromelain extraction. From TABLE 4, IL3 shows maximum extraction values. The gel filtration chromatography results give 11445 U/mg specific activity and 87% yield from IL3 based ATP system. The effect of various parameters on specific activity of IL based ATP system were analysed.

Effect of pH on specific activity:

Figure1 depicts that the interrelationship between the pH and specific activity of the ionic liquid based

TABLE 1 : Bromelian extraction- Ammonium sulphate precipitation

Stages	Specific Enzyme activity, U/mg	% Yield	Purity factor
Crude	145	100	1
40%Ammonium sulphate Precipitation	340	32	2.34
Gel-Filtration chromatography	520	24	3.58

TABLE 2 : Bromelian extraction-ATPs

Stages	Specific Enzyme activity, U/mg	% Yield	Purity factor
Crude	145	100	1
PEG4000+$(NH_4)_2So_4$	396	42	2.73
Gel-Filtration chromatography	548	30	3.8

TABLE 3: Bromelian extraction-RME

Stages	Specific Enzyme activity, U/mg	% Yield	Purity factor
Crude	145	100	1
Reverse micellar extraction	670	46	4.6
Gel Filtration chromatography	960	32	6.6

TABLE 4 : Bromelian extraction-IL based ATP system

Stages	Specific Enzyme activity, U/mg	% Yield	Purity factor
Crude	145	100	1
IL1+KH2Po4	4240	84	29.24
IL2+KH2Po4	3620	79	24.9
IL3+KH2Po4	5465	91	37.68
Gel-Filtration chromatography	11445	87	78.93

ATP system. As the pI of the protease enzyme is in the range of 7 to 9, the highest specific enzyme activity of the protease was observed at pH of 8.0. At higher and lower pH conditions there is tremendous decrease in the specific enzyme activity and there was no appreciable specific activity observed at pH below 4.0. The specific enzyme activity shows sharp decrease which depicts the acidic or alkaline strength which denatures the enzyme property.

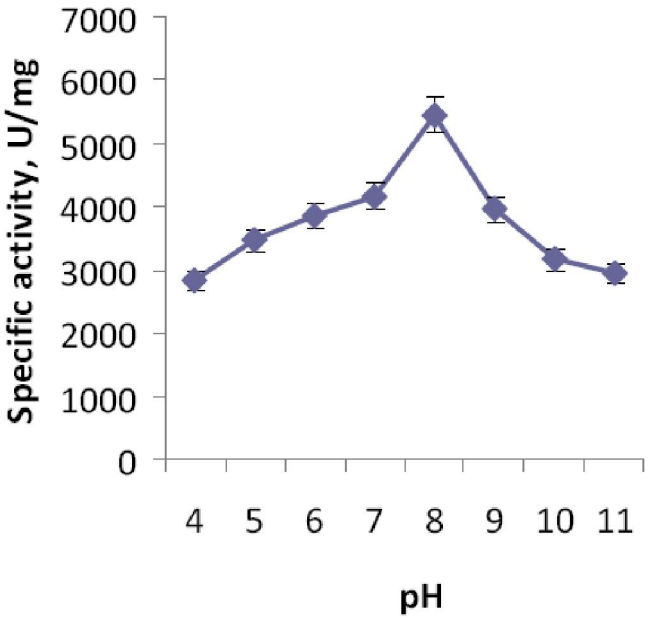

Figure 1 : Effect of pH on IL system

Effect of temperature on specific enzyme activity:

In an ionic liquid based ATP system, Figure 2 shows that the effect of temperature towards the specific enzyme activity. The specific enzyme activity was found to increase as the temperature of the ATP system increases. The specific activity of protease was found to be higher at 35 to 45°C where the maximum extraction was at 40°C which reveals the high extraction of enzyme at high temperature shows the endothermic nature of the process. Further, temperature increase affects the specific enzyme activity which underwent irreversible denaturation of the enzyme property. The study depicts the unfavour phase formation at low temperature range and protein denaturation at higher ranges of temperature which reduces the protein interactions towards ionic liquid.

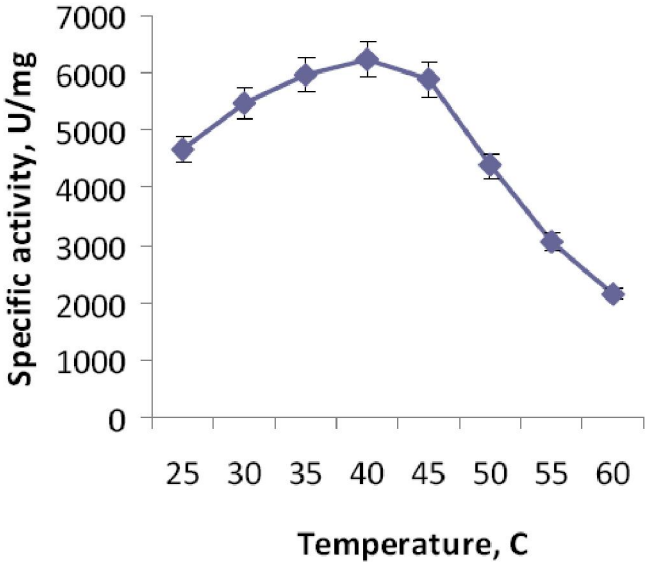

Figure 2 : Effect of Temperature on IL system

Effect of ionic liquid concentration on specific enzyme activity:

The influencing factor of enzyme extraction towards the top phase of ATP system is the ionic liquid concentration. The affinity of enzyme towards top phase improves the specific enzyme activity. The charge based interactions with the enzymes are carried out with the long chain cationic moieties in the Ionic liquids. The increase in IL concentration increases the enzyme bonding towards IL through which the high extraction of pro-

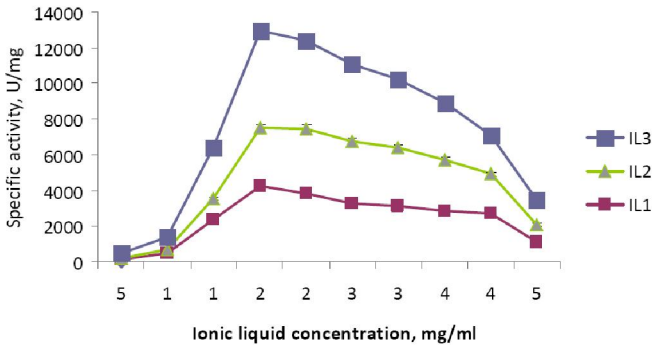

Figure 3 : Effect of Ionic liquid concentration

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tease enzyme is favoured. As the IL concentration exceeds the optimal level, the amount of anions in the sample increases which repels the enzyme binding. Figure 3 shows that the maximum specific enzyme activity at 11445U/mg. The specific activity was observed to be declining after the optimal concentration due to the lower hydrophobic interactions between the protease enzyme groups and ionic moieties of the IL.

Purity study on gel filtration chromatography

The resulting fractions were undergone purification on Gel filtration chromatography column. Based on the molecular weight the target proteins were eluted. The resulting peak from the GFC chromatogram confirms the purity range of the systems is shown in Figure 4. From the GFC fractions, ionic liquid based ATP system achieved high selective separation for bromelain extraction.

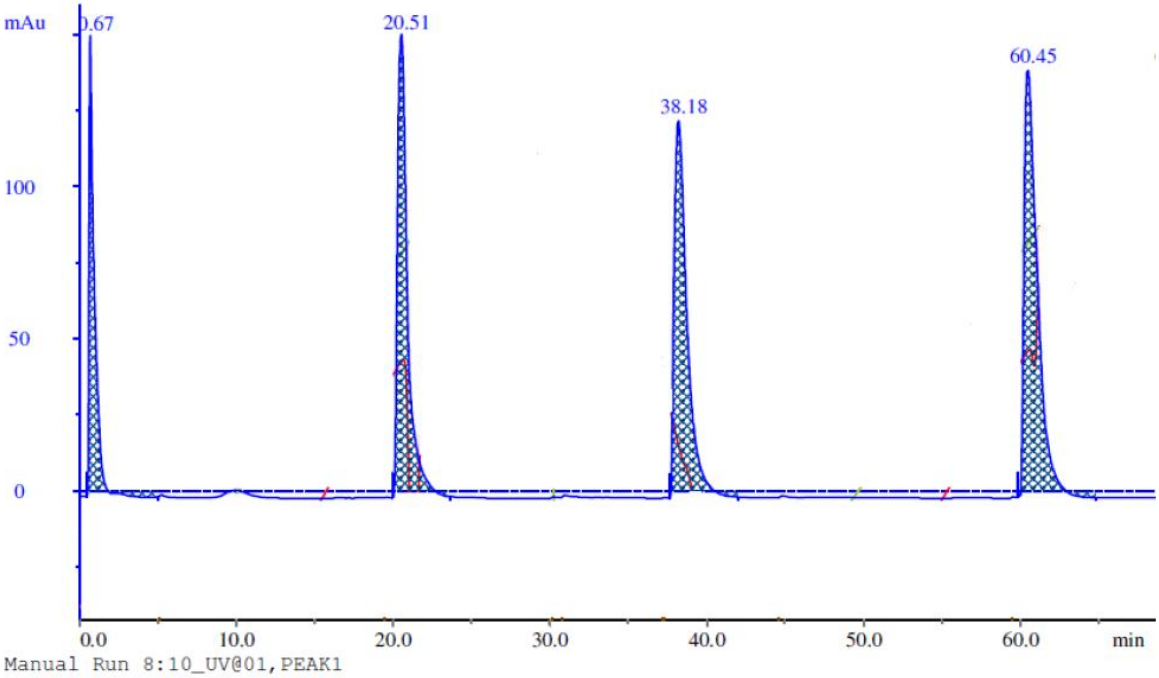

Figure 4 : Gel filtration chromatography of the fraction from IL systems.

CONCLUSION

This work depicts the resolving power of IL based ATP system for the separation of Bromelain from pineapple crude extract. This method provides a convenient method for pure enzyme extraction. The extraction of bromelain through ionic liquid based ATP system proves simpler and faster process compared with other conventional applications. Ionic liquids based ATP system shows high selectivity and easy scalability of the system which favours the large scale application of bromelain purification in industries.

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