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Partition coefficient studies of calotropain from its crude latex using ionic liquid based aqueous two phase extraction system

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ABSTRACT

Separation and purification of high value bio-molecules using Ionic liquid (IL) based aqueous two phase (ATP) extraction is an emerging green separation process. IL based ATP system 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 calotrpin from the latex of medicinal plant *Calotropis procera* (Family-Asclepiadaceae; The molecular mass and iso-electric point of the enzyme are 28.8 kDa and 9.32, respectively) and the same was compared with conventional ATP 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 conventional ATP system.

KEYWORDS

Calotropain;
Ionic liquid;
ATP;
Protease;
Partition.

INTRODUCTION

The aqueous two phase extraction (ATP) is a promising bioseparation technique, widely used in foodstuff processing industries. Simple in preparation, less energy requirement, ease of scale up and simple process steps are the major features that pays way for the selection of aqueous two phase extraction as a feasible methodology for the protein purification[1]. A polymer-polymer system or a polymer salt system is generally used for the preparation of an ATP. The phases were separated based on the existence of incompatibility between the two phases[2]. The separation of protein towards the top phase is influenced by various factors like polymer molecular weight, polymer concentration and system pH[3]. Ligands are applied for affinity partitioning in aqueous two phase extraction in most of the biological applications[4]. Selectivity and consistency of ATP system in Bioseparation process is enhanced through ionic liquid ligands and hence forth the name Integrated Aqueous Two phase extraction. Similar processing steps were employed in the partition of papain from its crude latex with metal ligand ATPi system[5].

The drawback faced in the ATP system is the recovery of polymers involved in the phase formation that affects the papain purity. An ionic liquid is employed as ligand to overcome the problems in ATP using 1-butyl phosphonium bromide[6]. The partition coefficient stud-

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ies were carried on both the polymer/salt ATP system and the integrated ATP system[7]. Ionic liquids composed of large nitrogen content cations (organic) and small amount of anion (inorganic), at normal temperature have the properties of hydrosolubility, stability in atmospheric condition and also possess inherent tunability[8]. Ionic liquids also known as clean solvents are environmentally friendly with negligible vapour pressure. An ionic liquid can be synthesized for particular application possessing expected properties[9].

Calotropis procera, is a plant seen in the tropic and sub-tropic regions. It produces latex from its broken plant parts and was utilized in traditional medicines and ayurveda[10]. Caloptropaine, a cysteine protease obtained from the latex of *Calotropis procera* has been widely used in medicinal and pharmaceutical industries[11]. In the present study, Caloptropaine from *Calotropis procera* latex was extracted by two different bioseparation methods like Aqueous two phase extraction and integrated aqueous two phase extraction with Ionic liquids as ligand[12]. These methods were compared for maximum yield. Purity tool evaluation improves the activity and functional applications of protease enzyme. This requires a high precise separation means along with economic operations. This ionic liquid based ATP system confirm not only the high selectivity but for low cost as well[13]. From the purity factor, the ATPi shows good resolving power for protease separation and it is a much simpler single step purification process in bioseparation in comparison to other methods[4,5].

The design of an integrated aqueous two phase extraction (ATPi) 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 and SDS page analysis for protein conformation. This present work also verifies the specific activity of the aqueous two phase extraction of protease enzyme, both with and without ionic liquid ligands.

MATERIALS

Latex of *Calotropis procera* was collected within the SASTRA university campus. Ionic liquid-1-butyl phosphonium bromide, Polyethylene glycol (PEG-1000), Sodium dodecyl sulfate (SDS), casein and bovine serum albumin (BSA), trichloroacetic acid (TCA) were purchased from Sigma Aldrich and all chemicals were of AR grade.

METHODOLOGY

Crude extract preparation

The latex was collected by breaking the stem of the plant and stored in a clean tube. The collected latex was mixed with distilled water at 1:1 ratio and centrifuged at 6000 rpm for 10 minutes at 4°C. The supernatant finally collected was referred as crude extract.

Aqueous two phase Extraction

PEG-6000 (8,10,12% w/w) at different concentrations and ammonium sulphate were taken for the ATP system formation. The concentrations were optimized by making the total system volume to 10 ml with buffer solution. The crude extract was added to the polymer salt mixture of the ATP system and mixed gently and centrifuged at 6,000 rpm for 6 minutes. Two phases formed were separated carefully to carry out estimations in both the top phase and bottom phases. The upper phase was taken for future analysis to carry backward extractions.

Integrated aqueous two phase system (ATPi)

An integrated ATP system was formed by applying ionic liquid as ligand to the optimized ATP system formed in the above process. Ionic liquid stock solution with 1-butyl phosphonium bromide was prepared separately with particular volume. Stock solution prepared was added with 22%w/v of PEG 6000 maintained at pH 6 using buffer and 18% w/v of salt incubated at 25°C for saturation. The crude latex was added with ATPi system. The phases were separated by centrifuging the mixture at 6000rpm for 10 minutes. The upper phase rich in ionic liquid and PEG 6000 were separated and used for further estimations.

Backward extraction

The backward extraction was carried out by adding 20% sodium chloride to the top phase of both the systems and centrifuged at 4000 rpm for 10 minutes. The two phases were formed and the bottom phase carefully separated was taken for further calculations.

The top phase was taken for PEG recovery.

Estimation of total protein analysis

Total protein was determined based on Lowry method[14], with BSA as standard for both the ATP and ATPi systems.

Enzyme activity assay and partition coefficient studies

Enzyme activity was verified by enzymatic hydrolysis of casein 2% (w/v) maintained at pH 7.5 with 37 C temperature.

The partition coefficient studies validate the selectivity and efficiency of the ATP method. Partition coefficient can be defined as the ratio of concentration of protein in top phase to the concentration of protein in bottom phase.

$$\text{Partition coefficient (K)} = \frac{\text{Concentration of protein in top phase}}{\text{Concentration of protein in bottom phase}}$$

Gel filtration chromatography

The bottom phase of the backward extraction for the ATP and ATPi systems were taken for purity studies. Hi Trap QFF(1ml) Chromatographic column (Akta Prime plus GE, Sweden) was used for the purification analysis.

RESULT AND DISCUSSION

The present work aims on the development of enzyme affinity towards the ionic liquid ligand to improve the protease extraction from the crude source. The specific activity was analysed by taking aqueous two phase system as reference. In the ATP system, the specific activity was found to be 3240 U/mg with 42% is yield shown in Tab.1. The ionic liquid concentration was optimized in the ATPi system for utmost enzyme separation is shown in Tab.2. The integrated ATP system gives an enhanced value of specific activity at 9642 U/mg and 82% yield. The results depict that ATPi system with ionic liquid as ligand was superior when compared to ATP system. The effect of various parameters on specific activity for ATPi system was studied.

Effect of pH on specific activity:

Figure 1 describes the relationship between the effect of pH and specific activity of the ATPi system. The pI of the protease enzyme lies in the range of 7 to 9, where the highest specific enzyme activity of the protease was found at pH of 7.0. The phase formation was not observed in pH value less than 4. The increase in pH values improves the specific activity of protease which influences the partition coefficient. The specific enzyme activity shows slender decline values at higher and lower pH values which indicate that the C.procera is a neutral protease. A sharp decrease in the specific enzyme activity values on pH illustrates the acidic or alkaline strength which affects the enzyme property.

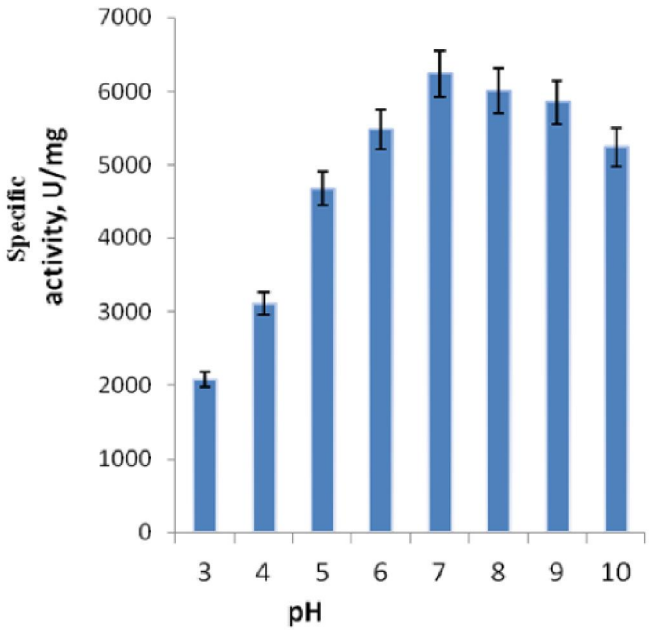

Figure 1 : Effect of pH on ATPi system

Effect of temperature on specific enzyme activity:

Figure 2 shows the effect of temperature towards the specific enzyme activity in an ionic liquid ligand ATP system. As the temperature of the ATPi system is increased, the specific enzyme activity was also found to increase which expose the endothermic nature of the process where higher values of temperature influences the protease separation. The specific activity of protease shows the maximum extraction at 55°C which gives high enzyme extraction at high temperature. Further increase in temperature above the optimized value affects the specific enzyme activity which undergoes denaturation of the enzyme property. There was no appreciable phase formations at lower temperature and at temperatures crossing the optimized values, protein

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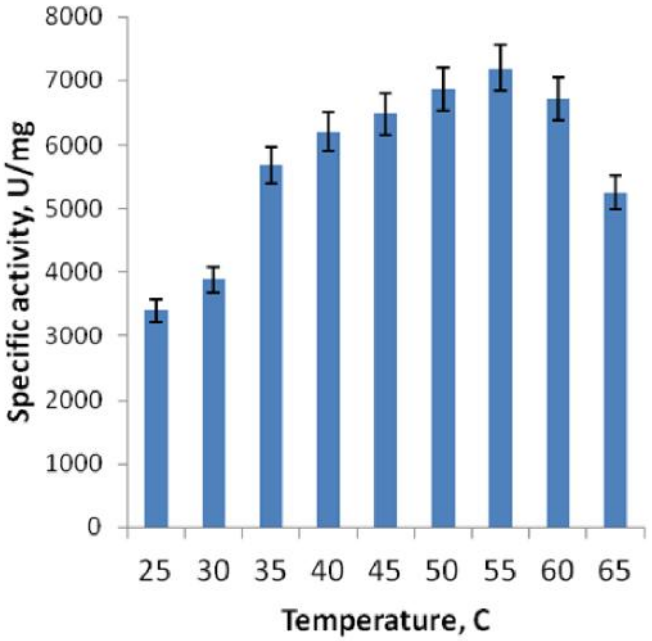

Figure 2 : Effect of Temperature in ATPi system

denaturation occur which spoils the protein extraction.

Effect of ionic liquid concentration on specific enzyme activity

The enzyme extraction of an ATPi system is highly controlled by the ionic liquid concentration. The affinity charge of the protease enzyme improves as the IL concentration increases which positively directs the partition coefficient of the system is shown in Figure 3. The addition of ionic liquid concentration improves the enzyme bonding towards the IL rich top phase and increases the enzyme extraction. The further addition of IL concentration above the optimized value increases the anion content and resists the protease separation towards the top phase. The maximum specific enzyme activity was found at 6 mg/ml. Due to lower hydrophobic interactions between the protease enzyme groups

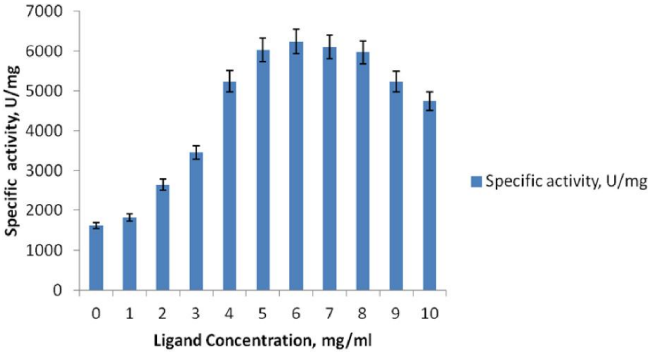

Figure 3 : Effect of Ligand concentration in ATPi system

TABLE 1 : Caloptropaine extraction by ATP

Stage	Specific Activity (U/mg)	Partition coefficient, K	% Yield	Purity Factor
Crude	420	----	100	1
ATP extraction	1615	0.82	54	3.85
GFC	3240	----	42	7.7

TABLE 2 : Caloptropaine extraction by ATPi

Stage	Specific Activity (U/mg)	Partition coefficient, K	% Yield	Purity Factor
Crude	420	----	100	1
ATPi extraction	6030	6.3	91	14.35
GFC	9642	----	82	22.95

and less ionic moieties of the IL, the specific activity value was observed to be decreasing after reaching the optimal concentration.

Gel filtration chromatography

The purity studies were carried out on the backward extraction of the ATP and ATPi systems were shown in Figure 4 and Figure 5. Depending on the molecular weight the proteins were separated and the peak length of the GFC shows the purity level of the

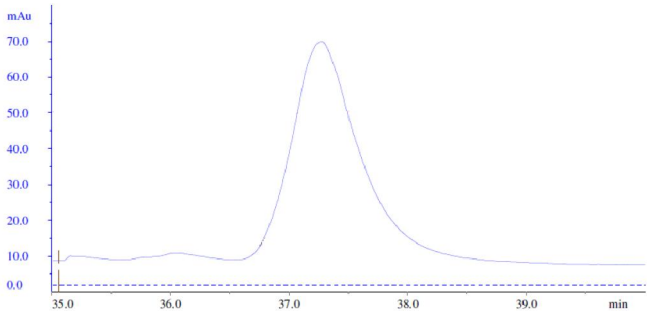

Figure 4 : Gel Filtration Chromatography_ATP

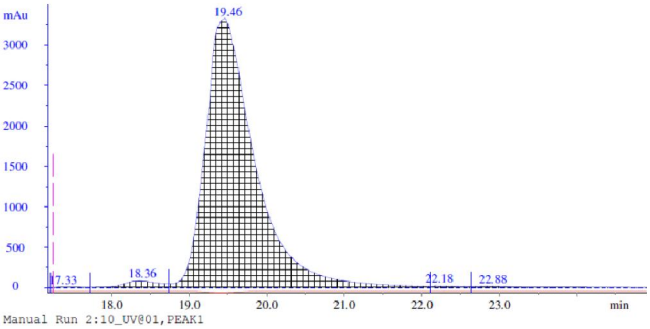

Figure 5 : Gel Filtration Chromatography_ATPi

resulting fraction. High pure peak was observed in the ATPi system than the conventional ATP system from the resulting GFC peaks. It shows that the selective extraction was efficient in the ATPi system only.

CONCLUSION

The development of an ATPi system with ionic liquid as ligand was proposed for maximum enzyme extraction compared with conventional system. The effect of various parameters was analysed for the effective extraction of the protein. Hydrophobic and electrostatic interactions, endothermic nature are some important driving factors that yields high pure protease enzyme. The results confirm the efficient extraction (82% yield) of the protease enzyme through the ATPi system than the traditional ATP extraction method.

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