# Design and development of single stage purification of papain using Ionic Liquid based aqueous two phase extraction system and its Partition coefficient studies

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**ABSTRACT**

As an emerging trend in bioseparation, aqueous two phase extractions based on phosponium ionic liquid have been utilized in this work to extract papain from Carica papaya fruit latex and the same was compared with conventional aqueous two phase extraction system. Factors affecting the partition coefficient of papain such as ionic liquid concentration, pH of the extraction system and temperature have been investigated. The optimization studies show that ionic liquid concentrations and pH are majorly influencing the phase formations and papain partitioning. It reveals the importance of electrostatic and hydrophobic interactions in the papain partitioning. Purification studies performed on Gel Filtration Chromatography shows that 96% of the papain enzyme could be extracted with the phosponium based ionic liquid in a single stage extraction. The final fraction containing papain enzyme was confirmed by SDS Page analysis.

Key words: ATP, IL, Papain, partition coefficient, Purity factor

**INTRODUCTION**

Protein separation industries are in high demand for its efficient protocol and its successive proceedings in order to meet the market challenges. High value bio-molecules need highly specific separation methods in order to meet the application standards. In the current scenario, emerging methods in the bioseparation protocols satisfies either the quality standards or commercial criteria. Aqueous two phase extraction is one of the biocompatible separation methods which satisfy almost in all the aspects. ATP itself is emerging as a separate phenomena as affinity partitioning, integrated ATP systems, ATP based on ILs, ATP based on detergents and dye liganded ATP systems[1-3]. In this work, the study deals with developing single step purification method for the target enzyme with high outcome.

Aqueous two phase extraction is the best and commercially important separation method for separating biologically important molecules[4]. It plays a vital role in biological and protein industries. In the present work, an attempt to develop the single step purification method for the desired enzyme is dealt. This study proposes a high specific extraction method which offers high affinity towards the target protein. The results revealed that it is equivalent to chromatographic separation of proteins. Aqueous two phase system is an effective implementation for the extraction of various proteins, enzymes, etc[5]. An immiscible aqueous two phases can be formed by a polymer/polymer combination or polymer/salt combination. PEG 6000 polymer and ammonium sulphate salt is used to form an ATP system. The recovery of phase forming polymers from the aqueous phase is a major drawback in ATP which in turn affects its purity[6]. In order to overcome these problems an ionic liquid based ATP system is proposed where the benzyl immidazolium chloride and butyl immidazolium chloride form ATPs with $K_2HPO_4$ salt. Here the ionic liquid can be an alternate to the polymer phase in ATP[7-9]. The partition coefficient studies are made on the polymer/salt ATP system and IL based ATP system[10].

Ionic liquids are compound composed of large nitrogen content cations (organic) and small amount of anion (inorganic) that are liquid salts at normal temperature[11-14]. Ionic liquids have the properties of hydrosolubility, they remain stable in atmospheric condition and also tunablility is inherent[15]. They have neglectable vapour pressure and are environmentally benign solvents so called clean manufacturing solvents[16, 17]. It is possible to synthesise an ionic liquid with aspired properties to set for an exact application[18, 19].

Papain is a commercially essential enzyme used as wool shrink proofer, tenderizer for meat, etc[20]. Enhancement of purity mechanism improves the activity and functional applications of papain enzyme for which highly precised separation tool with economic operations are required[21]. The purity factor shows that IL based ATP technique has high degree of resolution for extracting papain and it is single step purification in downstream processing[22, 23].

## MATERIAL

*Carica papaya* was locally collected (Tamilnadu, India). Ionic liquids - Tetra Butyl Phosphonium Bromide(TBPB) was purchased from Sigma Aldrich, India. All chemicals procured for the study were of AR grade.

## METHODS

### SAMPLE PREPARATION AND PRE-TREATMENT

Fresh milky latex was collected by making longitudinal incisions on the locally collected papaya fruit. The papain enzyme solution was prepared by dissolving the latex in a buffer solution maintained at ph 7 and centrifuged at 6000rpm for 10mins and the supernatant was stored at 4°c for further extraction studies.

### AQUEOUS TWO PHASE EXTRACTION USING POLYMER SALTS: (FORWARD EXTRACTION)

Polymer/salt combination of aqueous two phase system is prepared by dissolving 15% (w/v) polyethylene glycol (PEG) 6000 and 12% (w/v) ammonium sulphate to the enzyme solution for 15 minutes. Once the phase formation was observed the samples were centrifuged at 6000rpm for 5 mins. The resulting two phases were separated and were checked for protein content in both the phases. The top phase was taken into consideration for backward extraction by the addition of optimal amount of salt. To the top phase 20% NaCl was added and incubated for 40 minutes and then centrifuged at 8,000 rpm for about 10 minutes. The resulting bottom phase rich in papain enzyme was separated and used for further analysis.

### IONIC LIQUID BASED TWO PHASE EXTRACTION: (FORWARD EXTRACTION)

Preferred amount of Tetra Butyl Phosphonium Bromide (TBPB) was mixed with $K2HPO_4$ directly in the equal amount of diluted enzyme solution.The mixture was incubated at 25°C for 20 minutes to attain equilibrium. The incubated samples were centrifuged at 2000 rpm for a period of 5 minutes to obtain the phase separation. The sample was collected from the top phase rich in Ionic liquids and used for further analysis.

### IONIC LIQUID BASED TWO PHASE EXTRACTION: (REVERSE EXTRACTION)

The top phase from the forward extraction of the IL based ATP a system was treated for enzyme recovery. The ionic strength of the ATP systems was modified by the addition of 0.5M NaCl and then centrifuged to separate the two phases. The diluted enzyme rich bottom phase was collected separately and used for the further purification studies.

### OPTIMIZATION OF SYSTEM PARAMETERS FOR THE IL BASED ATP SYSTEM

The partition coefficient in the IL based ATP system are influenced by IL concentration, system pH, system temperatures was studied. By studying the effect of parameters in the IL based ATP system the optimal conditions for the papain recovery with high purity can be derived. In the present study, there are three parameters considered for the optimization. The IL concentrations are varied from 10% to 60% w/v for which the ATP extraction was repeated for all these levels. pH of the systems varied from 4 to 11for the optimal IL concentration for all three systems which were carried out. Finally the temperature of system varied from 20 to 40°C at the optimal IL concentration and optimal pH level were applied for all three IL based ATP systems. The resulting top phases of the all three systems were treated for enzyme recovery by carrying out the reverse extractions. The aqueous IL free bottom phase was taken for purification study.

### ANALYTICAL METHODS

Total protein concentrations were determined based on the Lowry's method[24]. Enzyme activity was determined by the casein digestion assay.

Partition coefficient is determined as the ratio of enzyme concentration in the top phase to the enzyme concentration in the bottom phase.

*Partition Coefficient*(K) = enzyme concentration in top phase / enzyme concentration in bottom phase

*Yield*(Y)

Yield for the ATP is defined as the ratio between total protein in the sample and the total protein in the feed.

*Purification factor* (PF)

Purification factor is determined as the ratio of specific enzyme concentration in the sample to the specific enzyme concentration in the feed.

### PURITY ANALYSIS ON GEL FILTRATION CHROMATOGRAPHY (GFC)

The purity analysis of the papain extracts from ATP and IL based ATP systems (Tetra Butyl Phosphonium Bromide) were performed on Sephadex G 100 (Akta prime plus, GE life sciences, Sweden). Previously the column was equilibrated with pH 7 buffer and then the papain extract from IL system was introduced into the column. The fractions were analysed for total protein and papain enzyme activity.

## RESULTS AND DISCUSSION

The present study involves the selective extraction of papain from its crude latex by ionic liquid based ATP system and a comparative study of same extraction with the conventional ATP system. In order to find the optimal conditions for the papain recovery, the effect of pH, Temperature of system and IL concentration in the forward extraction system were studied based on the partition coefficient. The resulting fractions were analysed for purification on Gel filtration column.

### EFFECT OF IL CONCENTRATION ON PARTITION COEFFICIENT

The ionic liquid concentration is an important factor which influences the enzyme extraction in the top phase of ATP system. An increase in IL concentration increases the affinity charge of enzyme towards itself and improves the partition coefficient of the system. Ionic liquids contain long chain of cationic moities which contribute to the charge based interactions with the enzymes. As the concentration of IL increases, there is a increase in the amount of enzyme that get bonded to IL because of which extraction of papain enzyme is favoured. When the concentration of IL increase higher than the optimal level,the amount of anion present in the sample increases which in turn repels the papain binding. In the forward extraction the concentration of IL was added in the range of 50 to 200mM. From Fig.1 it was observed that the maximum partition coefficient of papain at IL concentration of 150mM. The partition coefficient value was found to decrease after this ionic liquid concentration which reduces the enzyme activity due to lowering of hydrophobic interatcions between the papain surface groups and ionic moities of the IL.

### EFFECT OF PH ON PARTITION COEFFICIENT

From the Fig.2 it was observed that the partition coefficient of papain strongly depends on the pH of the Ionic Liquid based ATP system. There was no phase formation in the range of pH 5 and the pI value of the papain is in the range of 8.75 to 9.5. Therefore the study was carried out in the range of pH 5-11. It was observed that the increase in pH values of the extraction system positively influence the partition coefficient of papain. Increase in pH favours papain binding up to 7.5 and eliminates undesired proteins which improves purification factor. Beyond this range, papain interactions toward the ionic liquid is reduced due to the iso electric point of papain was reached. The surface charge of the papain enzyme and the cationic moiety of IL were in high interaction and plays key role in partition coefficient enhancement when pH variation.

### EFFECT OF TEMPERATURE ON PARTITION COEFFICIENT

Fig.3 shows the effect of temperature on the partition coefficient of papain in ionic liquid based ATP systems. The partition coefficient of papain enzyme slightly increases as the temperature of the system was increased from 20 to 30, which reveals the endothermic nature of the process where higher temperature favours the papain extraction.When the temperature was increased further, the enzyme extraction was affected and the partition coefficient value tend to decrease. The optimal temperature to obtain highest K was found to be 30°C. The lower temperature ranges unfavor the phase formation and the higher ranges reduces the protein ineractions towards the IL.

### PURITY STUDY ON GELFILTRATION CHROMTOGRAPHY

The fractions from the backward extractions were analysed for purification on Gel filtration chromatography(GFC) column . GFC elutes protein based on their molecular weight and the resulting peak length shows the purity range present in the fractions. Fractions of papain from convernional ATP system and IL based ATP system were analysed. From the GFC chromatograms Fig.4,5 it was found that fraction from IL based ATP is in high purity than the conventional ATP system. It reveals that the high selective separation method for papain was succesfully achieved. The resulting fraction from GFC were collected and analysed for SDS page.

### SDS Page analysis

Sodium docylsulphate (SDS) Page analysis was performed for the fractions from the conventional ATP system and IL based ATP system along with marker and it shown in the Fig.6

## CONCLUSION

In this research work, the high selective extraction of papain from its crude fruit latex was developed and the results were compared with the conventional ATP system. The extraction conditions were optimized and the IL concentration, pH were highly influenced the papain extraction. The major driving forces are hydrophobic interaction and electrostatic interaction between the papain enzyme and the IL cationic moities. Purification studies in Gel filtration chromatography shows that the IL based ATP yields high pure protein than the conventional ATP system and it shown in Tab.1. IL are tunable and designable solvents can be developed the same kind of single step extraction method for any commercial and medicinal grade biological products.

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Table and Figures

Table.1 Papain partitioning in ATP system and IL based ATP system:

| Stage | Partition Coefficient | Yield % | Purity Factor |
|---|---|---|---|
| Crude Latex | ------- | 100 | 1 |
| ATP | 3.86 | 33 | 3.22 |
| ATPi_IL | 15.24 | 96.22 | 9.55 |
| Gel filtration Chromatography | --------- | 98.67 | 11.35 |

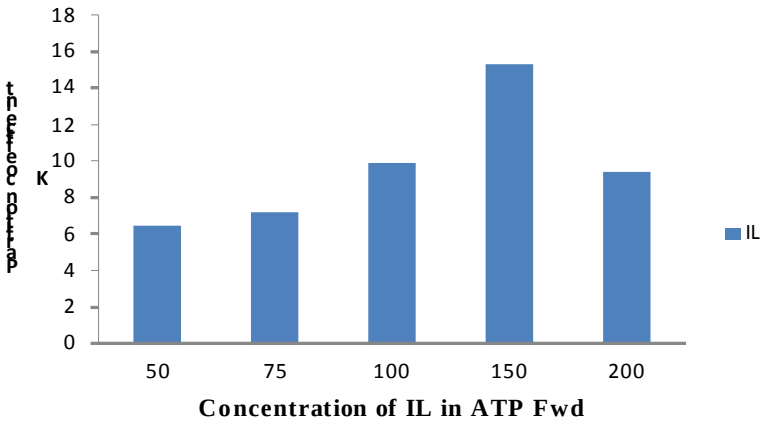


Fig.1 Effect of IL concentration on Partition coefficient in IL based ATP system:

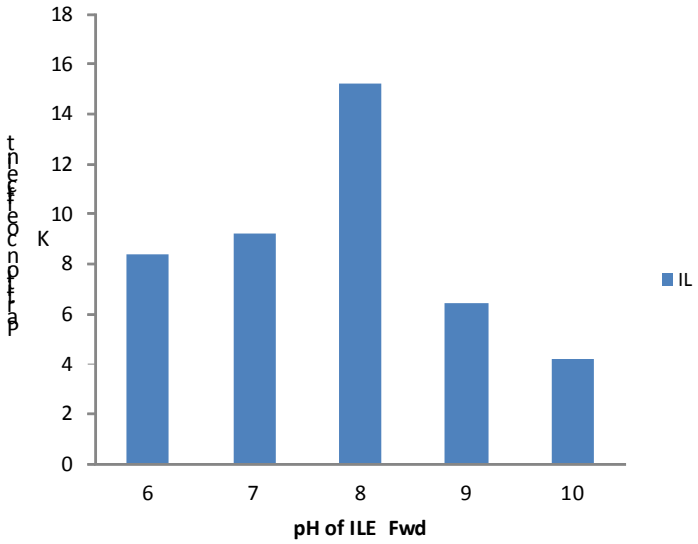


Fig.2 Effect of pH on Partition coefficient in IL based ATP system:

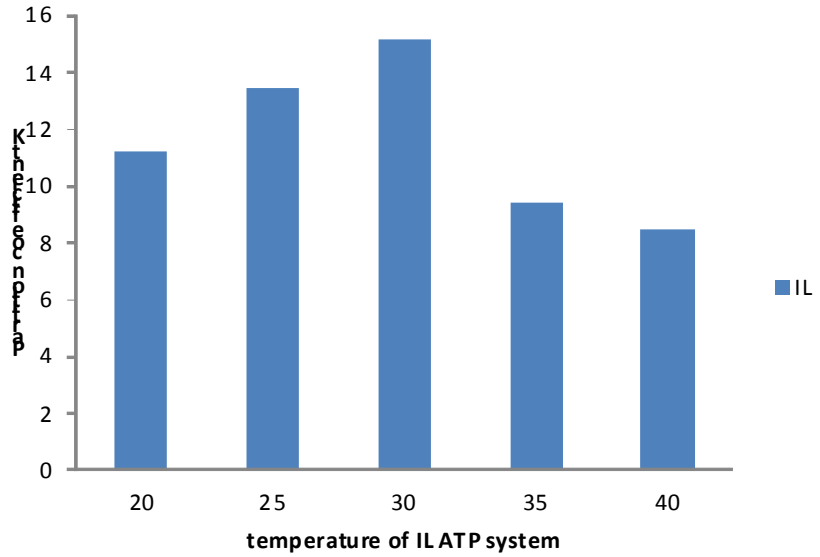


Fig.3 Effect of Temperature on Partition coefficient in IL based ATP system:

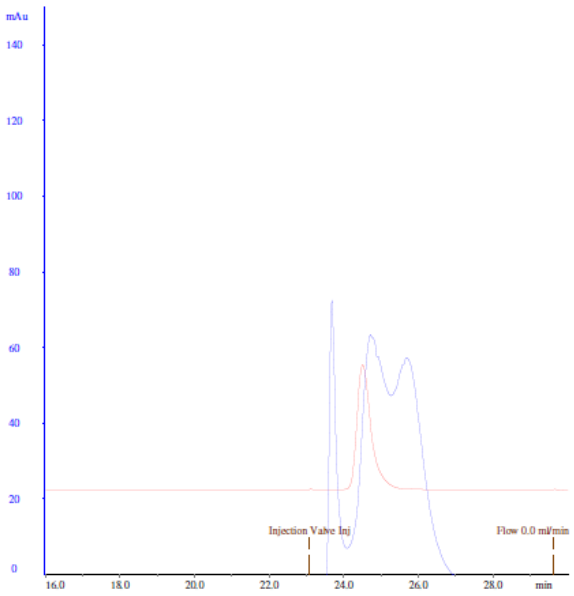


Fig.4 Gel filtration chromatogram of papain using conventional ATP system

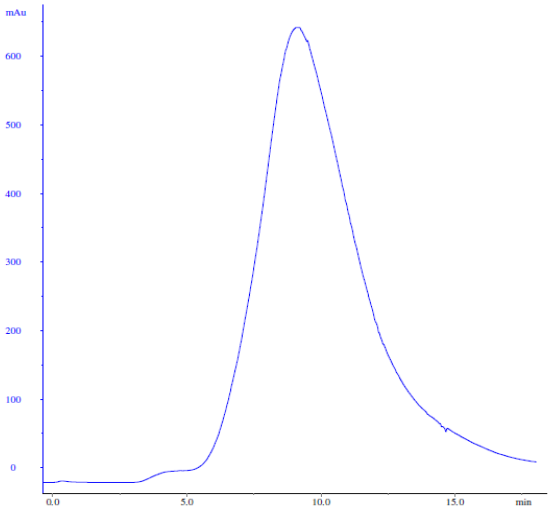


Fig.5 Gel filtration chromatogram of papain using conventional IL based ATP system

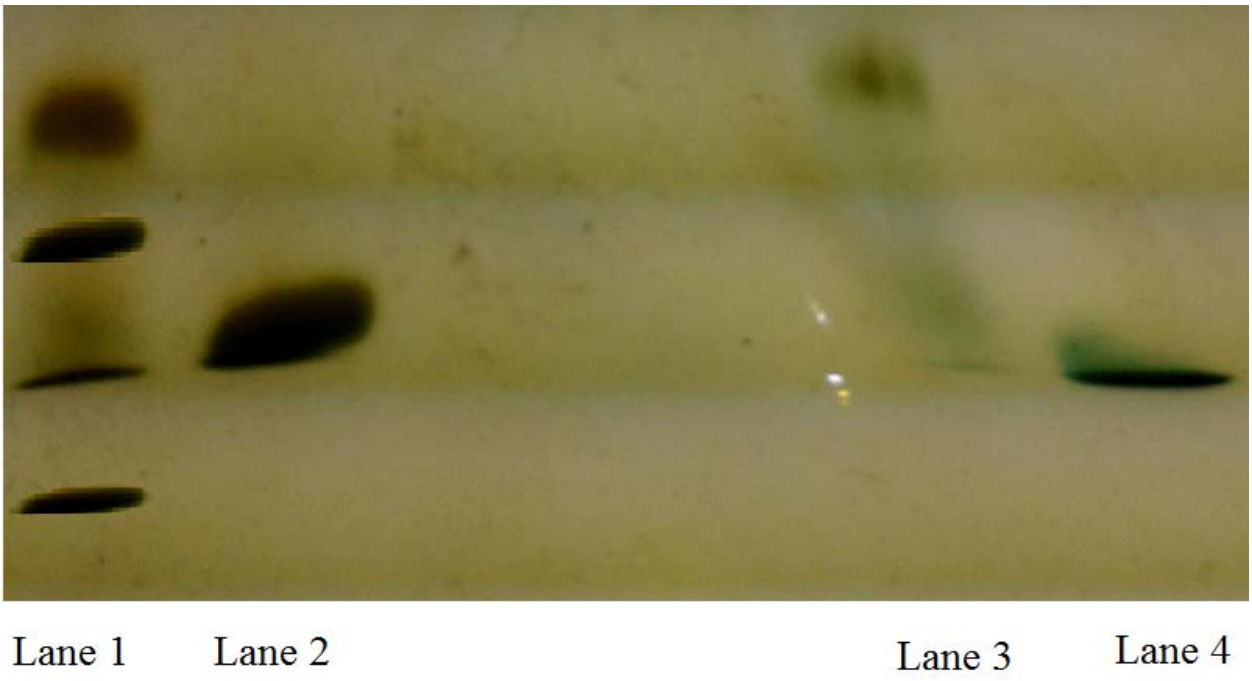


Fig. 6 SDS Page analysis: Lane 1:Marker , Lane 2: Papain from conventional ATP system, Lane 3: Crude latex, Lane 4: Papain from IL based ATP system.