

Integration and investigation of metal ligands in aqueous two phase extraction and its partition coefficient studies in papain extraction

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Affinity partitioning in aqueous two phase extraction is a single step purification process in downstream processing. Integrating metal ligands to the phase forming polymers in aqueous phase extraction known as ATPi ensures high selectivity and also promises good partition. In the present work, the effect of metal ligands in aqueous two phase extraction has been studied. This study has been validated in papain enzyme extraction from the fruit latex of papaya (*Carica papaya*) and compared with conventional aqueous two phase extraction (ATP). The metal integrated ATPi consisted of 20% w/v polyethylene glycol (PEG) and 15% w/v $(\text{NH}_4)_2\text{SO}_4$ along with ligand composition of 0 to 2% w/v of metal ions (CoCl_2 , ZnSO_4 , CuSO_4 , CdCO_3). This was compared with ATP extraction, which was composed of 20% w/v PEG and 15% w/v $(\text{NH}_4)_2\text{SO}_4$ without ligands. Purification studies were carried out in anion exchange chromatography. Highly pure papain was obtained from fruit latex of papaya using ATPi extraction. The partition coefficient of papain in ATPi extraction was found to be 2.56 times higher than conventional ATP extraction.

Keywords: ATP, ATPi, metal ligands, papain, partition coefficient

Introduction

Aqueous two phase extraction (ATP) is one of the key separation methods in downstream processing. Its biocompatibility is one of the advantages in protein partitioning with good selectivity at minimal protein loss¹. The choice of polymers and the salt is another advantage that provides a wide range of optimization for various applications². ATP can be applied as a selective technique for a particular protein separation by integrating with affinity ligands³⁻⁵. Affinity ligands can be the specific functional group or metal ions or some other charged compounds⁶⁻⁹. Affinity partitioning has been employed for single or multistep extraction of proteins in aqueous two phase systems from crude samples⁹. Usually the ligand is coupled to polyethylene glycol (PEG) because of the ease of steering the partition of proteins into the opposite phase^{6,10}, composed of other compounds (salt). Several ligands used in affinity partitioning are biospecific, such as, nucleotides, inhibitors, hormones, hormone receptors, antibodies and antigens¹¹⁻¹³. Alternatively, pseudo-biospecific (low cost) ligands, such as, reactive dyes, metal chelating groups and hydrophobic and hydrophilic ligands have also been often used⁹.

The use of conventional polymer-salt ATP system has many disadvantages, which includes low partition coefficient, low selectivity and low specific enzyme activity^{14,15}. These disadvantages with the conventional ATP system can be overcome with use of affinity ligands in ATP system¹⁶. These affinity ligands are mainly coupled with the PEG by the process known as PEGylation. The introduction of charged molecules into the system tends to increase the partition coefficient and the selectivity of the protein that is to be extracted^{5,17}. The charge possessed by the charged polymer plays a key role in attracting the counter charged proteins which accounts for the selectivity¹³.

Papain (EC 3.4.22.2) is an industrial enzyme, which belongs to cysteine protease family, and exists in papaya fruit latex as a minor constituent¹⁸⁻²⁰. This endopeptidase is widely used in food industries as a tenderizer and for several medicinal treatments. Papain was separated by many bioseparation tools like precipitation, aqueous two phase extraction, spray drying method and few chromatography techniques²¹. In present work, the enzyme separation is achieved by high selective partitioning so as to become single step purification.

Integrated aqueous two phase system (ATPi) is a single step purification process in which the

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protein is selectively extracted and also purified; whereas in conventional ATP system, the extraction is followed by purification methods like ion-exchange chromatography, affinity chromatography and so on²²⁻²⁴. In the present study, partition coefficient of papain extraction by conventional ATP process was compared with the extraction by metal affinity ATPi process. Four metal ligands, viz., Cu^{2+} , Cd^{2+} , Zn^{2+} and Co^{2+} , were utilized in the process of ATPi.

Materials and Methods

Materials

PEG 6000, ammonium sulphate, casein, cadmium carbonate, cobalt nitrate, zinc sulphate, copper sulphate and tri-chloro Acetic acid (TCA) (HiMedia Laboratories, Mumbai, India), hollow fiber membrane (30 kDa), His Trap Q and FF column 1 mL (GE Healthcare, Sweden). Papaya (*Carica papaya*) was collected from the local garden.

Sample Preparation and Pretreatment

Thin incisions were made on the fresh papaya fruit and milky latex was collected and 1 g latex was transferred into a buffer maintained at pH 7.5, and it was used as the starting material for the present study. Then the insoluble solid particles were separated by centrifugation processes and the clear latex was stored in the same buffer at 10°C.

ATP Extraction: Forward Extraction

Aqueous two-phase system without ligands was prepared with PEG 6000 and ammonium sulphate. Defined amount of PEG 6000 was taken in the range of 10 to 50% w/v with a constant amount of ammonium sulphate. Total system volume was made up to 10 mL with buffer solution and the concentrations were optimized. These compounds were mixed together with fruit latex and the mixture was allowed to get distributed completely, aided by gentle mixing. The mixture was centrifuged at 6000 rpm for 10 min. The phases were separated carefully and then the estimation was carried out in both the top and bottom phases. The maximum enzyme activity was found at 20% PEG and 15% ammonium sulphate. Top PEG rich phase was used for further analysis after carrying backward extraction for PEG recovery.

Integrated Aqueous Two Phase Extraction (ATPi): Forward Extraction

The ATPi system was prepared by adding metal ions as free ligands to the original ATP system

optimized in the above step. The stock solutions of metal ions, viz., cadmium carbonate, cobalt nitrate, zinc sulphate and copper sulphate, were prepared separately in the range of 0 to 2% w/v. Solution of each metal ion was added to the 20% PEG 6000 solution. The system was maintained at pH 6 using buffer solutions. Then, 15% ammonium sulphate was added to each of the above metal ligand+PEG solution and this mixture was incubated at room temperature for about 4 h for saturation. The fruit latex was added to metal ion integrated ATP system. Gentle mixing and stirring at regular intervals facilitates uniform distribution and ligand interaction. The phase separation was achieved by centrifugation at 6000 rpm for 10 min. The metal liganded PEG rich top phase was separated carefully and was utilized for further estimation.

Backward Extraction: ATP and ATPi

The top phase of both systems was added with 20% sodium chloride and then centrifuged at 10000 rpm for 10 min at 10°C. Without any interaction, both the phases were separated carefully for recovering PEG and the bottom phase was used for further purity analysis.

Estimation of Total Protein by Lowry's Method

Aliquots of the phases from the ATP and ATPi systems were taken for estimation of total protein content by Lowry's method²⁵ using Bovine Serum Albumin (BSA) as a standard.

Enzyme Assay by Casein Digestion Test

The papain activity was determined by casein digestion assay²⁴. 100 μL of sample was taken from the phases and analyzed for proteolytic activity. The sample was transferred to citrate buffer maintained at pH 7.2. 1 mL of 0.635% casein solution, maintained at pH 7.2, was added and incubated at 37°C for 10 min. The reaction was arrested with addition of 5 mL of 5% TCA and the mixture was allowed to incubate for 45 min. The reaction mixture was then centrifuged at 9000 rpm for 5 min. The supernatant from the above mixture was measured at 280 nm against TCA buffer solution as blank. The resulting amount of enzyme involved in this reaction at 280 nm was equivalent to 1 μmol tyrosine under assay conditions.

Partition Coefficient Studies

The selectivity and efficiency of the method can be validated by partition coefficient of the enzyme. This can be done by measuring the specific enzyme

activity of samples from both the phases of the system. Partition coefficient can be defined as the ratio of enzyme activity in top to the enzyme activity in bottom phase.

$$\text{Partition coefficient (K)} = \frac{\text{Enzyme activity of top phase}}{\text{Enzyme activity of bottom phase}}$$

Effect of pH in ATPi System

The effect of pH was studied by maintaining different pH conditions (pH 4-8) during the extraction step. The study was repeated and the enzyme activity of both the phases was studied at varying pH conditions. The maximum value attained by the partition coefficient was an indicator of the optimum pH of the system.

Purification Analysis: Protein Desalting and Anion Exchange Chromatography

The bottom phase from the backward extraction of respective systems was taken for purity analysis. The excess amount of ions present in the enzyme extract should be removed prior to performing liquid chromatography. This can be done by diafiltration using ultra-filter. The enzyme extract was washed with 6× volume of deionized water in diafiltration mode using 30 kDa MidGee Ultrafiltration Cross Flow Cartridge (GE Healthcare). The conductivity of retentate was measured for ensuring the removal of ions. These ion-free enzyme extracts were used for further chromatography studies.

Hi Trap QFF (1 mL) chromatographic column (Akta Prime Plus GE, Sweden) was utilized for the purification study. All the buffers and samples were prefiltered with 0.45 µm ultra filtration cartridge and then equilibrated with binding buffer. The column was pre-equilibrated with 20 mM Glycine buffer of pH 10.5, for about 10 column volumes (CV) in order to attain zero baselines in the chromatogram. 0.5 mL of the enzyme sample was injected in to the column. The column was washed with the same buffer, about 5 CV was used. The bound protein was eluted using 0.5 M NaCl buffer pH 10.5 in gradient mode, which was followed by fraction collection. Chromatographic peaks of the protein were observed at 280 nm followed by peak integration.

FTIR Spectroscopy Analysis

The purity of the papain purified was confirmed by FTIR (Perkin Elmer Spectrum 100) analysis.

Results and Discussion

The present study focuses the progression of enzyme affinity towards the metal ligand to enhance

the extraction of papain from its crude latex. The aqueous two phase system has been taken as the reference where the partition coefficient studies were analysed. The phase formation of polymer-salt ATP system gave maximum papain extraction at 20% PEG+15% (NH₄)₂SO₄ (Table 1). The protease enzyme extraction was observed higher in top phase with a partition coefficient of 1.25 and 33% yield. In the metal integrated ATPi system, the concentrations of the metal ions were optimized for maximum enzyme separation (Fig. 1). The results show that ATPi system with metal ions was superior in comparison to ATP system in terms of partitioning coefficient and enzyme yield (Tables 2-4). Moreover, 0.2% (w/v) CuSO₄ and ZnSO₄ as free ligands in 20% PEG+15% (NH₄)₂SO₄ system exhibited the maximum partition coefficient (3.20), followed by 0.16% (w/v) CoCl₂ (3.14) and 0.08% (w/v) CdCO₃ (2.73). The enzyme yield was 53.33% in case of CuSO₄ and ZnSO₄; while in case of CoCl₂ and CdCO₃, it was 73.29 and 74.54%, respectively (Table 2). Nitswang *et al*¹⁹ reported 87% of papain extraction using extraction in comparison with the conventional two step precipitation method. In the present study, the aqueous two phase (ATP) extraction system was further optimized with the use of metal as free ligands (ATPi) (Tables 3-4). In metal liganded ATPi system, the coupling of metal ion with linear polymer enhanced the partitioning of papain towards the polymer rich phase and resulted in better

Table 1—Partition coefficient of ATP extraction

ATP*	Total protein conc. (mg/mL)	Protease enzyme (U/mL)	Specific enzyme activity (U/mg)	Partition coefficient K _L	Yield (%)
Top phase	0.0923	0.0666	0.7222	1.25	33.33
Bottom phase	0.0615	0.0488	0.5772	-	-

*20% PEG + 15% (NH₄) SO₄

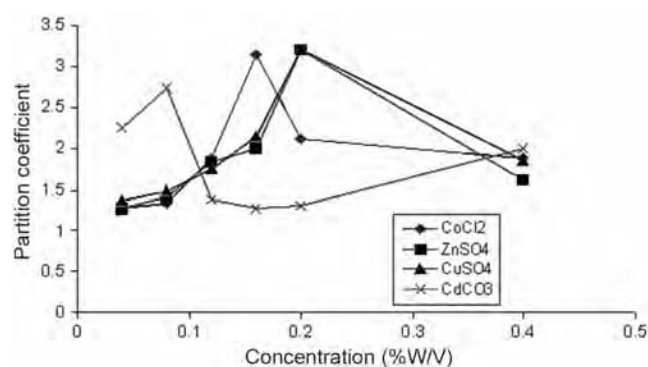


Fig. 1—Effect of metal ligand concentration on ATPi extraction.

Table 2—Effect of metal ligands in ATPi extraction

ATPi system	Enzyme activity (U/mL)	Specific enzyme activity (U/mg)	Partition coefficient (K_L)	Yield (%)
20% PEG+15% $(\text{NH}_4)_2\text{SO}_4$ + CdCO_3 (0.08% w/v)	0.1822	2962.00	2.73	74.54
20% PEG+15% $(\text{NH}_4)_2\text{SO}_4$ + CoCl_2 (0.16%w/v)	0.0977	907.9	3.14	73.29
20% PEG+15% $(\text{NH}_4)_2\text{SO}_4$ + CuSO_4 (0.2% w/v)	0.0711	690.29	3.20	53.33
20% PEG+15% $(\text{NH}_4)_2\text{SO}_4$ + ZnSO_4 (0.2 % w/v)	0.0711	770.3	3.20	53.33

Table 3—Overall process tabulation using ATP extraction

Steps	Protein conc. (mg/mL)	Protease enzyme (U/mL)	Specific enzyme activity (U/g)	Partition coefficient (K_L)	Yield (%)
Crude papaya latex	0.2000	0.1333	666.5	-	100
ATP	0.0923	0.0666	722.2	1.25	33.33
Dialysis	-	0.0266	-	-	20
Anion exchange chromatography	.00136	0.0044	3221.22	-	3.33

Table 4—Overall process tabulation using ATPi extraction

Step	Protein conc. (mg/mL)	Protease enzyme (U/mL)	Specific enzyme activity (U/g)	Partition coefficient (K_L)	Yield (%)
Crude papaya latex	0.2000	0.1333	666.5	-	100
ATPi	0.1030	0.0711	690.29	3.2	53.33
Dialysis	-	0.0400	-	-	30.00
Anion exchange chromatography	0.0029	0.0177	6084.34	-	13.33

yield. As copper and zinc exhibited the yield of 53.33%, consistent values were observed in the copper and it was selected for the further purification process. Since much work has not been reported so far for the papain extraction using metal ligands in aqueous two phase system, a preferable improvement in partition coefficient of metal ligand ATPi over conventional ATP system at least resolves the lower selectivity, interface deposition and backward extraction complication problems.

In the ATPi system, the partition coefficient value increased with the increase in metal ion concentration (Fig. 1) because of affinity of enzyme towards the metal-polymer bonding. As the metal ion concentration increased, the hydrophobicity was also increased, leading to more papain entry towards the metal ion rich top phase. The *pH* of the system was maintained at 5 using phosphate buffer. Each ligand has a specific saturation concentration, up to which increase in ligand concentration leads to an increase in the partition co-efficient values. The cadmium

(0.08% w/v), cobalt (0.16 w/v), copper (0.2% w/v) and zinc (0.2% w/v) exhibited their peak values at 2.73, 3.14, 3.20 and 3.20, respectively. After the saturation point, the spatial resistance increases which affects the partition coefficient. The better preference for the metal ligand ATPi system was CuSO_4 (0.2 % w/v) which showed highest partition coefficient value constantly and also have maximum enzyme activity (Table 1).

The partition behaviour of papain with different *pH* values ranging from 4 to 8 was analyzed in (0.2% w/v) copper ligand ATPi system containing 20% PEG+15% $(\text{NH}_4)_2\text{SO}_4$ (Fig. 2). The *pH* range was selected based on the isoelectric point of the papain enzyme. *pH* 5 was recorded to be the optimal *pH* and further increase in *pH* decreased the recovery of papain. This occurs due to the influence of *pH* on the papain and the alteration of protein surface charges by the ionizable groups, which in turn reduces the affinity of proteins towards the metal ion rich top phase. The metal ion partition proportion was

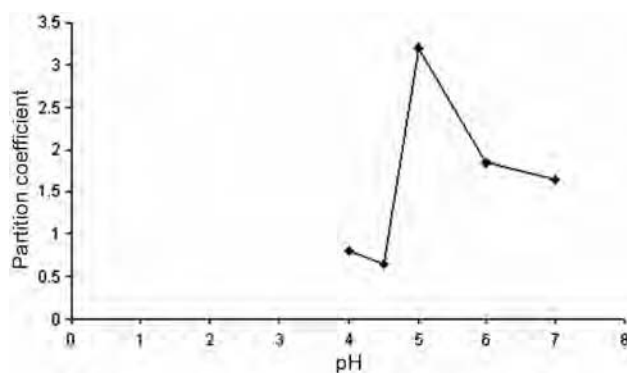


Fig. 2—Effect of pH on ATPi extraction.

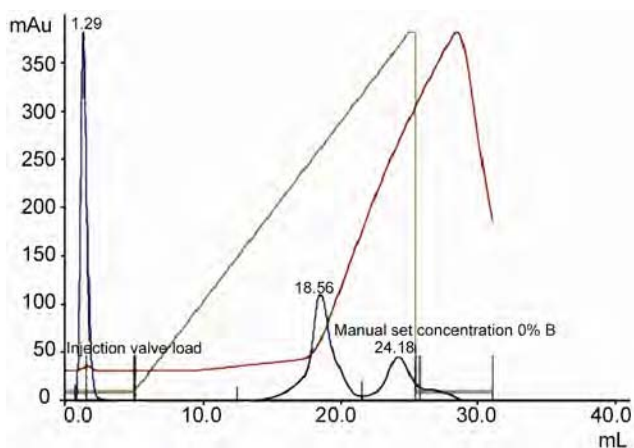


Fig. 3—Chromatogram for ATP extraction. [Hi Trap QFF (1mL) (Acta Prime Plus GE, Sweden)]

changed in the ligand ATPi system, which varied the potential difference between the phases.

Enzyme samples from ATP and copper integrated ATPi system were analyzed for purification and chromatograms of papain extracted from both systems are shown in Figs 3 and 4. A perusal of both the chromatograms shows that the amount of undesired proteins was drastically reduced in ATPi in comparison with ATP system without ligands. This is yet another evidence of the purity level present in ATPi system, where no traces of metal ions were found in the final sample. The papain fraction from the ATPi system was found to have both purity and partition coefficient, ensuring high enzyme selectivity of the system. Purity analysis from the chromatogram shows that fractions from ATP system had low activity and contains high amount of impurities present as unbound. The FTIR analysis confirmed the presence of papain in both the conventional ATP and affinity based ATPi systems (Fig. 5).

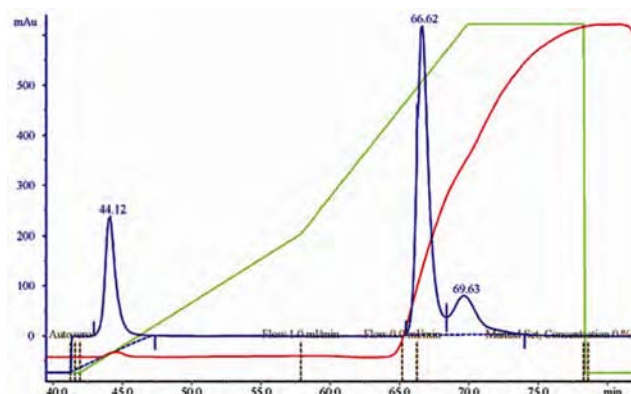


Fig. 4—Chromatogram for ATPi extraction. [Hi Trap QFF (1mL) (Acta Prime Plus GE, Sweden)]

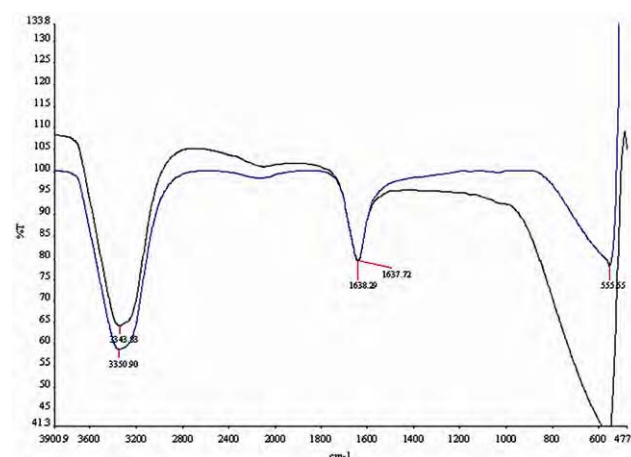


Fig. 5—FTIR spectrum of ATPi system.

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