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Lactoperoxidase Extraction from Caprine Milk using Conventional and Ionic Liquid Based ATP System

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Abstract: Lactoperoxidase is an enzyme, which is naturally present in fresh raw milk that functions as a natural antibacterial agent. Its concentration is fairly constant throughout the lactation. The objective of the present study was to compare the extraction and partition coefficient of LPO present in caprine milk using Ionic liquid (IL) based aqueous two phase (ATP) and Polymer based ATP system. Separation and purification of high value bio-molecules using IL based ATP extraction is an emerging green separation process. Here the Phosphonium based IL ATP system was used and it is the best alternative for the conventional organic solvent extraction and polymer based ATP systems. The resulting protein was purified and confirmed on gel filtration chromatography column. From the results it was found that IL based ATP system is highly selective and yield higher than the conventional system.
Keywords Ionic liquid, Caprine milk, Lactoperoxidase, Aqueous Two phase.

Introduction

Lactoperoxidase (LPO) is the most important enzyme which is present in caprine milk. It belongs to peroxidase family and it is the second most abundant enzyme after xanthine oxidase. It is exclusively found after cheese processing. Its concentration is about 30mg/lt constituting 1% of the whey protein[1]. It is an oxido reductase present secreted from milk and plays important role in protecting lactating mammary gland and intestinal tract of new born infants against pathogenic microorganism. Lactoperoxidase is a glycoprotein which carries Fe3+ Hemoprotein found in several mammalian biological fluids such as milk, saliva and tears[2]. Lactoperoxidase catalyses the oxidations of a number of inorganic and organic substrates by hydrogen peroxide. These substrates include bromide and iodide and therefore Lactoperoxidase can be categorized as a halo peroxidase.

A mutual incompatibility between two phase components results in the formation of aqueous biphasic system [3, 4]. It is a Liquid-Liquid extraction technique which has significant advantages over other conventional system such as short time consumption, quick phase separation, selective extraction, improved purity and yield[5-8]. The flexibility of the ABS has already been reported for the extraction and purification of biological products just as plant proteins, enzymes, antibodies and drug molecules by polymer/polymer or polymer/salt system [9-11]. Despite, the characteristic features of polymers like high viscosity, density and restricted polarity interfere with the effective phase formation. In this regard, it is essential to develop a new and efficient ABS system.In recent trends, a green separation technique have been introduced in to Aqueous

biphasic system by the application of "greener solvents" namely Ionic Liquids (IL)[12]. Ionic liquids are salt like materials which has melting point of below 100 °C. Ionic liquids are usually present in liquid state which is called as green solvent because of its negligible volatility and non-flammability characteristics[13]. The main property of aqueous two phase formation is hydrophobicity which can be greatly influenced by Ionic liquids. The literature studies reveals that the formation of two phase region in an IL ABS is greatly influenced by the operating parameters like pH, Temperature, concentration and nature of the phase components etc. The previous literature outcomes implies that the selectivity towards the protein extraction by IL based ATP can be improvised with evaluation of different types of ILs[14]. Hence, the present work was performed with an objective of designing new IL based AB system of Tetra butyl phosphonium bromide ($P_{4444}Br$) +K_2HPO_4 for partitioning of Lactoperoxidase. The resulting undergoes gel filtration chromatography technique for purity range. This study moreover investigates a specific activity of the aqueous two phase extraction of lactoperoxidase, conventional Vs Ionic liquid based ATP system.

Experimental Details

Materials and Purity

Tetra butyl phosphonium bromide (98%) was purchased from Sigma Aldrich and Di potassium hydrogen phosphate (99.5%) was purchased from Hi media and used without further purification. Poly ethylene glycol 6000 (98%) and Ammonium sulphate (97%) was purchased from Hi media. Double distilled water was used for solution preparations throughout the experiment.

Apparatus

Analytical balance (BL-220H, Shimadzu Corporation, Japan). Hot Plate (Technical made in SUNBIM, India). Magnetic stirrer (Remi Laboratory, India). PC based Double beam spectrometer (Model2202 Systronics, India).

Crude preparation and Characterization

Crude Caprine milk has been obtained in the local area of Karur. The gathered samples were stored at -4°C and defrosted at room temperature before the process. Afterwards, the fat present in the milk sample was removed by centrifugation at 4500 rpm for 30 min at 4®C. After centrifugation, the pH of the skimmed milk was maintained at 4.2 by the addition of 2N HCl and centrifuged at 4500 rpm for 30 min at 4®C to precipitate casein. Further, Casein was removed by treating the whey with 1.2 g/l of $CaCl_2$ at 3 to 5®C. Then the pH of the sample was elevated to 7.3 with 6N NaOH and temperature was maintained at 55 ®C. The above operating condition was seized for 8 min to settle down the complex calcium lipid particles. After that the clarified whey isolate was acquired in supernatant and precipitate of calcium phosphate was removed. The crude whey sample was analyzed for both protein concentration and enzyme activity.

Purification of LPO in Conventional ATP system

LPO partitioning was carried out with PEG6000 and ammonium sulphate ATP system. Crude extract was added to the aqueous two phase mixture and mixed gently. Centrifugation was performed at 6000rpm for 30 mins at 4◦C for effective phase separation. Two phases formed were separated carefully. Backward extraction was done with top phase separated by adding 150mM NaCl and allowed for phase separation. Then, the resulting bottom phase was subjected for gel filtration chromatography.

Purification of Lactoperoxidase in Ionic Liquid based ATP system

Forward extraction

Caprine whey protein isolate which were stored in phosphate buffer pH 6.5 were mixed with and inorganic salt K_2HPO_4. The concentrations of ionic liquid and salt used for the forward extraction were considered from the binodal curve. At defined concentration, the ionic liquid and salt were mixed in a graduated centrifuge tube with clarified whet isolate. The temperature of the system was adjusted to 25◦C .And the two phase separation as shown in **fig.1** was carried out by performing centrifugation at 6000 rpm for 10 minutes, then the resulting top phase was carefully separated to perform backward extraction.

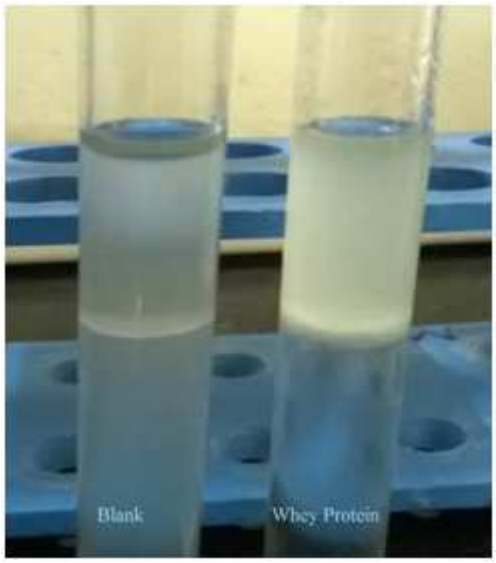

Fig.1 Extraction of Lactoperoxidase in $P_{4444}Br+K_2HPO_4ATP$ system

Backward extraction

In the backward extraction process, aqueous two phase system was formed by blending 20% of NaCl salt with the top phase separated from the forward extraction. The centrifugation of the mixture at 8000 rpm for 10 minutes resulted in two phase formation and the salt rich bottom phase containing the target LPO enzyme was separated by micropipette and the Gel filtration chromatography analysis was carried out to perform purification studies on LPO enzyme.

Determination of Total Protein

The total protein estimation was determined for the enzyme fraction by the Lowry's method with respective protein as a standard. In Lowry's protein estimation 0.5 ml of the sample solution was mixed with 4.5 ml of Lowry's reagent. Lowry's reagent of 50 ml was prepared by mixing 48 ml of Reagent A(2% Na_2CO_3 in 0.1N NaOH), 1 ml of Reagent B(1% sodium potassium tartarate in distilled water) and 1 ml of Reagent C (0.5% copper sulphate Pentahydrate in distilled water). The reactive mixtures were well stirred and incubated for 10 minutes at room temperature. After incubation, 0.2ml of Folin-Ciocalteu reagent was added and mixed well. Absorbance was measured at 580 nm after 30 minutes incubation at room temperature. The protein concentration was determined from the BSA calibration chart.

Spectrophotometric Lactoperoxidase activity

The enzyme (LPO) activity was measured by using potassium iodide (KI) as a substrate. Initially, the reaction mixture was prepared by mixing 0.15 ml of 0.09M hydrogen peroxide in 30 ml of 0.005M potassium iodide. Then, 0.01 ml of whey sample was added with 3.0 ml of reaction mixture. The absorbance of the sample mixture was measured at 350 nm for every 1 min. The reaction results in the formation of yellow color due to the oxidation of KI by H_2O_2. The concentration of LPO was measured by calibration chart which was referred from previous reports and shown in Appendix. The quantity of LPO which can capable of producing change in absorbance of 1 per second is called as one enzyme unit.

$$\text{Enzyme activity of Lactoperoxidase} = \frac{\Delta A_{350/\min}}{\in / mlofreactionmixture}$$

Whereas, ΔA350/min is the change in absorbance per min at 350 nm, ε is the molar extinction coefficient (i.e. 26.6 mM−1 cm−1).

Gel Filtration Chromatography

Whey component was identified by using Gel Filtration Chromatography (GFC) or size exclusion chromatography. A Sephadax G -250 column (AKTA Prime Plus, GE, and Sweden) was used for protein purification with the column size of 5ml. The column was previously equilibrated with pH 6.8 buffer solution at

flow rate of 2.5ml/min. Sodium phosphate buffer was prepared with 0.05% sodium azide to identify the occurrence of Lactoperoxidase The samples isolated from the backward extraction were injected to remove the ions present in the salt solution. A sample volume of 0.5 ml was introduced into the column through an injection port. The sample elution occurred at the same flow rate were collected separately with fraction collector. The elution was monitored by UV-Vis spectrophotometer at 280nm and the data obtained were interpreted with prime view software. The final fraction was collected and the presence of LPO was confirmed by assay.

Results and Discussion

The extraction of Lactoperoxidase from crude sample was carried out through conventional ATP system and compared with Ionic liquid base ATP system. The results show that the maximum extraction was found at ionic liquid based ATP system. The parameters like pH and temperature were analyzed against specific activity.

Polymer ATP system Vs Ionic liquid based ATP system

The specific activity was found to be 258 U/mg in Conventional ATP system where IL based ATP system gives maximum specific activity of about 1215 U/mg were shown in **Table 1 and 2. The** gel filtration chromatography results give 11523 U/mg specific activity and 85% yield from the IL based system.

Table 1 Lactoperoxidase Extraction – Conventional ATP system

System	Specific Enzyme Activity, U/mg	% yield	Purity factor
Crude	137	100	1
PEG 6000 + $(NH_4)_2SO_4$	258	54	2.72
GFC	523	40	3.9

Table 2 Lactoperoxidase Extraction – Ionic Liquid based ATP system

System	Specific Enzyme Activity, U/mg	% yield	Purity factor
Crude	137	100	1
$(P_{4444}Br)$ +K_2HPO_4	1215	94	35.6
GFC	11523	85	80.2

Effect of pH on Specific activity

Figure 2 depicts the interrelationship between the pH and Specific activity of the ionic liquid based ATP system. As the pI of the peroxidase enzyme is in the range of 3- 9, the highest activity was found to be at pH 5.At higher and lower pH conditions there is tremendous decrease in the specific activity and there was no appreciable activity observed at pH below 4. The specific activity shows sharp decrease which depicts the acidic or alkaline strength which denatures the enzyme property.

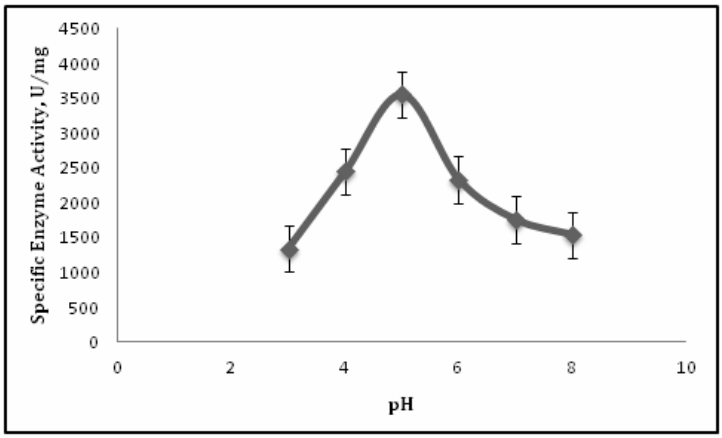

Fig 2: Effect of pH on specific enzyme activity of Lactoperoxidase

Effect of Temperature on specific activity

In an ionic liquid based ATP system, Figure 3 shows that the effect of temperature towards the specific enzyme activity. The specific enzyme activity was found to be higher at 30 C which reveals the high extraction of enzyme at optimum temperature shows the endothermic nature of the process. Further increase in temperature affects the 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.

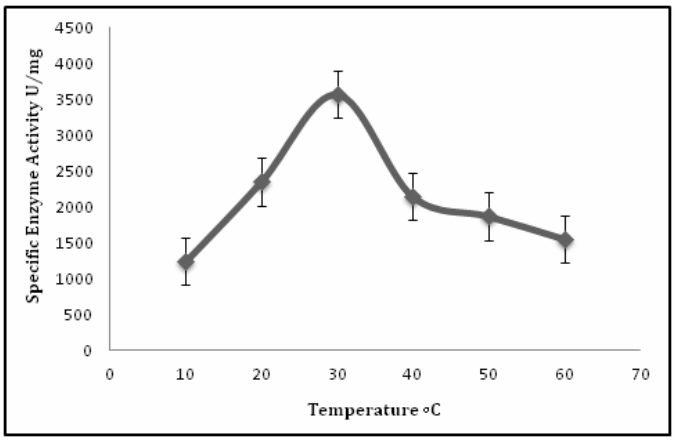

Fig 3: Effect of temperature on specific enzyme activity of Lactoperoxidase

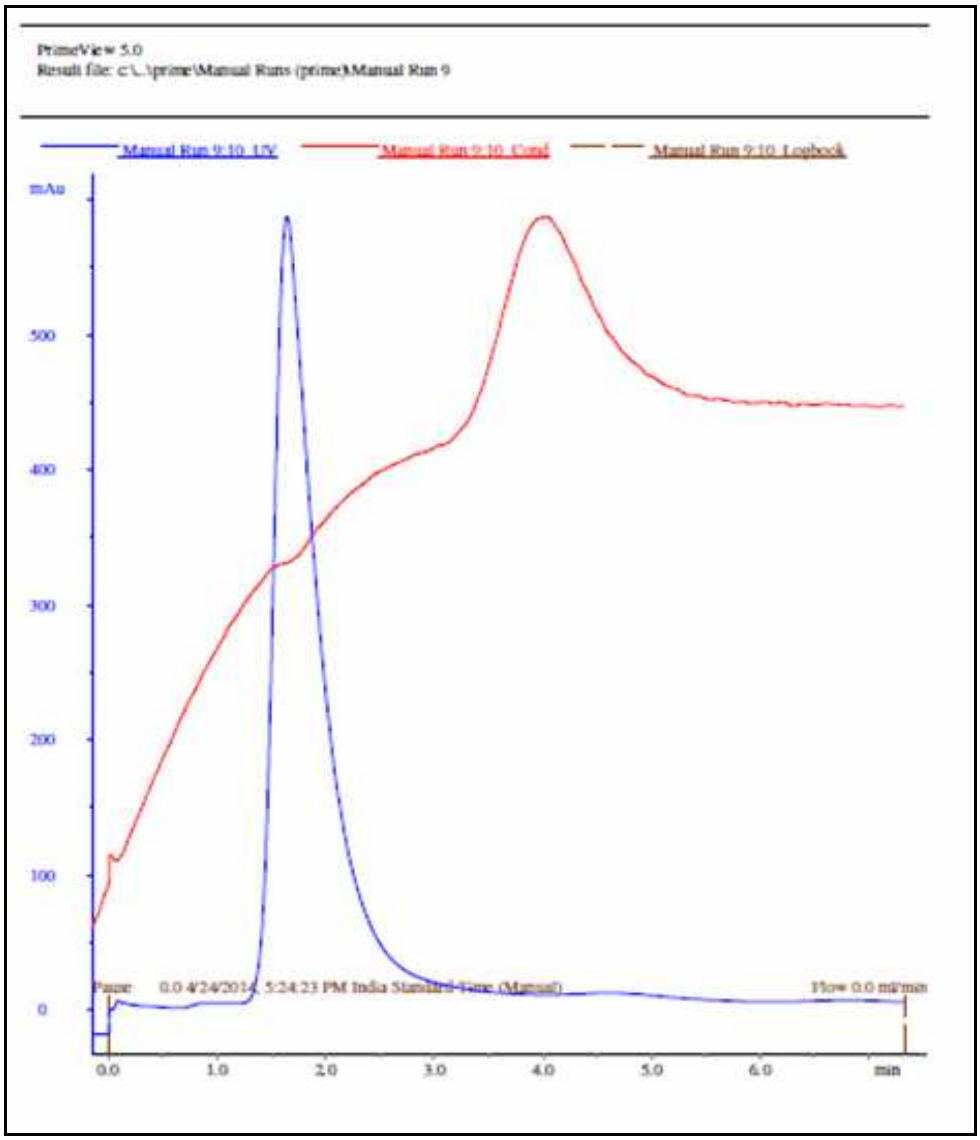

Fig 4: GFC for LPO from backward extraction on Sephadex G 100 column, 5ml @ pH 7.5 in Conventional ATP system with a flow rate of 2.5ml/min.

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 and 5. From the GFC fractions, ionic liquid based ATP system achieved high selective separation for LPO extraction.

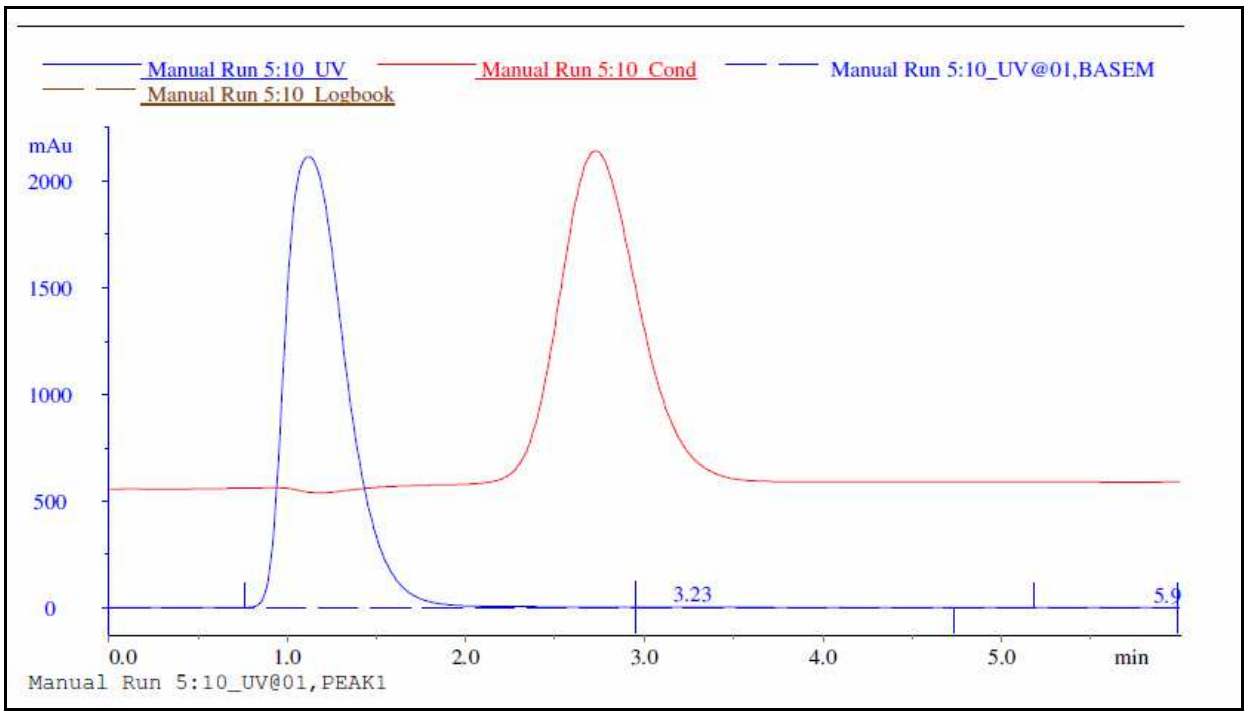

Fig 5: GFC for LPO from backward extraction on Sephadex G 100 column, 5ml @ pH 7.5 in IL based ATP system with a flow rate of 2.5ml/min.

Conclusion

This work reveals the advantages of IL based ATP system for the purification of Lactoperoxidase from caprine milk. This method provides a convenient method for pure enzyme extraction. The extraction of lactoperoxidase through ionic liquid based ATP system proves a simple and rapid process compared with other conventional methods. Ionic liquid base ATP system shows high selectivity and easy scalability of the system favours the large scale application of LPO purification in industries.

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