Research Journal of Chemical Sciences _____ ISSN 2231-606X
Vol. **2(7),** 47-52, July **(2012)** *Res.J.Chem. Sci.*

Potential Recovery of Protein from Shrimp Waste in Aqueous two Phase System

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Available online at: www.isca.in
Received 7th April 2012, revised 17th April 2012, accepted 18th April 2012

Abstract

Shrimp waste is an important source of bioactive molecules and it undergoes rapid disintegration which leading to environmental pollution. It is necessary to preserve the material adopting the environmentally safe techniques, prior to recovery of bioactive components such as proteins and carotenoids. Aqueous two-phase system (ATPS) partitioning has been used to recover and concentrate proteins from Shrimp Waste and offers many advantages along with biomass removal. Hence, a feasible protocol for the recovery of protein from Shrimp Waste has been established. The best conditions of partitioning were achieved using ATPS composed of PEG 4000, ammonium citrate salt, pH8, 1M addition of sodium chloride, and 36.28 Tie line length. The maximum percentage yield of protein extracted from shrimp waste was found to be 74.50%.

Key words: Aqueous two phase system, shrimp waste, protein partitioning.

Introduction

The India shrimp aquaculture industry has developed to a great extent today because of the huge demand for shrimp especially in the European Union, USA and Japan. India with a fishery production 6.57 million metric tons from both captured and cultured resources is ranked 3rd among the largest fishing producing countries and occupies the 17th position among the seafood exporting countries of the world. The export of Indian marine products mainly consisted of dried items like Frozen fish, Frozen shrimp, chilled items etc. The export of Indian marine products was 6, 22,291 tonnes in 2010-11. Major contribution of export in seafood products Frozen fish is 38% and the second highest is Frozen shrimp 20% ie nearly 1, 26,625 tonnes in 2010-11. About 85 species of shrimp are known exist in Indian waters of which 55 species are reported either as commercially important or having considerable demand in the local as well as International market. There is scope for extensive Indian shrimp culture were found in places like Andhra Pradesh, Tamilnadu, Karnataka, Kerala, Orissa, Maharastra, Goa, Pondicherry and West Bengal. There are 399 seafood freezing plants along the Indian coast, with a built in capacity of 7284 tonnes per day. Tamilnadu has about 48 freezing plants with capacity 525 tonnes per day.[1] Normally Agricultural wastes are using for adsorption studies only but now we are recovery wealth from waste[2,3].

Shrimp processing for freezing normally involves removal of head and body carapace. Processing of shrimps generates large quantities of solid wastes. The solid shrimp waste contains head and body shell accounts approximately to 40-50% of whole shrimp weight[4]. The tropical shrimps the head generally constitutes 34-45% and body shell constitutes 10-15% [5]. These wastes contain protein (35-40%), chitin (10-15%) minerals (10–15%) and caroteinds[6]. Shrimp bio waste is usually dried on the beaches that encourages not only environmental pollution but also reduces the recoverable components in this bio waste. A better economic use of the shrimp head would minimize the pollution problem and at the same time maximize the profits of the processor. With pollution control norms becoming more stringent, processors are looking at utilization of these byproducts for recovery of marketable byproducts as an alternative to disposal. Utilization of byproducts for recovery of value added products, not only minimizes the pollution but also improves the economy of the plant. However, for the industry to develop processes for byproduct recovery and utilization it has to be more economically feasible than discarding by-products. Shrimp head waste is a rich source of chitin and also good source of protein, nutritive components and enzymes, making use of such wastes has drawn much interest from researchers in recent years. Few attempts have been made to utilize Shrimp waste as a source of protein, pigments (e.g. astaxanthin), flavour compounds[7] and chitin[8], Chitosan[9], Protein and pigments found in shrimp waste have been proven to be an excellent animal feed supplement[10].

Partitioning in aqueous two phase systems (ATPS) is a good alternative method to separate and purify mixture of proteins. ATPS, a liquid – liquid separation step, has been used as a first purification step in the removal of contaminants by a simple and economic process. Aqueous two-phase systems (ATPS) have been widely applied in the separation and purification of biomolecules, such as proteins, enzymes, and nucleic acids, is a capable alternative to many conventional processes because of its evident advantages: ATPSs provide a suitable environment to maintain biological activity and protein solubility[11]. This is due

to the high biocompatibility, high water content and low interfacial tension of these systems that minimizes product degradation[12]. On the other hand, good resolution and yields can be obtained simply by varying certain experimental conditions such as pH, ionic strength, and polymer molecular weight. ATPS systems can be formed by two polymers or by a polymer and a phase-forming salt. Polymer–salt systems are less expensive[13], and the phases are less viscous, which results in faster phase separation. Finally ATPSs are an ideal technology where clarification, concentration, and partial purification can be integrated in one step.

The objective of this study was to investigate nutritional quality of shrimp processing waste in order to explore possibilities for their utilization. The effect of the polymer molecular weight, pH, Tie line length, and addition of neutral salts on the partitioning behavior of protein has been investigated. The present paper will also report the optimization of the ATPS and to determine the optimum conditions for the next step of protein purification directly from the crude waste.

Material and Methods

Fresh shrimp waste was collected from the local market in India. Known weight of shrimp waste was ground in cell lysis buffer, centrifuged and the supernatant was collected. The shrimp extract was stored at 4°C and required quantities were taken as and when required for different experiments and directly subjected to aqueous two phase system (ATPS). Polyethylene glycol with molecular weights of 4000, 6000 and 10000 was obtained from Merck-Schuchardt (Munich,Germany) and bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). tri-ammonium citrate monohydrate was obtained from Loba, (with a minimum purity of 99 %). The polymer and salts were used without further purification. Milli pore water was used throughout the experiments.

The binodal curves were determined by the cloud point method[14]. Ammonium citrate of 30 % (w/w) and poly ethylene glycol 4000, 6000 and 10000 of 40 % (w/w) was prepared. A known amount of the PEG solution was taken and titrated against salt, To ensure the end point, salt was added in drops till the appearance of turbidity, which indicates the two-phase formation. Water was then added until the disappearance of turbidity. The procedure was repeated to get the other binodal points. The determination of tie lines, samples were prepared (50g) by mixing polymer, salt, and water in appropriate proportions in a centrifuge tube. The samples were thoroughly mixed and then placed at room temperature (30°C) for 24 h. After separation of the two-phases (PEG rich top phase and salt rich bottom phase), the concentration of PEG in both phases was determined by refractive index measurement using an Abbe- type refractometer with a precision of ± 0.0001. The concentration of Sodium citrate in the top and bottom phase was determined by using a flame photometry. The tie-line length (TLL) was determined by the square root of the sum of the squares of the difference in PEG and citrate concentrations between the top and bottom phases.

$$\text{TLL}= \sqrt{\left(C_P^T - C_P^B\right)^2 + \left(C_S^T - C_S^B\right)^2}$$

Where C_P^T and C_P^B are PEG concentrations (%, w/w) in the top and bottom phases, respectively, and C_S^T and C_S^B are salt concentrations (%, w/w) in top and bottom phases, respectively. Partitioning of soluble proteins from shrimp waste was carried out in PEG + ammonium citrate + water system. BSA was used as model protein for partitioning. All partition experiments were carried out at different pH (6, 7 and 8) values at constant temperature (30°C). Phase systems were prepared in 50 ml graduated centrifuge tubes by weighing out appropriate quantities of the PEG of desired molecular weight and sodium citrate stock solutions and added to crude shrimp extract to make the total weight of the system 100 % (w/w). The pH of the system was maintained by using citric acid monohydrate and the contents were mixed thoroughly. Complete phase separation was achieved by centrifugation at 3000 rpm for 20 min to speed up the phase separation, and then placed at room temperature (30°C) for 24 h to ensure complete equilibration. After equilibration, estimates of the volumes of top and bottom phases were made in graduated centrifuge tubes. In order to determine the concentration of proteins in each of the co-existing phases, samples from each solution phase was collected using a syringe. The top and bottom phases were withdrawn separately. Due to high viscosity of the polymer solution, it was necessary to dilute the sample prior to estimation of protein. Total protein was quantified by the Bradford method using a Coomassie assay reagent supplied by Pierce Rockford, IL, USA). To avoid interference from phase components, samples were analysed against blanks containing the same phase composition but without proteins. Bovine serum albumin (BSA) was used as a protein standard absorbance was monitored at 595 nm. An average of three replicates was considered. The error in the analysis was within ±1%.The partition coefficient is defined as the ratio of equilibrium concentration of protein extracted in top phase (C_T) to equilibrium concentration of protein extracted in bottom phase (C_B), and was determined using results from the Bradford protein assay.i.e.,

$$K = \frac{C_T}{C_B}$$

The partition coefficient (K) is used to quantify the degree of separation reached in an extraction process. The phase volume ratio (R) is defined as ratio of volume of the top phase to volume of the bottom phase,

$$R = \frac{V_T}{V_B}$$

Where V_T and V_B are the upper and lower phase volumes, respectively. Yield percentage was calculated by using the following equation:

$$Y = \frac{100}{(KR)^{-1} + 1}$$

Results and Discussion

The binodal curve describes the border between the single-phase area and the two-phase area. The area above the binodal describes all compositions giving rise to two phase systems. Binodal curve is a border line between the homogeneous phase and heterogeneous two phase of ternary mixture. The two phase region formation is due to the incompatibility of system components. The tie-line describes the compositions of the two phases in equilibrium. Phase diagrams were determined for different PEG molecular weights (figure- 1). It can be noted from this figure that the binodal lines became more asymmetric and close to the origin with the increase in polymer molecular weight. This happens because as the polymer molecular weight increases, the components of the system become more different and so lower concentrations are required for phase separation. This may be caused by the increase in the hydrophobical character of PEGs of higher molecular weight[15].

Experiments were carried out four different TLL and three different molecular weight of PEG/ammonium citrate system at pH 6 without addition of NaCl and the results are shown in Figure- 2. In this study, the partition coefficient K and extraction yield Y% was increased with the increase of PEG molecular weight from 4000 to 10,000 as well as increasing TLL. The partition behaviour of total protein depended on the PEG molecular weight in ATPSs. The molecular weight of polymer influences the protein partition by changing the number of polymer–protein interactions. This is usually attributed to hydrophobic interactions between the chains of PEG and the hydrophobic area of protein. At high molecular masses the preferential interaction between the PEG and the protein is decreased due to increase in the hydrophobic character of PEG and this leads to a decrease in partition coefficient. On other hand increase in PEG molecular weight results is reduction of excluded volume, meaning less space available for the protein accommodate in the top phase[16-17]. Another tendency that affects the protein partitioning is the high PEG molecular weight which will strength the viscosity of system and thus unsuitable for processing. In this study it has been observed that for increasing values of TLL, the partition coefficient increases. On increasing TLL, salt concentration in bottom phase increases which effectively salts out the biomolecules as their solubility limits are reached. It promotes the partition of protein from the bottom phase to the top phase[18]. The system pH is another important factor which influences partitioning of biomolecules. The influence of pH on partition coefficient of protein partitioning in two phase system was shown in figure- 3. The pH could affect the partitioning, either by changing the charge of the solute or by altering the ratio of the charged species present. At low pHs, the protein has a net positive charge because the amine gains an extra proton and at high pHs, it has a net negative charge because the carboxyl loses its proton. The intermediate pH at which protein has a net charge of zero is called the isoelectric point. At higher pH, the protein is more negatively charged than at low pH, and therefore, the partition coefficient of the protein increases with increasing the pH, which may be caused by the electrostatic interactions between the protein and PEG units. Several authors reported that negatively charged proteins partition to the top phase (PEG rich) and positively charged protein to the bottom phase in the ATPS[19-20]. The globular proteins aggregate and attain negative charge on increasing the pH of the system. This provides better partitioning of the proteins in the top phase on increasing the pH from 6 to 8.

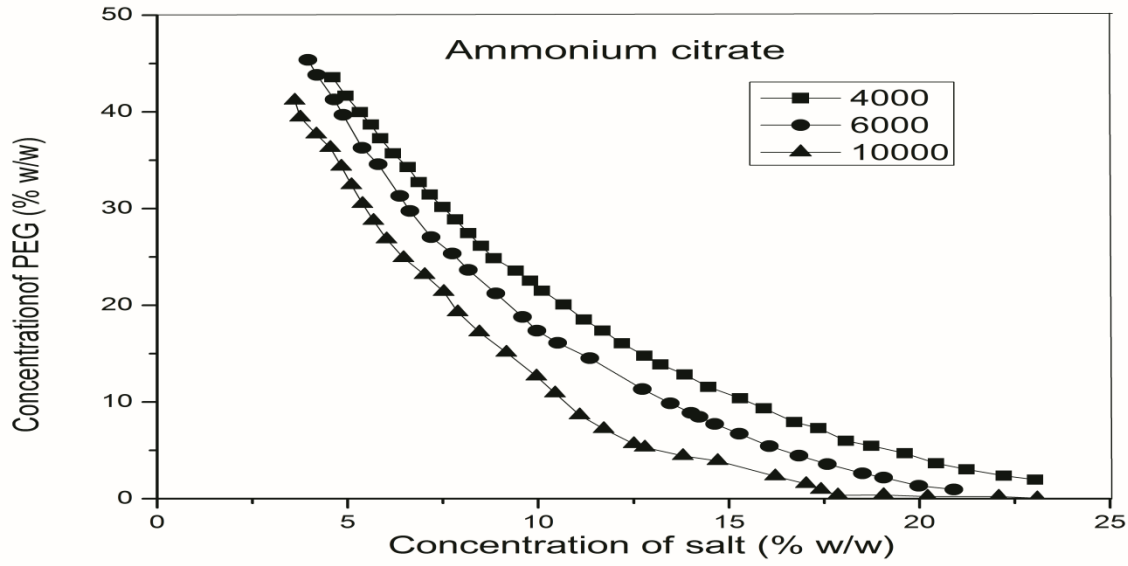

Figure- 1
Effect of molecular weight on Binodal Curve of PEG/Ammonium citrate aqueous two phase system

It is well known that, added salt type and its concentration alter the partition behavior of biological materials. Generally, the addition of sodium chloride salts affects partitioning in ATPS by speeding up phase separation, by influencing the phase potential or by decreasing protein hydrophobicity. Partitioning of protein as a function of varying NaCl concentration for studied at pH 8 and the results was depicted in Figure- 4.The addition of NaCl is known to generate an electrical potential difference between the two phases, thus affecting the partitioning of proteins in ATPS[21]. Moreover NaCl are added to rinse the hydrophobic difference between the phases addition and consequently enhanced the partition process. The positive effect of NaCl on driving biomolecules partitioning to the top phase has been already demonstrated[22].

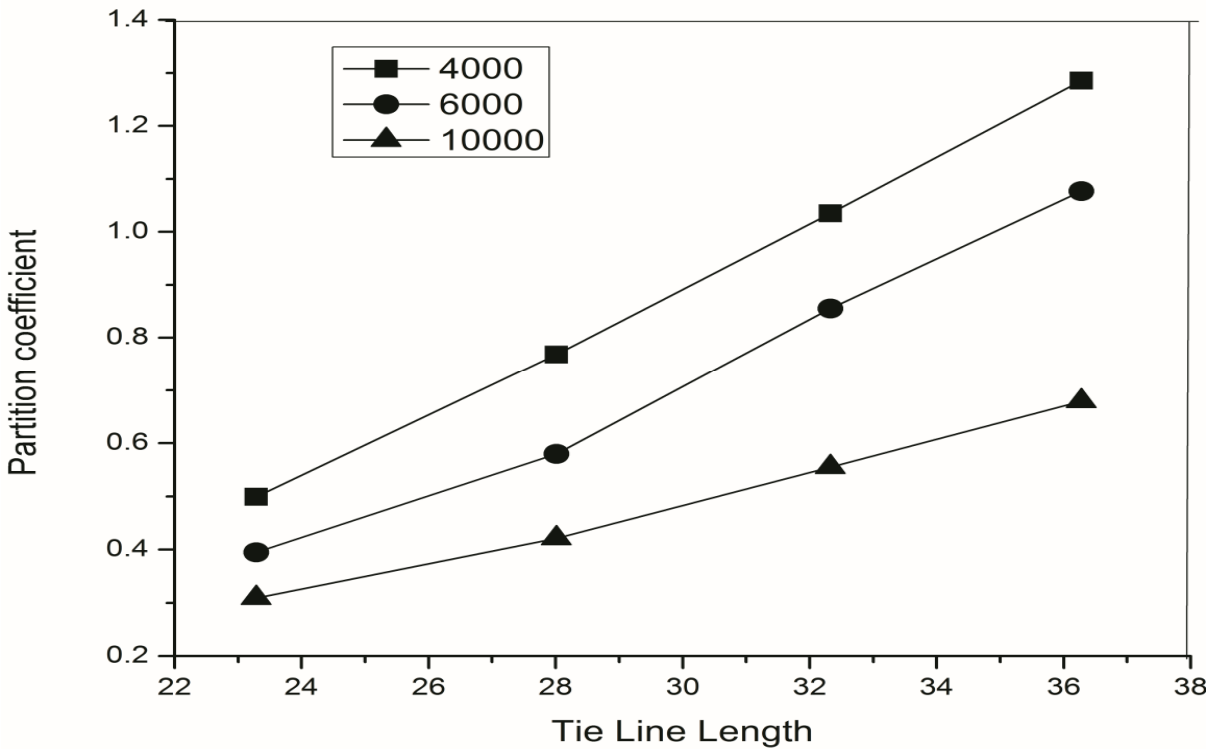

Figure- 2
Influence of PEG molecular weight and TLL on protein partitioning in PEG-Ammonium citrate system

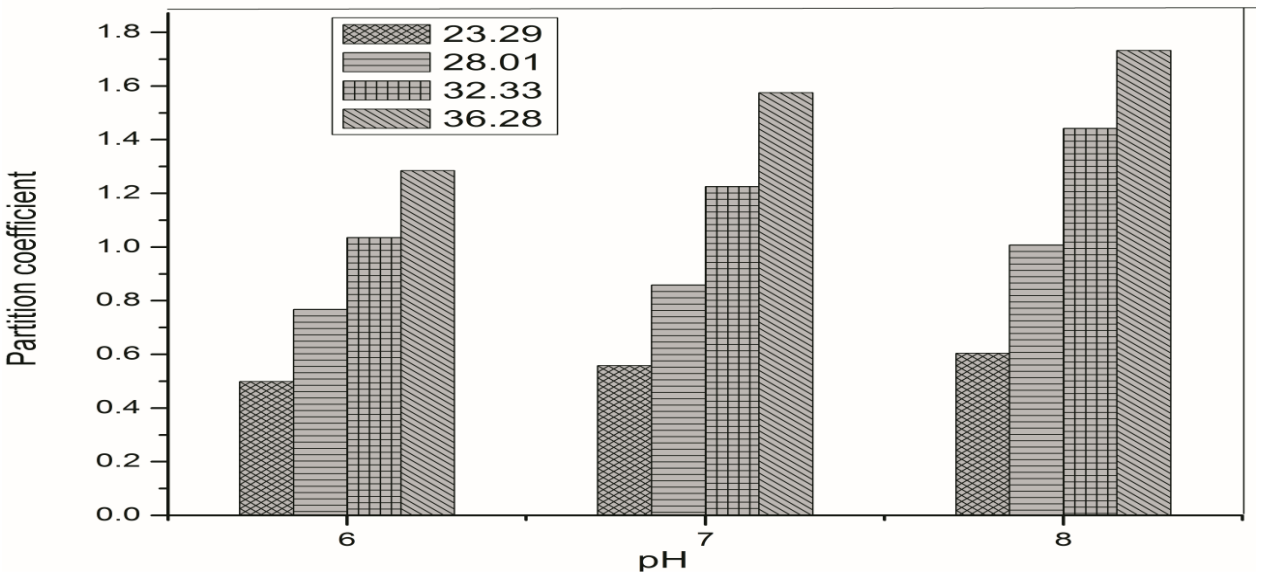

Figure- 3
Influence of pH on protein partitioning in PEG- Ammonium citrate system

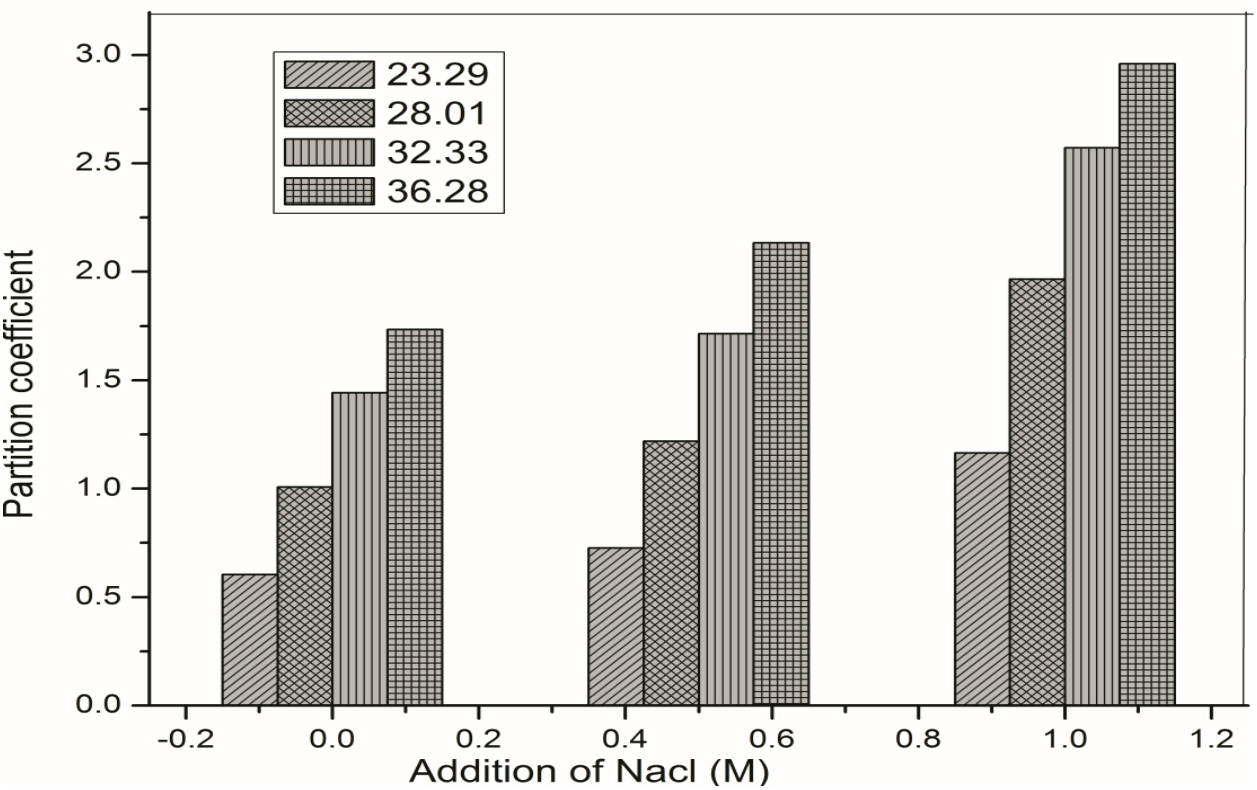

Figure- 4
Influence of Addition of NaCl on protein partitioning in PEG- Ammonium citrate system

Conclusion

Conventional purification methods usually need long processing time and expensive also. Therefore, new approaches are necessary to minimize time and costs. In the present study, PEG-4000 and ammonium citrate ATPS was used for the partitioning of protein. According to above results, protein was extracted from shrimp waste by using an ATPS composed of PEG-4000 and ammonium citrate with addition of 1M NaCl at pH8.The optimal system gave partition coefficient of 2.960 and yield of 74.50% in the top phase of the system. Overall results obtained here demonstrated the feasibility of ATPSs for the purification of protein. Further purification of the particular protein is essential in order to remove the other contaminant proteins, which could be achieved by integrating the ATPS with chromatographic technique like ion-exchange chromatography. Protein partitioning needs to be thoroughly investigated for other different ATPSs. It is necessary for a continuous mode of operation in order to make the technique commercially viable.

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