〈Original Article〉

Optimisation of ammonium sulfate precipitation method to achieve high throughput concentration of crude alkaline phosphatase from Brown shrimp (*Metapenaeus monoceros*) hepatopancreas

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Summary Effectiveness of various pH and ammonium sulfate saturation level in the pancreatic samples that has been homogenised at 3,000 rpm for 10 min at 4℃ and clarified at RCF of 1681.1× g for 5min at 4℃ to concentrate alkaline phosphatase with optimum yield and purity was determined. Precipitation performed using 0.1 M Tris-HCl buffer of pH 8.4 containing 0.1 M $MgCl_2$ and $ZnCl_2$ produced optimum yield and activity in comparison to the precipitation performed using 2 M KCl solution of pH 7, and hence components and pH of the buffer plays a major role during concentration. The salt saturation level of 65% at pH 8.4 reduced the volume of the precipitate to one third, fractionated half of the proteins away from the precipitate, purified the enzyme by 5.24±0.06 folds, and increased the enzyme yield by 95.32±0.04%, compared to the initial homogenates, as it achieves optimum concentration along with partial purification of the enzyme with optimum yield and activity. Below 65% saturation level enzyme yield was reduced and above this level enzyme purification was inefficient.

Key words: Alkaline phosphatase, Shrimps, Insoluble, Clarification, Precipitation

1. Introduction

Hepatopancreas of shrimps is an important organ functions in food absorption, transport, secretion of digestive enzymes, and storage of lipids, glycogen, and a number of minerals, hence is a very good source of varieties of commercially important products such as enzymes adapted to various environmental conditions[1-3]. However, these impurities with narrow variation in their physico-chemical properties are the bottle neck during the purification of alkaline phosphatase to commercially exploit it, including its use in dairy, medicine, leather processing and bioprocess industries. Concentration of the alkaline phosphatase by exploiting physico-chemical properties of the components of clarified hepatopancreatic tissue

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Abbreviation: RCF = Relative Centrifugal Force, AMP = 2-amino-2-methyl-1-propanol, ANOVA = Analysis of variance, *p*NPP = *p*-nitrophenylphosphate, *p*NPP = *p*-nitrophenol, Tris-HCl = Tris (hydroxymethyl) aminomethane hydrochloric acid solution

homogenate by ammonium sulfate precipitation is critical at the initial stages of enzyme purification itself as it efficiently reduces the bulk of the volume and partially purifies the enzyme, even though partial purification of the enzyme is incidental[4, 5]. Reducing the bulk volume of the homogenate is crucial at the initial stage of the purification itself as this on the other turn reduced the requirement of space, quantity of the ingredients, scale of the operation, and associated cost of purification[4]. Hence it is need of the hour to investigate the specific parameters required to precipitate alkaline phosphatase into a smallest volume of the two phase developed during precipitation with optimum yield and activity while retaining other proteins with similar physico-chemical properties in the bulk of the volume through experimental setup[6-8]. This is possible because proteins with few hydrophilic regions are precipitated at lower saturation level and some other proteins with more hydrophilic region are precipitated at higher saturation level without changing its native conformation of the precipitate[9-14]. Even though parameters of ammonium sulfate precipitation is well researched and widely discussed, no single set of standard operating conditions to concentrate enzyme from the clarified tissue homogenate of the shrimps in terms critical factors such as of ammonium saturation level and pH have been defined. Moreover, the saturation level of the ammonium sulfate required to precipitate various proteins in biological mixture varies from one protein to other protein under given condition and various external factors influence the pattern of protein precipitation, we have designed strategy to remove contaminating protein and water from the enzyme mixture optimally using a set of standard operating conditions that could be used for isolating alkaline phosphatase from the hepatopancreatic tissues of Brown shrimp (*Metapenaeus monoceros*) with optimum yield and activity.

2. Materials and methods

2.1. Chemicals

All the chemicals and reagents used were of analytical grade and were obtained from Merck Limited (Mumbai, India). Solutions were prepared with reagents according to the current American Chemical Society specifications[15]. Tris-HCl buffer or KCl solution was used as working buffers during the homogenisation and precipitation of hepatopancreatic tissues of shrimp. Working buffer, 0.1 M Tris-HCl buffer of pH 8.4 was prepared by adding 12.111 g of the free Tris base to 900 mL of deionized water. This mixture is then titrated with 1 M HCl solution to pH 8.4, and volume was made up to 1,000 mL. The buffer was added with $MgCl_2$ and $ZnCl_2$ to respective final concentration of 1 mM. Whereas, 2 M potassium chloride (KCl) solution was prepared by dissolving 149.1 g of potassium chloride using deionised water, and pH was adjusted to 7 using 0.1 M NaOH solution. Assay buffer, 2-amino-2-methyl-1-propanol (AMP) buffer of pH 10.3 was prepared by dissolving 78 g of AMP in 500 mL of deionized water. To this resulting mixture 200 mL of 1 M HCl is added. Subsequently, volume of the mixture is made up to 1,000 mL using deionized water in 1,000 mL volumetric flask. Assay buffer is used to determine the alkaline phosphatase activity. Working buffer and assay buffer preparations were filtered and sterilized at 121℃ for 20 min[16].

2.2. Sample collection

Brown shrimp (*Metapenaeus monoceros*) caught using trawl nets from the Arabian Sea were obtained from the fishing boats landed in 'Bunder area', Mangalore between the months of July and December. The time elapsed between catching and landing may not exceed over four to six hours. The material was kept in an insulated container after adequately icing them in the proportion of 1:1 shrimp to ice, and transported to the laboratory within two hours. Brown shrimp available along the coastal Karnataka was identified and used for the present study[17]. Freshly caught Brown shrimp with the weight range of 20-25 g were washed and dissected to remove the hepatopancreas. The hepatopancreas and attached tissues were sorted out, and weighed. Hepatopancreatic tissues were packed in plastic bags, labeled, frozen at -40℃, and stored at -20℃ in a deep freezer (JHBio, Chennai, India) until further use.

2.3. Homogenization

The samples were thawed at room temperature of about 28℃, weighed and homogenised using a Potter-Elvehjem homogenizer (RH-2 Homogenizer, Rotek Instruments, Kerala, India) with a sample holding tank mounted in a cooling jacket. The samples were homogenized in the homogenizer at homogenization speed of 3,000 rpm for 10 min at the temperature of 4℃ using 0.1 M Tris-HCl buffer of pH 8.4 or 2 M KCl solution of pH 7 at 1:10 tissue to buffer ratio[18].

2.4. Centrifugation

The crude homogenate with highest protein content were centrifuged at relative centrifugal force (RCF) of 1681.1×g for 5 min at 4℃ in C-24BL/CRP24 model microprocessor controlled low volume high speed refrigerated centrifuge (Remi Laboratory Instruments, Mumbai, India)[19].

2.5. Ammonium sulfate precipitation

Ammonium sulfate crystals were dried overnight at 120℃ and ground finely using pestle and mortar. Infranatant obtained after centrifugation were taken in 3 mL centrifugation tubes and placed in a cooling jacket maintained at 0℃. While keeping the samples in ice jacket, 0.066, 0.144, 0.209, 0.277, 0.351, 0.430, 0.516, or 0.608 g/mL of finely ground ammonium sulfate crystals were added gently with intermittent agitation to dissolve all the added crystals to achieve 15, 25, 35, 45, 55, 65, 75 and 85% saturation level, respectively. This step took around 15 min and the mixture was further stirred for 20 min at 0℃. Subsequently, mixture was centrifuged at 15,124.8×g for 30 min at 0℃. Supernatant was decanted and precipitate was reconstituted in 0.1 M Tris-HCl buffer of pH 8.4 or 2 M KCl solution of pH 7 at 1:1 pellets to buffer ratio, and samples were estimated for protein content and alkaline phosphatase activity. The ammonium sulfates from the precipitates were removed by the process of dialysis.

2.6. Dialysis

Dialysis-tube of 10 kDa was prepared by boiling the tubes in 10 mM sodium bicarbonate containing 1 mM ethylene diamine tetra acetic acid (EDTA) for 20-30 min. The tubes were cooled and washed extensively in distilled water, and was stored at 4℃. One end of the dialysis tube was closed using leak proof clamps (Himedia, Mumbai). Respective reconstituted pellets were dispensed into the tubing using pipette. The other end of the dialysis tubes were clamped with clamps while keeping sufficient space above the sample and placed in the beaker containing more than ten times volume of the 0.1 M Tris-HCl buffer of pH 8.4 maintained at 4℃. Magnetic stirrer was used to stir the buffer gently to improve solute exchange. The dialysis buffer was changed once in three hours. Respective samples were drawn at 6, 12, 16 and 24 h of dialysis and estimated for total protein content and alkaline phosphatase activity.

2.7. Proximate analysis

Samples were drawn at different intervals of experiment was performed in quadruplicates. The protein content was estimated as per the Folin-Ciocalteau method of Lowry and others[20], using bovine serum albumin (BSA) as a standard. Total protein content of the hepatopancreatic tissues were done by incubating 0.4 mg of tissues with 0.5 mL of 4 M NaOH at 100℃ for 5 min, and the resulting homogenate was cooled and assayed for total protein by Folin-Ciocalteau method. Similarly, protein content in the hepatopancreatic tissue homogenate during homogenization or in three phases after centrifugation was estimated using Folin-Ciocalteau method.

2.8. Enzyme assay

The procedure used for alkaline phosphatase analysis was based on the method of Bomers and McComb[21]. The substrate was prepared by dissolving 83.5 mg of disodium paranitrophenyl phosphate (*p*NPP) in 1.0 mL of 1.5 mM magnesium chloride solution and stored at 4℃. This solution was colourless and its absorbance was measured at 410 nm< 0.800. A stock solution of 10.8 mM of *p*NP was prepared by dissolving 150 mg of *p*NP in about 80 mL of 0.25 M NaOH solution and stored at room temperature of about 28℃ in amber colored bottle. A working solution of 54 mM of *p*NP was freshly prepared by pipetting 0.5 mL of *p*NP stock solution in 100 mL volumetric flask and the volume was made up

to the mark using 0.25 M NaOH solution. Enzyme assay incorporates AMP buffer. About 1.4 mL of buffer was mixed with the solution and incubated at 37℃ for 5 min. Then 0.05 mL of the hepatopancreatic tissue homogenates was added. To this mixture, 0.1 mL of the substrate was added, mixed and incubated at 37℃ for 15 min. Then, 4 mL of the 0.25 M NaOH was added to each tube in sequence maintaining timed intervals to terminate enzyme activity. Then, the solutions were mixed and cooled to room temperature (28℃). Colourless *p*NPP gets hydrolyzed by alkaline phosphatase at a given buffer pH and incubation temperature of 37℃ to form yellow colored free *p*NP, which shows maximum absorbance at 410 nm in a spectrophotometer that was set to zero with the blank. In our alkaline phosphatase assay, 0.05 mL of tissue homogenate was mixed with reagent and incubated for 15 min and the total volume was made up to 5.55 mL. However, the total volume in the case of each standard was 5.0 mL. Hence, *p*NP in mM or alkaline phosphatase activity in units/L in the tissue homogenate = (Test absorbance × 0.027 × 5.55 × 1,000)/(Standard absorbance × 15 × 5.0 × 0.05). Alkaline phosphatase activity in units/L is the liberation of 1 mM of *p*NP per min at 37℃ incubation temperature per liter of tissue homogenate in respective buffers. We made no corrections for the slight variation of molar absorptivity of *p*NP with pH and (or) buffer concentration.

2.9. Statistical analysis

The analysis of enzyme recovery was carried out in quadruplicate. The results were treated by analysis of variance (ANOVA), followed by Tukey's test, using the software Statistica 6.0 (Statsoft, Tulsa, OK, USA). The results were expressed as averages ± standard deviations followed by corresponding letters which indicates the significant differences. All analyses were performed considering a confidence level of 95% ($p<0.05$).

3. Results

Clarified hepatopancreatic tissue homogenates of Red shrimp were subjected to ammonium sulfate precipitation at various saturation levels under various conditions to determine the effectiveness each of these parameters to reduce the bulk of the medium and improve the enzyme yield.

3.1. Optimisation of pH during ammonium sulfate precipitation

When the precipitation was carried out at pH 8.4, alkaline phosphatase activity in the pellets obtained at ammonium sulfate saturation level of 15, 25, 35, 45, 55, 65, 75, and 85% was, respectively, 7.04±0.01, 10.57±0.01, 14.01±0.01, 17.61±0.02, 59.57±0.03, 98.65±0.06, 98.62±0.06, and 98.62±0.02% activity of its initial homogenates (Fig. 1). However, when the pH of the precipitation medium was decreased to 7, the saturation level of 15, 25, 35, 45, 55, 65, 75 and 85% was able to fractionate only 1.64±0.01, 2.52±0.01, 5.03±0.01, 18.87±0.09, 30.19±0.09, 26.42±0.09, 13.84±0.09, and 11.32±0.08% of the alkaline

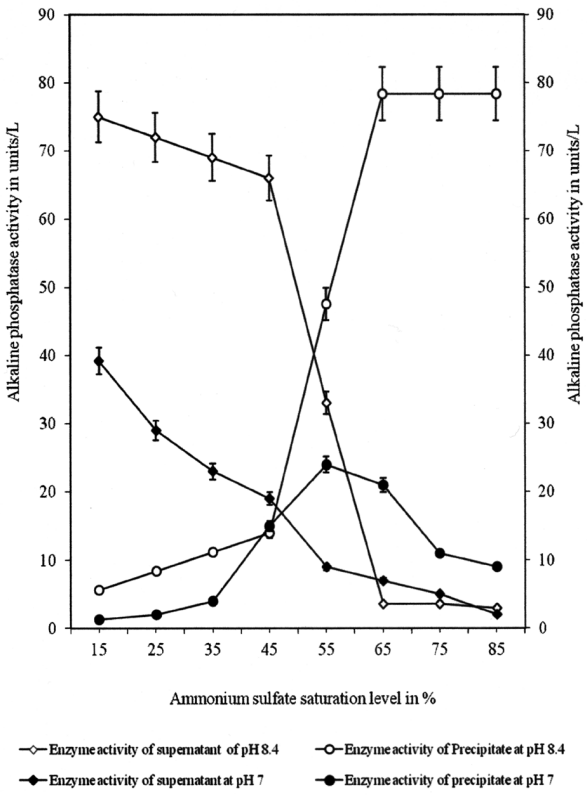

Fig. 1 Alkaline phosphatase activity of Brown shrimp supernatant and precipitate at different salt saturation and pH

phosphatase of its respective homogenate, respectively. It is very interesting to note here that, residual alkaline phosphatase activity in the supernatant at pH 7 was only 49.31±0.09, 36.48±0.09, 28.93±0.12, 23.90±0.09, 11.32±0.01, 8.81±0.01, 6.29±0.02, and 2.52±0.02%, whereas at pH 8.4 the activity was 92.96±0.09, 89.43±0.09, 85.91±0.12, 82.39±0.09, 40.13±0.01, 1.38±0.03, 1.38±0.01, and 1.38±0.02% of its initial homogenate, respectively, at 15, 25, 35, 45, 55, 65, 75 and 85% saturation level. Pellets obtained at these saturation levels at pH 7 were showing lower levels of alkaline phosphatase activity in comparison to the pellets obtained at the same saturation levels of ammonium sulfate but at pH 8.4. The overall significant effect of pH of the medium on alkaline phosphatase activity of the pellets at all saturation level remained at 5% level of significance, as indicated by one-way ANOVA with *post hoc* Tukey's test.

When the precipitation was carried at pH 8.4, increase in ammonium sulfate saturation level from 15% to 65% significantly (p<0.05) increased the precipitation of alkaline phosphatase into the pellets. Here, the results of analysis of variance with p-values demonstrate significance for the regression model for both ammonium sulfate saturation level and precipitation of alkaline phosphatase. Beyond 65% saturation at pH 8.4 one-way ANOVA with *post hoc* Tukey's test was not able to establish a significant difference in the alkaline phosphatase activity amongst the pellets obtained at different saturation. Here at pH 7, increase in the ammonium sulfate saturation level from 15 to 55% significantly (p<0.05) increased the precipitation of the proteins with alkaline phosphatase activity into the pellets. However, beyond 55% saturation level at pH 7 significant (p<0.05) decrease in the fractionation of the proteins with alkaline phosphatase activity took place in comparison to its activity at 55%. Hence, 0.1 M Tris-HCl buffer of pH 8.4 with $MgCl_2$ and $ZnCl_2$ to respective final concentration of 1 mM was used as a working buffer for subsequent precipitation experiments.

3.2. Optimisation of ammonium sulfate saturation level

Of the estimated 1833.75±1.02 mg total protein per liter of clarified homogenate of Red shrimp was ammonium sulfate saturation level of 15, 25, 35, 45, 55, 65, 75, and 85% was able to fractionate proteins at various ratio into pellets and supernatant as represented in the figure 2. The saturation level of 15, 25, 35, and 45% was able to precipitate only 1.15±0.01, 6.71±0.01, 9.98±0.01, and 17.01±0.07% of total protein from the respective clarified homogenate. However, the saturation levels of 55, 65, 75, and 85% ammonium sulfate was able to precipitate 54.31±0.13, 57.26±0.14, 62.99±0.13, and 68.55±0.13% of the total protein in the respective clarified homogenate. Here, one-way ANOVA with *post hoc* Tukey's test was able to establish significant (p<0.05) difference in the protein content amongst pellets obtained at 15, 25, 35, 45, 55, 65, 75, and 85% the saturation level.

As the saturation level was increased from 15 to 55%, alkaline phosphatase activity in the pellets were increased from 7.04±0.05% to 98.62±0.06%, and

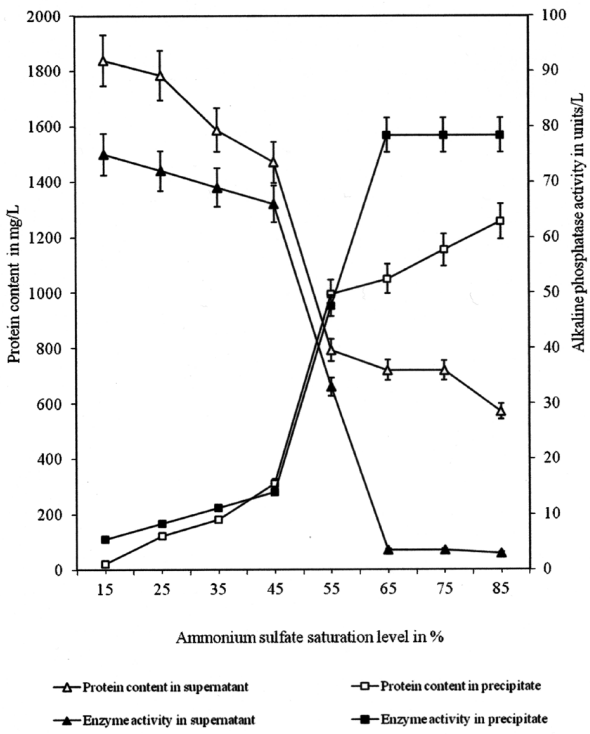

Fig. 2 Changes in protein and alkaline phosphatase activity in Brown shrimp supernatant and precipitate at different salt saturation

subsequently remained at a level of 98.61±0.22% of the activities in the clarified homogenate even up to 85% saturation level. One-way ANOVA with *post hoc* Tukey's test was able to establish higher (p<0.05) level of fractionation of the total protein and alkaline phosphatase into the precipitate at 55, 65, 75, and 85%, compared to 15, 25, 35, and 45% saturation levels. Beyond 65% saturation level no significant change (p>0.05) in the alkaline phosphates activity was registered among the precipitates, Even though increase in ammonium sulfate saturation level from 15% to 85% significantly (p<0.05) increased the precipitation of protein. Here, beyond 65% saturation level, significant (p<0.05) quantity of proteins without alkaline phosphatase were also fractionated into the precipitates when there was no significant change (p>0.05) in the alkaline phosphatase activity was observed in the pellets as established by one-way ANOVA with *post hoc* Tukey's test. The salt saturation level of 45% was able to precipitate only around one third of the alkaline phosphatase activity, and the saturation level 55% was able to precipitate only around three fourth of the activity, and at saturation level of 65% or more was able to precipitate more than 95% of the activity of clarified tissue homogenate. In one hand, the saturation level of the ammonium sulfate required to precipitate various proteins in biological mixture varies from one protein to other protein and should be to be done empirically, and on the other hand pH of the medium has a very important role to play.

3.3. Enzyme fractionation and concentration during precipitation

We have used 818.53±0.81 mL of the clarified homogenates for precipitation experiment at various ammonium sulfate saturation levels. The salt saturation level of 15, 25, 35, 45, 55, 65, 75, and 85% was able to fractionate 97.62±0.16, 96.06±0.19, 95.05±0.17, 94.78±0.14, 87.91±0.19, 81.25±0.11, 81.25±0.15, and 79.70±0.18% of the volume as a supernatant with 92.96±0.09, 89.43±0.09, 85.91±0.12, 82.39±0.09, 40.13±0.01, 1.38±0.03, 1.38±0.01, and 1.38±0.02% alkaline phosphatase activity of its initial homogenate, respectively (Fig 3). Specific activity of the pellets significantly (p<0.05) reduced as the ammonium sulfate saturation level increases from 15% to 35%, as established by one-way ANOVA with *post hoc* Tukey's test. Subsequent increase in the ammonium sulfate saturation level significantly (p<0.05) increased the specific activity, even up to 65% saturation level. However, significant (p<0.05) fall in the specific activity was observed as the saturation level increased from 75% to 85%. Pellets exhibited highest specific activity for alkaline phosphatase at 15% saturation levels of ammonium sulfate, though registering lowest of the enzyme yield at this saturation level compared to the other pellets. Nevertheless, pellets obtained at 65% saturation level produced lower specific activity in comparison to the pellets obtained at 15% saturation level, but highest alkaline phosphatase activity amongst all the pellets. Here, highest specific activity in the pellets produce at 15% saturation level is due to the lowest protein content in the pellets compared to the precipitates

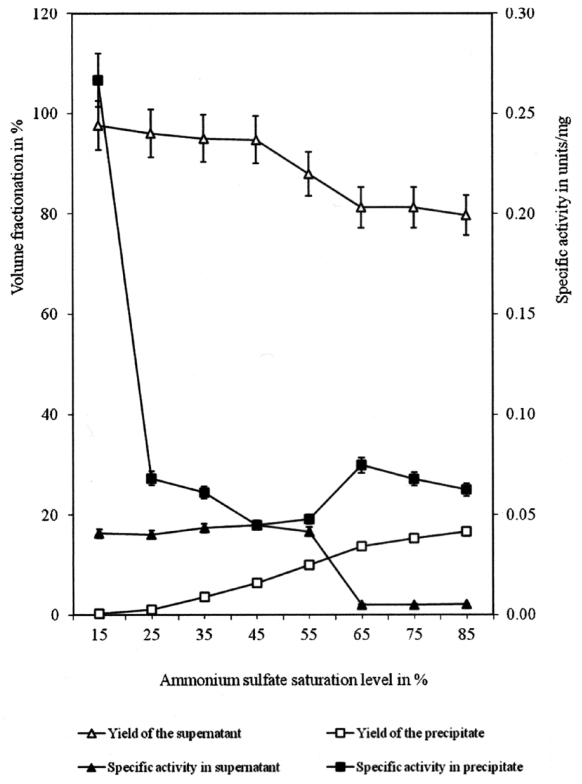

Fig. 3 Changes in volume and specific activity in Brown shrimp homogenate at different salt saturation.

produced at higher saturation level. Significant increase in protein precipitation even at insignificant change of alkaline phosphatase activity is responsible for the reduction in the specific activity beyond 75% saturation level. Optimum specific activity of alkaline phosphatase was observed at ammonium saturation level of 65%, and above and below this level specific activity was decreased, with the exception of at 15% saturation level.

The clarified tissue homogenates of the shrimp divided into supernatant with more than four fifth the volume and precipitates with less than one fifth of the volume at these ammonium sulfate saturation level. However, volume of the supernatant increased as the saturation levels of the ammonium sulfate reduced from 85 to 15%. Volume of the reconstituted pellets nearly doubled during the dialysis of the reconstituted precipitates using ten times the volume of the 0.1 M Tris-HCl buffer of pH 8.4 at 4℃ for 6, 12, 18, and 24 h, (Fig 4). No significant change in the specific activity was observed in each pellets obtained at different saturation levels, even when the volume of the samples doubled during the entire 24 h of dialysis of the reconstituted pellets. This is because during entire 24 h of dialysis the dilution of the reconstituted pellets resulted in simultaneous dilution of both total protein and alkaline phosphatase. Enzyme yield was only 6.81±0.03% of the initial homogenate used, even though the alkaline phosphatase was purified by 18.72±0.04 folds during the entire process of enzyme recovery at 15% saturation level. Nevertheless, when saturation level was increased to 65% ammonium sulfate enzyme yield was increase to 95.32±0.06% and purification was increased by 5.24±0.06 folds of the initial tissue. One-way ANOVA with *post hoc* Tukey's test was not able to establish significant (p>0.05) difference in enzyme yield and purification fold amongst the samples precipitated at 65, 75, and 85% saturation level.

4. Discussion

In one hand, alkaline phosphatase activity of the pellets obtained at different saturation levels of ammonium sulfate in 2 M KCl solution of pH 7 were at lower levels in comparison to the activity shown by pellets obtained at respective saturation levels of ammonium sulfate but 0.1 M Tris-HCl buffer of pH 8.4. On the other hand, nevertheless alkaline phosphatase activity remained at constant level beyond 65% saturation level of ammonium sulfate at pH 8.3, alkaline phosphatase activity reduced drastically beyond 55% saturation level of ammonium sulfate at pH 7. Previous work shows that ammonium sulfate at 60% saturation level efficiently removes high molecular weight proteins[22]. However, alkaline phosphatase activity of the pellets obtained at a given ammonium sulfate saturation level is influenced by the components and pH of the precipitation medium, as even at 55% alkaline phosphatase activity as reduced at pH 7 in comparison to pH 8.4[13]. This is supported by various reports showing that alkaline phosphatase isolated from the hepatopancreas of Indian white shrimp having optimum pH of 8.4[16, 18, 19]. Hence, components and the pH of the buffer used for

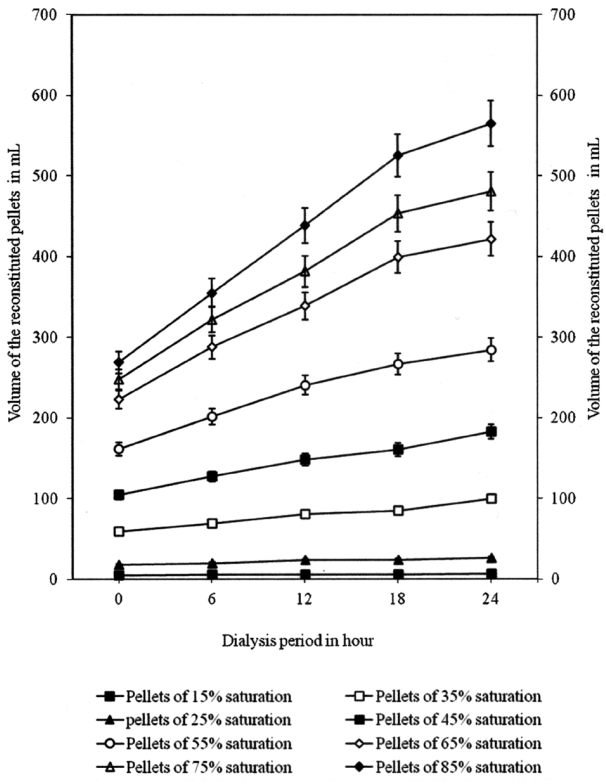

Fig. 4 Changes in volume of the reconstituted pellets during dialysis.

ammonium sulfate precipitation plays a major role in alkaline phosphatase activity, because of the three metal binding sites in the active sites of the enzyme, two zinc ions are shown to play a direct role in catalysis[23]. Hence, 0.1 M Tris-HCl buffer of pH 8.4 with $MgCl_2$ and $ZnCl_2$ to respective final concentration of 1 mM is the suitable buffer.

In one hand, increase in the saturation level of the ammonium sulfate increases the quantity of the protein precipitated from the clarified homogenate, because some proteins with few hydrophilic regions precipitate at lower ammonium sulfate saturation level and some other proteins with more hydrophilic region precipitate higher saturation of ammonium sulfate[12, 13]. In this study ammonium sulfate saturation level of 65% or more is able to precipitate more than 50% of the protein of the clarified hepatopancreatic homogenate. This is supported by the previous report that ammonium sulfate saturation levels of more than 60% reduce substantial high molecular weight proteins and majority of the hydrophobic protein[22, 24]. Protein precipitation by ammonium sulfate is the high-throughput method to concentrate proteins compared to other process alternatives such as ultrafiltration because proteins in mixture can be separated from one another efficiently based on their relative hydrophilicity by gradually increasing the concentration of ammonium sulfate[14]. On the other hand increase in the ammonium sulfate saturation level increase the precipitation of ammonium sulfate reaching more than 98% fractionation of alkaline phosphatase into pellets. However, beyond 65% saturation level, contaminating proteins gets fractionated into the pellets even though no precipitation of the alkaline phosphatase was possible. This is supported by the previously reported research that optimum alkaline phosphatase and maximum protein precipitation can be achieved at 20-50% fractions compared to 1-20% or 50-80% saturation level[24, 25].

To study the efficiency of the saturation level to retain maximum quantity of the contaminating proteins in the bulk of the volume and to fractionate maximum quantity of the insolubilised alkaline phosphatase into a minimal volume as possible resulting concentration and to some extent purification of the enzyme. Even though all the ammonium sulfate saturation levels were able to concentrate the alkaline phosphatase into less than one fifth of the volume of the clarified homogenate, degree of the enzyme yield and purification varied from one saturation level to other saturation level. Ammonium sulfate saturation at 15% was able to achieve highest purification of alkaline phosphatase in comparison to the purification achieved at 45% saturation levels of ammonium sulfate, because 15% saturation level of ammonium sulfate is able to precipitate some proteins with few hydrophilic regions precipitate at lower ammonium sulfate saturation level[12, 13]. However, the saturation level of 65% was not able to produce highest enzyme yield and least enzyme yield was achieved at 15% saturation level, because ammonium sulfate saturation level of 56% was able to precipitate some other proteins with more hydrophilic region precipitate higher saturation of ammonium sulfate[12, 13]. Even though ammonium sulfate saturation level of more than 65% was able to maintain same level of enzyme yield in comparison to the enzyme achieved at 65% saturation level, the saturation level of more than 65% was not able to maintain the enzyme purification level. Hence, it makes more sense to optimize yield rather than purification with batch methods such as ammonium sulfate precipitation[25]. These findings are in conformity with the previous works stating that ammonium sulfate saturation level of 60-89% purifies alkaline phosphatase by around 2-5 folds[26, 27]. Subsequent increase in ammonium sulfate saturation level beyond 75% significantly (p<0.05) decreased both the enzyme yield and purification. Similarly even though dialysis nearly doubled during the dialysis of the reconstituted precipitates, specific activity was unaffected due to the simultaneous dilution of both alkaline phosphatase and other protein precipitates.

5. Conclusion

Components and the pH of the buffer used during the alkaline phosphatase precipitation plays a major role in recovery, because ammonium sulfate precipitation performed using 0.1 M Tris-HCl buffer of pH 8.4 containing 0.1 M $MgCl_2$ and $ZnCl_2$ produced

optimum yield and activity in comparison to the 2 M KCl solution of pH 7 both at 0℃. It is more practical to optimize yield rather than purification at this stage because alkaline phosphatase yield is very poor even when ammonium sulfate saturation of 15% is efficient in enzyme purification. Ammonium sulfate saturation level of 65% efficiently reduces the volume of the homogenate by one third, effectively fractionates half of the contaminating proteins away from the precipitate and recovers more than 95% of the alkaline phosphatase activity into the precipitates.

References

1. McComb RB, Bowers Jr GN, Posen S: Alkaline phosphatase, Plenum Press, New York, USA (1979).
2. Hanpongkittikun A, Siripongvutikorn S, and Cohen DL: Black tiger shrimp (*Penaeus monodon*) quality changes during iced storage. ASIAN food J, 10, 125-130, 1995.
3. Chuang NN, and Yang BC: A comparative study of alkaline phosphatases among human placenta, bovine milk, hepatopancreases of shrimp *Penaeus monodon* (Crustacea: Decapoda) and clam *Meretrix lusoria* (Bivalvia: Veneidae): to obtain an alkaline phosphatase with improved characteristics as a reporter. Comp Biochem Physiol B, 96: 787-789, 1990.
4. Nooralabettu KP: Enzyme technology-Pacemaker of biotechnology. Prentice Hall (India), New Delhi (2011).
5. Arakawa T, Timasheff SN: Theory of protein solubility. Methods Enzymol, 114: 49-77, 1985.
6. Chiew YC, Kuehner D, Blanch HW and Prausnitz JM: Molecular thermodynamics for salt-induced protein precipitation. AIChE J. 41: 2150-2159, 1995.
7. Hatti-Kaul R and Mattiasson B: Isolation and purification of proteins, Marcel Dekker, New York (2003).
8. Wheelwright SM: Protein purification: design and scale up of downstream processing. John Wiley and Sons, New York (1991).
9. Nooralabettu KP: Downstream processing-New horizon in biotechnology. Prentice Hall (India), New Delhi (2012).
10. Aukhil I, Joshi P, Yan Y and Erickson HP: Cell- and heparin-binding domains of the hexabrachion arm identified by tenascin expression proteins. J Biol Chem, 268: 2542-2553, 1993.
11. Cutler P: Protein Purification Protocols. Humana Press Inc., Totowa, New Jersey (2004).
12. Mirica KA, Lockett MR, Snyder PW, Shapiro ND, Mack ET Nam S and Whitesides GM: Selective precipitation and purification of monovalent proteins using polyvalent ligands and ammonium sulfate. Bioconjug Chem, 23, 293-299, 2012.
13. Ko KY and Ahn DU: Preparation of Immunoglobulin Y from egg yolk using ammonium sulfate precipitation and ion exchange chromatography. Poult Sci, 86: 400-407, 2007.
14. Moore PA and Kery V: High-throughput protein concentration and buffer exchange: comparison of ultrafiltration and ammonium sulfate precipitation. Methods. Mol Biol, 498, 309-314, 2009.
15. American Chemical Society (ACS): Lab Guide, American Chemical Society. Washington, D.C. (1999).
16. Puttige K and Nooralabettu KP: Alkaline phosphatase activity during homogenisation of hepatopancreatic tissues of shrimps using sodium acetate, KCl solution, Tris-HCl and glycine-NaOH buffer. IJSER, 2: 1-7, 2011.
17. Racek AA: Littoral penaeinae from New South Wales and adjacent Queensland waters. Aust J Mar Freshwat Res, 6: 209-241, 1955.
18. Puttige K and Nooralabettu KP: Effect of homogenization speed and time on the recovery of alkaline phosphatase from the hepatopancreatic tissues of shrimps. Food Sci Biotechnol, 21: 461-466, 2012.
19. Nooralabettu KP and Puttige K: Primary recovery of alkaline phosphatase from the hepatopancreatic tissues of Indian white shrimp at varied centrifugal force and time. Food Sci Biotechnol, 22: 1-7, 2013.
20. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ: Protein measurement with the folin phenol reagent. J Biol Chem, 193: 265-275, 1951.
21. Bowers Jr GN and McComb RB: Measurement of total alkaline phosphatase activity in human serum. Clin Chem, 21: 1988-1995, 1975.
22. Akita EM and Nakai S: Immunoglobulins from egg yolk: Isolation and purification. Food Sci, 57, 629-634, 1992.
23. Bortolato M, Besson F and Roux B: Role of metal ions on the secondary and quaternary structure of alkaline phosphatase from bovine intestinal mucosa. Proteins Struct Funct Genet, 37: 310-318, 1999.
24. Kumar P, Kumar A, Kumar T and Singh BN: Purification of alkaline phosphatase (ALP), from *Streptomyces Sp Js 20*, isolated from mangrove sediment. Asian J Exp Biol Sci, 3: 236-242, 2012.
25. Ward WW and Swiatek G: Protein purification. Curr Anal Chem, 5: 85-105, (2009).
26. Bhattacharjee S, Das, A.K and Mandal, S.K., Isolation and characterization of alkaline phosphatase of

Saccharopolyspora erythraea from fermentation broth of erythromycin production. Ind J Biotechnol, 3: 558-562, 2004.

27. Mizobutsi GP, Finger FL, Ribeiro RA, Puschmann R, Neves LLM and Mota WF: Effect of pH and temperature on peroxidase and polyphenoloxidase activities of litchi pericarp. Sci Agric (Piracicaba, Braz.), 67: 213-217, 2012.