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High throughput Diethylaminoethyl cellulose chromatographic purification of Alkaline Phosphatase from Hepatopancreas of *Brown shrimp, Metapenaeus monoceros.*

Krishna Prasad Nooralabettu

Department of Biotechnology, P.A. College of Engineering, Nadupadavu, Mangalore-574153, Karnataka, India

ABSTRACT

Alkaline phosphatase was isolated from Brown shrimp hepatopancreas by homogenisation at 3,000 rpm for 10 min at 4^{o}C, clarification at RCF of 1681.1 x *g* for 5 min at 4^{o}C, and precipitation at 65% ammonium sulfate saturation level at 0^{o}C. Alkaline phosphatase purification was optimised in DEAE-cellulose columns using binding buffer of pH 7.6, 8.0, 8.4, or 8.8 and ionic strength of 0.00, 0.05, 0.10, or 0.15 *M* NaCl, and elution buffers of gradient 0.10-0.25, 0.10-0.35, 0.10-0.45 or 0.10-0.55 *M* NaCl in 25 min and flow rate of 0.5, 1.0, 1.5, or 2.0 mL/min. Binding buffers of pH 8.4 and ionic strength 0.1 *M* NaCl at flow rate of 1 mL/min is suitable for optimal binding of alkaline phosphatase to the column, and elution buffer of ionic gradient of 0.10-0.35 *M* NaCl in 25 min at a flow rate of 1.5 mL/min is suitable for optimal resolution.

KEYWORDS; Alkaline phosphatase, *Brown shrimp*, anion exchange chromatography, enzyme purification, DEAE-cellulose.

Short title: Optimization of alkaline phosphatase purification

INTRODUCTION

Shrimps exposed to varying environmental is a very good source of commercially important enzymes such as alkaline phosphatase with unique physico-chemical properties adapted to various environmental conditions can be recovered from the hepatopancreas of shrimps [1-4]. However, presence of protein impurities in the pancreas are the bottle neck during the final purification of alkaline phosphatase from other protein impurities for its commercial exploitation because this is an important organ functions in enzymes secretion, food absorption, transport and storage of lipids, glycogen and minerals, [5-7]. Careful selection of chromatographic parameters is an important strategy that to be adopted to exploit their physico-chemical properties by discriminating their surface charges based on their type, density and distribution that inturn affects their degree of interaction on charged chromatography media [8]. Net surface charge of the protein molecules that is unique for each protein species of the homogenate can be exploited selectively binding the protein of interest to weekly charged anion exchange resins such as popularly used DEAE-cellulose with high resolution, group separation and loading capacity under given condition that in turn depends on pH and ionic strength [9]. DEAE-cellulose chromatographic parameters such as pH and ionic strength of the binding buffer, and gradient slop and flow rate of the elution buffer can be favourably controlled in order to favor binding or elution of specific molecules and achieve separation of alkaline phosphatase from other protein impurities [10]. Number of research works was carried out to resolve of alkaline phosphatase from different sources in DEAE-cellulose columns [11-13]. Since, number and types of protein impurities varies from one enzyme source to other source, and no single set of chromatographic operating conditions were standardized in terms of the pH and ionic strength of the binding buffer, and gradient slop and flow rate of the elution buffer to isolate alkaline phosphatase from hepatopancreatic tissue homogenate of Brown shrimp (Metapenaeus monoceros). We have made an effort to determine the effect of pH and ionic strength of the binding buffer, and gradient slop and flow rate of the elution buffer in the DEAE-cellulose columns to optimize the purification of alkaline phosphatase from other protein impurities.

MATERIALS AND METHODS

Chemical and reagents

Analytical grade chemicals and reagents and were obtained from Merck Limited (Mumbai, India), and solutions were prepared according to the current American Chemical Society specifications [14]. Alkaline phosphatase assay buffer used for the present study was 2-amino 2-methyl 1-propanol (AMP) buffer of pH 10.3, which was prepared by dissolving 78 g of AMP in 500 mL of deionised water, and then 200 mL of 1 *M* hydrochloric acid (HCl) was added, and subsequently made up to 1000 mL in 1 L volumetric flask using deionised water. For chromatography, series of binding buffer such as, 0.1*M* Tris-HCl buffer of pH 7.2, 7.6, 8.0,

* **Corresponding Author:** Krishna Prasad Nooralabettu, Department of Biotechnology, P.A. College of Engineering, Nadupadavu, Mangalore-574153, Karnataka, India. E-mail: lodhariad1@hotmail.com

8.4, and 8.8 were prepared. First, 0.1 *M* Tris (hydroxymethyl) aminomethane solution was prepared by taking 12.111 g of the Tris (hydroxymethyl) aminomethane in 1000 mL volumetric flask and making up the volume to 1000 mL using deionised water. Similarly, 0.1 *M* Tris hydrochloric acid solution was prepared by taking 15.76 g of the Tris hydrochloric acid in 1000 mL volumetric flask and making up the volume to 1000 mL using deionised water. Binding buffer of pH 7.6, 8.0, 8.4, and 8.8 at 25^{o}C were prepared by mixing 6.06, 4.44, 2.64, and 1.23 mL of 0.1 *M* Tris (hydroxymethyl) aminomethane solution with 1.39, 2.65, 4.03, and 5.13 mL of 0.1 *M* Tris hydrochloric acid solution, respectively. The buffers were added with $MgCl_2$ and of $ZnCl_2$ to respective final concentration of 1 m*M*. Elution buffer used for chromatographic process was prepared using a second series of buffers with same pH values, but including 1*M* NaCl in gradient during the elution. To make 1 *M* NaCl, 58.44 g of NaCl was dissolved using deionised water in a final volume of 1000 mL using 1000 mL volumetric flask. All the binding buffer preparations were filtered and sterilized at 121^{o}C for 20 min.

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 material was brought in an insulated container after adequately icing them in the proportion of 1:1 shrimp to ice ratio, to the laboratory within two hours. The time elapsed between catching at the fishing ground and processing at the laboratory may not exceed over four to six hours and temperature recorded during the catching and the processing did not exceed 4°C. Freshly caught Brown shrimp with the size group of 86-120 mm in length and weighing around 30-55 g were sorted on a sanitized stainless steel working table, washed using chilled running water system maintained between 2-4°C, and identified as per Racek [15]. The shrimp was dissected to remove the hepatopancreas, hepatopancreas and attached tissues were packed in plastic bags, labeled, frozen at –40^{o}C, and stored at –20^{o}C in a deep freezer (JHBio, Chennai, India) until further use.

Homogenization

Brown shrimp samples were thawed at room temperature of about 28^{o}C, 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^{o}C 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 [16].

Centrifugation

The hepatopancreatic homogenates were clarified at relative centrifugal force (RCF) of 1681.1 x *g* for 5 min in C-24BL/CRP24 model microprocessor controlled low volume high speed refrigerated centrifuge (Remi Laboratory Instruments, Mumbai, India) at 4^{o}C [17].

Ammonium sulfate precipitation

The clarified homogenate were concentrated and partially purified by ammonium sulfate precipitation at 65% saturation level at 0^{o}C [18, 19]. Subsequently, mixtures were centrifuged at 15,124.8 x *g* for 30 min at 0^{o}C. Supernatant was decanted and precipitates were reconstituted in 0.1 *M* Tris-HCl buffer of pH 8.4 at 1:1 pellets to buffer ratio. The ammonium sulphate salts from the reconstituted precipitates were removed by the process of dialysis using 10 kDa prepared dialysis tubes (Himedia, Mumbai, India) in the beaker containing more than ten times volume of the 0.1 *M* Tris-HCl buffer of pH 8.4 maintained at 4^{o}C for 24 h at constant stirring and intermittent change of buffer for every three hours [20]. Dialyzed samples were removed from the dialysis bags and centrifuged at 6724.3 x *g* for 2 min to remove any dissolved debris. After filtering the solution into a microcentrifuge using syringe filter containing 0.22 µm filter, volume of the filtrate is measured and used for subsequent purification steps.

Ion exchange chromatography

Optimization of the purification of the alkaline phosphatase from the hepatopancreatic tissues of Brown shrimp using anion exchange chromatography was done by running Diethylaminoethyl (DEAE)-cellulose column using binding buffers of varied pH and ionic strength, and elution buffer of varied gradient slope and flow rate [21]. DEAE-cellulose (anion exchanger) was commercially purchased and slowly 5 g DEAE-cellulose was added to 300 mL of 0.1*M* NaOH with gentle stirrer for 30 min, when pH reached to 13. Sodium hydroxide solution was discarded and resin was washed with double distilled water until pH reached to pH 8.0. Then the solution was replaced with 0.1 *M* HCl with gentle stirrer for 30 min, when pH reached to 1.0. Resin was washed with the double distilled water until pH reached to 3.0. Distilled water was discarded and replaced it with 10× buffers (500 m*M* Tris-HCl of respective pH) and stirrer gently for 30 min. The 10× buffer was discarded and then the resin was equilibrated with 50 m*M* Tris-HCl pH 8.0, and after degassing fines were removed before the suspension of DEAE-cellulose resin was transferred into a glass column.

Setting columns for suitable pH of the binding buffer

A series of four chromatographic columns of size 8×80 mm (internal diameter × height) for DEAE-cellulose to be tested were set up. Flow rate of the mobile phase was run by two Scigenics Model 4735 peristaltic pump, and controlled by Honeywell DC 1040 controller (Scigenics, Chennai). DEAE-cellulose weighing around 100 g was washed thoroughly twice in 2000 mL of binding buffers, 0.1 *M* Tris-HCl buffer of pH 7.6, 8.0, 8.4, or 8.8. Resins were allowed to settle, and excess buffer was aspired to produce thick slurry. Approximately 25 mL of slurry was poured into the column up to 60 mm height, allowing the medium to settle as the column fills, without allowing the column to dry. Each column was equilibrated to a respective pH by washing five times using 5 mL each at the flow rate of 1 mL/min using a 0.1 *M* Tris-HCl buffer of pH 7.6, 8.0, 8.4, or 8.8. Eluent pH was checked for stability. Dialysed hepatopancreatic tissue homogenate of volume 0.2 mL was loaded carefully using syringe after adjusting the pH of the dialysed tissue homogenate to that of the binding buffer to each column at the flow rate of 1 mL/min, and the sample was allowed to soak into the resin and displaced buffer was collected. Column was washed using a binding buffer of respective pH at the rate of 1 mL/min, until no protein appear in the eluent. Bound materials were eluted using elution buffer of respective pH but gradient ionic strength of 0-0.5*M* NaCl in 25 min at the flow rate of 1 mL/min. All eluents were assayed for alkaline phosphatase activity and total protein content. The most suitable pH that allows alkaline phosphatase to bind to resins, where the enzyme is absent from the eluent, but as close to the point of release as possible, but the first pH at which the protein appears in the eluent was selected for subsequent experiments.

Setting columns for suitable ionic strength of the binding buffer

Here, series of four DEAE-cellulose columns were set up and was equilibrated using binding buffer of pH proven to be optimum in the previous experiment, but at ionic strengths of 0.00, 0.05, 0.10, or 0.15 *M* NaCl. To all the columns 0.2 mL of the dialysed tissue homogenates were applied while collecting the eluent. Eluent assayed for the maximum ionic strength that permits binding of the alkaline phosphatase and the minimum ionic strength required for complete elution. The highest ionic strength that permits binding and the lowest ionic strength for elution are used as binding and elution buffers, respectively, during subsequent gradient elution.

Setting columns for suitable gradient slope of the elution buffer

Similarly, series of four DEAE-cellulose columns were set up and was equilibrated using binding buffer of pH and ionic strength proven to be optimum in the previous experiments. To all the columns, 0.2 mL of the dialysed tissue homogenates was applied while collecting the eluent. But bound proteins were eluted at gradient slope using elution buffer of 0.10-0.25, 0.10-0.35, 0.10-0.45 or 0.10-0.55 *M* NaCl in 20 min at the flow rate of 1 mL/min. Eluents were assayed for the gradient slops that permits optimum peaks for alkaline phosphatase. The gradient slope that optimizes the resolution of alkaline phosphatase was determined and used for the next round of the experiment.

Setting columns for suitable flow rate of the elution buffer

In this part of the experiment, series of four DEAE-cellulose columns were set up and was equilibrated using binding buffer of pH and ionic strength proven to be optimum in the previous experiments. To all the columns 0.2 mL of the dialysed hepatopancreatic tissue applied while collecting the eluent. The efficient gradient slop proven to be efficient in the previous experiment was used to elute bound protein at flow rate of 0.5, 1.0, 1.5, or 2.0 mL/min. Fractions collected were assayed for the optimum flow rate that produces efficient resolution of alkaline phosphatase. The flow rate of the elution buffer used to elude the alkaline phosphatase optimally was determined. Chromatographic fractions with alkaline phosphatase activities were pooled and dialysed in dialysis bag at constant stirring using ten times volume of the 0.1 *M* Tris-HCl buffer of pH 8.4 maintained at 4^{o}C for 24 h with change of buffer at every 3 h of dialysis period and used for subsequent process.

Proximate analysis

Samples were collected at different intervals of experiment was performed in quadruplicates. The protein content was estimated as per the Folin-Ciocalteau method of Lowry and others [22], 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 4M NaOH at 100^{o}C 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 every stages of recovery was estimated using Folin-Ciocalteau method. Homogeneity of the preparation was determined in Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gel was prepared according to standard protocol of Laemmli [23].

Enzyme assay

Alkaline phosphatase was assayed based on the method of Bowers and McComb [24]. The substrate was prepared by dissolving 83.5 mg of disodium paranitrophenyl phosphate (*p*NPP) in 1.0 mL of 1 m*M* magnesium

chloride solution and stored at 4°C. This solution was colourless and its absorbance was measured at 410 nm< 0.800. A stock solution of 10.8 m*M*/L of *p*-nitrophenol (*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°C in amber colored bottle. A working solution of 54 m*M*/L 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 of pH 10.3. About 1.4 mL of buffer was mixed with the solution and incubated at 37°C 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°C 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°C). Colourless *p*NPP gets hydrolyzed by alkaline phosphatase at a given buffer pH of 10.3 and incubation temperature of 37°C 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 m*M*/L or alkaline phosphatase activity in units/L in the tissue homogenate = (Test absorbance × 0.027 × 5.55 × 1000)/ (Standard absorbance × 15 × 5.0 × 0.05). Alkaline phosphatase activity in units/L is the liberation of 1 m*M* of *p*NP per min at 37°C 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.

Statistical analysis

The analysis of samples 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% ($p<0.05$).

RESULTS AND DISCUSSION

Optimum pH and ionic strength of the binding buffer conducive for optimum binding of the hepatopancreatic alkaline phosphatase into the DEAE-cellulose, where there is no alkaline phosphatase activity in any fractions in the eluent, but as close to the point of release as possible, and the most suitable linear ionic gradient and flow rate of the elution buffer at which alkaline phosphatase activity first appears in the eluent was selected.

Optimising pH of the binding buffer

Of the total 7, 6, 5, and 4 protein peaks collected, respectively, using buffers of pH 7.6, 8.0, 8.4, and 8.8, three protein peaks with no alkaline phosphatase were recorded in the binding stage and rest of the protein peaks were observed during elution stage (Figure 1). This supports the view that regardless of the variation in the pH of the binding buffer, optimum binding of the alkaline phosphatase took place as supported by the finding that the three protein peaks of the elution stage did not exhibit any alkaline phosphatase activity, and only a protein peak of alkaline phosphatase was seen only in the elution stage. DEAE is a weak ion exchange resin that is positively charged below pH 8.5 (pKa~10.0) binds to shrimp alkaline phosphatase that is negatively charged at pH above its isoelectric point (pI) of 7.6 at low ionic strength such as 0.1 *M* NaCl, but eluted at high ionic strength [9, 25, 26].

Reducing the pH of the buffer from 8.8 to 7.6, in one hand, increased the resolution of the peaks in the elution stage, and on the other hand, decreased the time required for the appearance of the first peak of alkaline phosphatase activity. The ANOVA indicates the overall effect remained at 5% level of significance. Here, when a binding buffer of pH 8.8 was used the trailing peak is only a shoulder, but at pH 8.4 trailing peak is more completely separated. Even though, reducing the pH of the buffer below 8.4, increased the resolution, elution buffer of pH 8.4 produce a peak of alkaline phosphatase activity with maximum cumulative activity compared to the peaks obtained at other pH levels. Adjusting the pH of both binding buffer and elution buffer between 7.5 and 8.5 affects the protonation of histidine and affects the resolution between protein impurities and the enzyme by anion-exchange chromatography (27, 28). Alkaline phosphatase can be purified to near homogeneity using DEAE-Cellulose chromatography (29).

Optimising ionic strength of the binding buffer

Ionic strength of the binding buffers upto 0.15 *M* NaCl at pH 8.4 permits optimum binding of alkaline phosphatase to the DEAE-Cellulose and on the other hand is not favorable for the protein impurities to bind, supported by the observation that shrimp homogenates lost 39.34±1.19% of the total proteins of no alkaline phosphatase activity in the binding stage itself (Figure 2). Eventhough One-way ANOVA with *post hoc* Tukey's test was not able to establish any significant ($p>0.05$) difference amongst the elution profile of the proteins using elution buffer of ionic strength 0.00, 0.05, 0.01 and 0.15 *M* NaCl at pH 8.4, increase in the ionic strength of the binding buffer significantly ($p<0.05$) decreases the time required for the appearance of the first major peak of

alkaline phosphatase activity. However, increase in the ionic strength of the binding buffer depleted the resolution of the protein peaks with alkaline phosphatase activity, which is supported by the observation that the proteins bound using binding buffer of ionic strength 0.15 *M* NaCl started eluting even at the onset of the binding stage itself, which was proved in the chromatogram in which the last protein peak of the binding stage was shouldering the major peak of alkaline phosphatase activity.

Here, binding buffer with 0.1 *M* NaCl at pH 8.4 is appropriate for optimum binding, as this is the most suitable pH and ionic strength of the binding buffer that allows the proteins of interest to bind to the resins, but as close to the point of release as possible from the resins when eluted using elution buffers of increasing ionic strength [30]. Both ionic strength and pH of the binding buffer is very important in order to achieve the most effective high resolution of the alkaline phosphatase from other protein impurities, because efficient binding behavior of proteins to ion exchange resins is depends on various factors such as net charge, surface charge distribution, protein hydrophobicity, van der Waals interactions and choice of the adsorbent materials, that inturn influenced by pH and ionic strength of the buffer [31-36].

Optimising gradient slope of the elution buffer

Linear ionic gradient of 0.10-0.55 *M* NaCl in 25 min at the flow rate of 1 mL/min, chromatogram showed a major peak shouldering a minor protein peak and an independent minor peak of no alkaline phosphatase activity in the elution stage (Figure 3). Whereas, the gradient of 0.10-0.45 *M* NaCl at this rate produced one more minor peak that is trailing a major protein peak, where trailing peak was only shoulder of major protein peak at gradient slope of 0.10-0.55 *M* NaCl. Once again, the gradient of 0.10-0.35 *M* NaCl was able to produce one major protein peak and two independent minor protein peaks. Both, salt gradient of 0.10-0.25 *M* NaCl and 0.10-0.35 *M* NaCl was able to produce a major protein peak of alkaline phosphatase activity without any merger of minor protein peaks. Nevertheless, salt gradient of 0.10-0.45 *M* NaCl or 0.10-0.55 *M* NaCl in 25 min was not able resolve minor peaks from major peaks. At the selected pH, the proteins with the lowest net charge will be the first ones to elute from the column as ionic strength increases, and the proteins with the highest charge will be most strongly retained and will be eluted last and proper ionic gradient is crucial for achieving high resolution and efficient elution [31].

Increasing the ionic gradient slope of the elution buffer in one hand decreased the resolution of the major protein peak and on the other hand reduced the time required for the major protein peak with alkaline phosphatase to first appear during elution stage. This is because, as the concentration of the NaCl increases, the salt ions such as Na^+ or Cl^- compete with the bound proteins for charges on the surface of the ion exchange resins and one or more of the bound protein species begin to elute and move down the column [26, 31, 36]. However, in either of these two cases protein peaks of the binding stages and elution stage did not merge each other. Here, NaCl gradient slope of 0.10-0.35 *M* NaCl in 25 min was able to produce separate, narrow and symmetrical major protein peak with alkaline phosphatase activity from other two minor protein peaks of no alkaline phosphatase activity in the elution stage of the binding stage.

Optimising the flow rate of the elution buffer

Very low flow rates such as 0.5 mL/min was inefficient in resolving shrimp alkaline phosphatase from other protein impurities, and chromatogram showed a single trailing major peak shouldering two minor peaks, and increasing the flow rate to 1.0 mL/min produced a distinct highly resolved major peak clearly isolated from two minor peaks (Figure 4). Subsequent increasing the flow rate of the eluting buffer was efficient in resolving the major peak from other impurities and reduced resolution time by 0.5 min for every incremental flow rate of 0.5 mL/min. Elution buffer at a flow rate of 1.5 mL/min was able to clearly separate the fractions of single major peaks with alkaline phosphatase and three other minor peaks without any alkaline phosphatase activity. For eluted protein, increasing the flow rate for a given linear salt gradient is also performed has significant effect as indicated by low *p*-value. Alkaline phosphatase was resolved optimally from other impurities that were confirmed in the chromatogram showing clearly separated single major peaks of alkaline phosphatase from other three minor peaks with no alkaline phosphatase activity. Here, excluding at very low flow rate, increase in flow rate increases the resolution in anion exchange chromatography [39]. However, elution buffer above flow rate of 1.5 mL/min peaks with the alkaline phosphatase activity was shouldering with the minor peaks of protein impurities.

Volume of the samples during the chromatographic separation was increased by around six folds in comparison to the volume of the respective shrimp homogenates loaded to the column. DEAE-Cellulose chromatography was able to retain more than 50% of the alkaline phosphatase, and was able to purify the enzyme by more than 20 folds in comparison to the enzyme in the initial homogenate. SDS-PAGE analysis of the pooled samples of the major protein with alkaline phosphatase showed the presence of a major band and no minor bands were observed indicating homogeneity. It is impossible to affirm that the preparation is equally pure in absolute terms, for the purity of the alkaline phosphatase can be judged only in a relative way, and cases are known in which a product found to be homogeneous by electrophoresis is not so when examined by another analytical techniques. Nevertheless, high specific activity and the result of electrophoresis analysis lead us to

suppose that the preparation is practically pure. Accurate and reproducible flow control of the buffers along with the ionic strength and pH is critical for good resolution as these parameters has the greatest effect on enzyme purification. Hence, these factors are tested at various levels to arrive at the final separation condition for the isolation of alkaline phosphatase from other protein impurities of hepatopancreas.

Conclusion

Binding buffer of pH of 8.4 and ionic strength of 0.10 *M* NaCl at flow rate of 1mL/min optimally binds alkaline phosphatase to the DEAE-Cellulose in such a way that little change in the ionic strength of the elution buffer is sufficient to elute it from the column. Elution buffer at linear ionic gradient of 0.10-0.35 *M* NaCl at flow rate of 1.5 mL/min optimally elutes the bound alkaline phosphatase with maximum cumulative activity from the resins with high resolution. Optimising the pH and the ionic strength of the binding buffer, and the linear ionic gradient and the flow rate of the elution buffer is crucial to facilitate the optimum binding of the shrimp alkaline phosphatase to the DEAE-cellulose but at the verge of release from the resins without being present in the eluent, and on the other hand this pH and the ionic strength is not favorable for other protein impurities of the shrimp hepatopancreas to bind to the resins, where these impurities present in the eluent.

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