〈Original Article〉

Effective anion exchange chromatographic purification of hepatopancreatic alkaline phosphatase of Red shrimp, *Solenocera choprai*

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Summary Hepatopancreas of Red shrimp was homogenised at 3,000 rpm for 10 min at 4℃, clarified at RCF of 1681.1 × g for 5 min at 4℃, and concentrated at 65% ammonium sulfate saturation level at 0℃ to isolated alkaline phosphatase. Parameters of chromatographic purification of the alkaline phosphatase 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. Suitable binding buffer is of pH 8.4 and ionic strength 0.1 M NaCl at flow rate of 1 mL/min and effective elution buffer is of ionic gradient of 0.10-0.35 M NaCl in 25 min at a flow rate of 1.5 mL/min.

Key words: Alkaline phosphatase, Red shrimp, Anion exchange chromatography, Enzyme purification, DEAE-cellulose

1. Introduction

Marine organisms adapted to diverse harsh fluctuating environmental conditions are very good source of commercially important enzymes such as hepatopancreatic alkaline phosphatase of shrimps with novel physico-chemical properties recovered that can be commercially exploited in medicine, agriculture, leather processing, and dairy industry[1, 2]. Since, the hepatopancreas of the shrimp is an important organ functions in enzymes secretion, food absorption, transport and storage of lipids, glycogen and minerals, is the bottle neck during the purification of alkaline phosphatase[3]. Separation of alkaline phosphatase from other protein impurities of the hepatopancreas demands carefully designed strategy involves careful selection of chromatographic parameters to exploit their physico-chemical properties by discriminating alkaline phosphatase from other protein impurities based on their type, density and distribution of surface charges that in turn affects their degree of interaction on DEAE-cellulose resin[4]. Depending on their structure and environment charged group within the protein molecule that contributes to the net surface charges possess different pKa values. Purification of alkaline phosphatase from other impurities with high

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resolution are popularly achieved in DEAE-cellulose columns with efficient group separation and loading capacity, by exploiting net surface charge that is unique for each protein species of the homogenate under given condition that in turn depends on pH and ionic strength[5]. Chromatographic parameters in DEAE-cellulose columns such as pH and ionic strength of the binding buffer, and gradient slop and flow rate of the elution buffer can be controlled in order to favor binding or elution of specific molecules so as to achieve efficient separation of alkaline phosphatase from other protein impurities[6]. Number of efforts was made to recover alkaline phosphatase from different sources using DEAE-cellulose chromatography[7, 8]. Nevertheless, 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 Red shrimp (*Solenocera choprai*). This study is focused to arrive at optimum parameters such as pH and ionic strength of the binding buffer, and gradient slop and flow rate of the elution buffer in the DEAE-cellulose columns conducive for the purification of alkaline phosphatase from other protein impurities.

2. Materials and methods

2.1. Sample collection

Freshly caught Red shrimp with the size group of 34-66 mm in length and weighing around 15-25 g using trawl nets from the Arabian Sea were brought in an insulated container maintained at 4℃ within two hours and identified as per Racek[9]. The shrimp was dissected to remove the hepatopancreas, and frozen at -40℃, and stored at -20℃ in a deep freezer (JHBio, Chennai, India) until further use.

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

2.2. Cell disruption

Frozen tissues were thawed at room temperature of about 28℃, weighed and homogenised using a Potter-Elvehjem homogenizer (RH-2 Homogenizer, Rotek Instruments, Kerala, India) at the 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.

2.3. Clarification

The tissue homogenates were clarified at relative centrifugal force (RCF) of 1681.1 × g for 5 min in C-24BL/CRP24 model microprocessor controlled low volume high speed refrigerated centrifuge (Remi Laboratory Instruments, Mumbai, India) at 4℃.

2.4. Concentration

The clarified homogenate were precipitated by ammonium sulfate at 65% saturation level and precipitates were removed at relative centrifugal force of 15,124.8 × g for 30 min at 0℃. 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 sulfate 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℃ for 24 h at constant stirring[10].

2.5. Purification method

A series of four chromatographic columns of size 8 × 80 mm (internal diameter × height) for DEAE-cellulose to be tested were set up. Slowly 5 g of DEAE-cellulose-52 with the charge density of 0.90-1.20 mEq/g (Himedia, Mumbai, India) was added to 300 mL of 0.1 M NaOH with gentle stirred 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 stirred 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 mM Tris-HCl of respective pH) and stirred gently for 30 min. The 10× buffer was discarded and then the resin was washed twice with 100 mL of 0.1 M Tris-HCl buffer of respective pH and ionic strength as indicated in the subsequent experiment. Resins were allowed to settle, and excess buffer was aspired and entrapped bubbles were removed to produce thick slurry. Approximately 4 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 buffer by washing five times using 5 mL each at the flow rate of 1 mL/min using respective binding buffer. Dialysed homogenate of volume 0.4 mL was loaded carefully using syringe after adjusting the pH and ionic strength of the 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[11]. Mobile phase was run by two Scigenics Model 4735 peristaltic pump, and flow rate was controlled by Honeywell DC 1040 controller (Scigenics, Chennai).

1) Setting columns for suitable pH of the binding buffer

Series of four DEAE-cellulose columns were set up and was equilibrated using binding buffer of pH 7.6, 8.0, 8.4, or 8.8 at the flow rate of 1 mL/min. To all the columns, dialysed tissue homogenates was applied while collecting the eluent. Bound materials were eluted using elution buffer of respective pH but gradient ionic strength of 0.0-0.5M NaCl in 25 min at the flow rate of 1 mL/min.

2) Setting columns for suitable ionic strength of the binding buffer

Similarly, 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 strength of 0.00, 0.05, 0.10, or 0.15 M NaCl. To all the columns, dialysed tissue homogenates was applied while collecting the eluent. Bound materials were eluted using elution buffer of respective pH but gradient ionic strength of 0.0-0.5M NaCl in 25 min at the flow rate of 1 mL/min.

3) 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, 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.

4) Setting columns for suitable flow rate of the elution buffer

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, dialysed tissue homogenates was applied while collecting the eluent. The efficient gradient slope 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.

2.6. Reagents

All the chemicals and reagents used were of analytical grade and were obtained from Merck Limited and Himedia (Mumbai, India). Solutions were prepared using chemicals and reagents according to the current American Chemical Society specifications[12]. Alkaline phosphatase assay buffer used for the present study was 2-amino 2-methyl 1-propanol (AMP) buffer of pH 10.3. Working buffer used for the recovery was 0.1 M Tris-HCl buffer of pH 8.4. Binding buffer of pH 7.6, 8.0, 8.4, and 8.8 at 25℃ 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. Elution buffer used for chromatographic process was prepared using a second series of buffers with same pH values, but including 1M NaCl in gradient during the elution. The buffers were added with $MgCl_2$ and of $ZnCl_2$ to respective final concentration of 0.1 mM. All the

binding buffer preparations were filtered and sterilized at 121℃ for 20 min.

2.7. Proximate analysis

Samples drawn at different intervals of experiment were performed in quadruplicates. The protein content was estimated as per the Folin-Ciocalteau method of Lowry and others[13], using bovine serum albumin (BSA) as a standard. Total protein content of the hepatopancreatic tissues was done by incubating 0.4 mg of tissues with 0.5 mL of 4M NaOH at 100℃ for 5 min, and the resulting homogenate was cooled and assayed for total protein by Folin-Ciocalteau method. Homogeneity of the preparation was determined in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gel was prepared according to standard protocol of Laemmli[14].

2.8. Enzyme assay

The procedure used for alkaline phosphatase analysis was based on the method of Bowers and McComb[15] using disodium *p*-nitrophenyl phosphate (*p*NPP) as a substrate using *p*-nitrophenol (*p*NP) as a standard. Alkaline phosphatase activity is expressed as 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 correc-

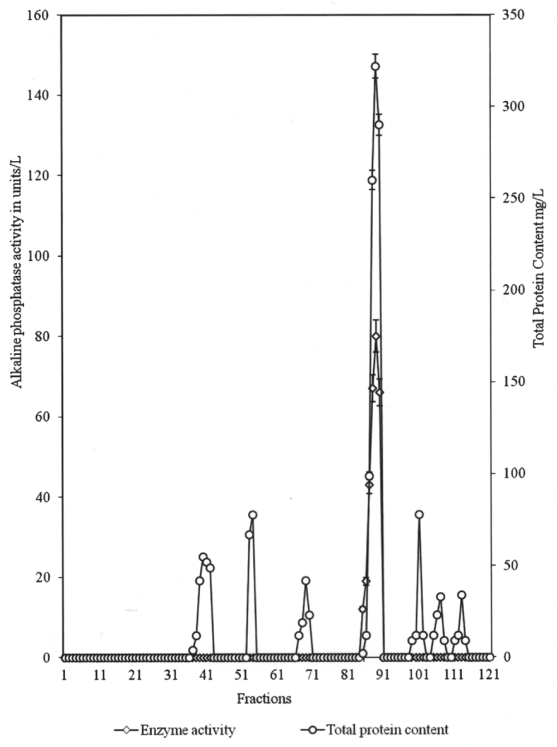

Fig. 1 Elution profile of the proteins in DEAE-cellulose column using binding buffer of pH 7.6.

Fractions collected during binding stage using binding buffers of pH 7.6 and elution stage using elution buffer of respective pH at NaCl gradient of 0.0-0.5 M in 25 min at flow rate of 1 mL/min produced 7, protein peaks. Of the total number of protein peaks, 3 protein peaks were recorded in the binding stage without any alkaline phosphatase activity and 4 protein peaks were observed in elution stage with alkaline phosphatase activity between 92-96 fractions.

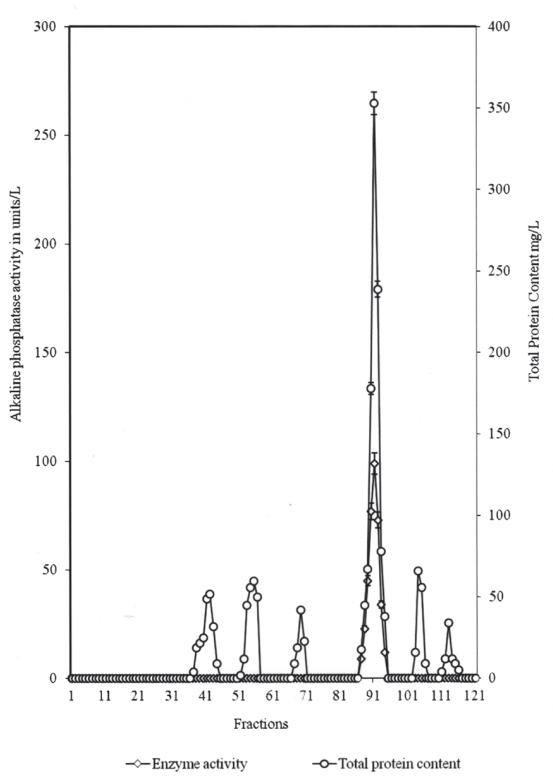

Fig. 2 Elution profile of the proteins in DEAE-cellulose column using binding buffer of pH 8.0.

In a binding buffers of pH 7.6 and elution buffer of respective pH at NaCl gradient of 0.0-0.5 M in 25 min at flow rate of 1 mL/min chromatogram showed 6 protein peaks. Here, 3 protein peaks were recorded in the binding stage without any alkaline phosphatase activity and 3 protein peaks were observed in elution stage with alkaline phosphatase activity between 94-100 fractions.

tions for the slight variation of molar absorptivity of *p*NP with pH and (or) buffer concentration.

2.9. 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 analysis were performed considering a confidence level of 95% ($p<0.05$).

3. Results

Optimum Ionic strength and pH of the binding buffer favorable for binding of the alkaline phosphatase into the DEAE, 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 gradience and flow rate of the elution buffer at which alkaline phosphatase activity first appears in the eluent was selected.

3.1. Optimising pH of the binding buffer

Of the total 7, 6, 5, and 4 protein peaks shown in the Figure 1-4, respectively, using binding buffers of

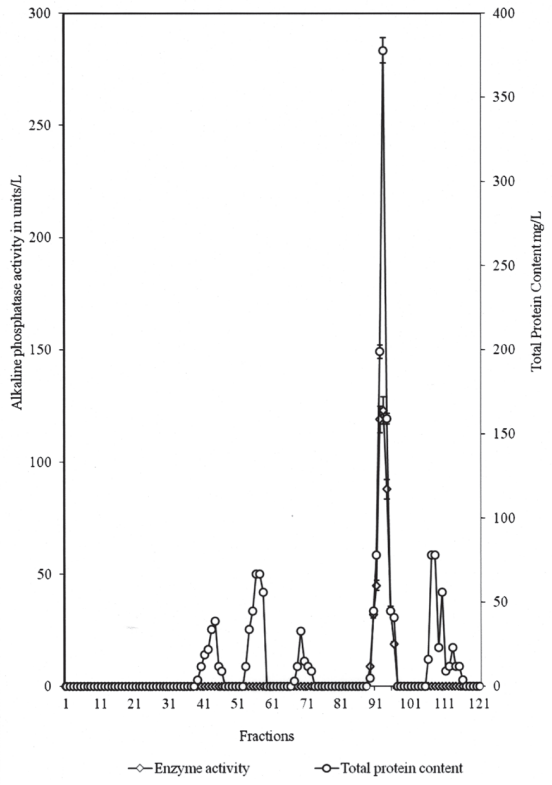

Fig.3 Elution profile of the proteins in DEAE-cellulose column using binding buffer of pH 8.4.

When the fractions collected during binding stage using binding buffers of pH 7.6 and elution stage using elution buffer of respective pH at NaCl gradient of 0.0-0.5 M in 25 min at flow rate of 1 mL/min produced 5, protein peaks. In the chromatogram, 3 protein peaks were recorded in the binding stage without any alkaline phosphatase activity and 2 protein peaks were observed in elution stage with alkaline phosphatase activity between 96-106 fractions.

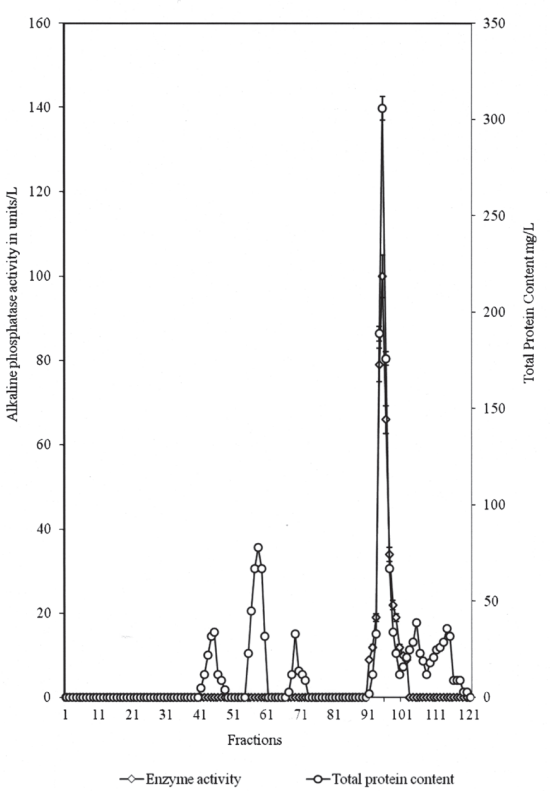

Fig. 4 Elution profile of the proteins in DEAE-cellulose column using binding buffer of pH 8.8.

During binding stage using buffers of pH 7.6 and elution stage using buffer of respective pH at NaCl gradient of 0.0-0.5 M in 25 min at flow rate of 1 mL/min produced 4, protein peaks. Of the total number of protein peaks, 3 protein peaks were recorded in the binding stage without any alkaline phosphatase activity and 2 protein peaks were observed in elution stage with alkaline phosphatase activity between 98-108 fractions.

pH 7.6, 8.0, 8.4, and 8.8, three protein peaks with no alkaline phosphatase activity were recorded in the binding stage and rest of the protein peaks were observed during elution stage. Here, 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. 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 significant 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 (Fig. 4), but at pH 8.4 trailing peak is more completely separated (Fig. 3). 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.

3.2. Optimising ionic strength of the binding buffer

Ionic strength of the binding buffers up to 0.15 M NaCl at pH 8.4 in one hand permits optimum binding of alkaline phosphatase to the DEAE-cellulose and on the other hand is not favorable for the protein impurities to bind, the view supported by the observation that shrimp homogenates lost 40.00±3.22% of the total protein with no alkaline phosphatase activity in the binding stage itself (Fig. 5). Even though 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, 0.10 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 deteriorated 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

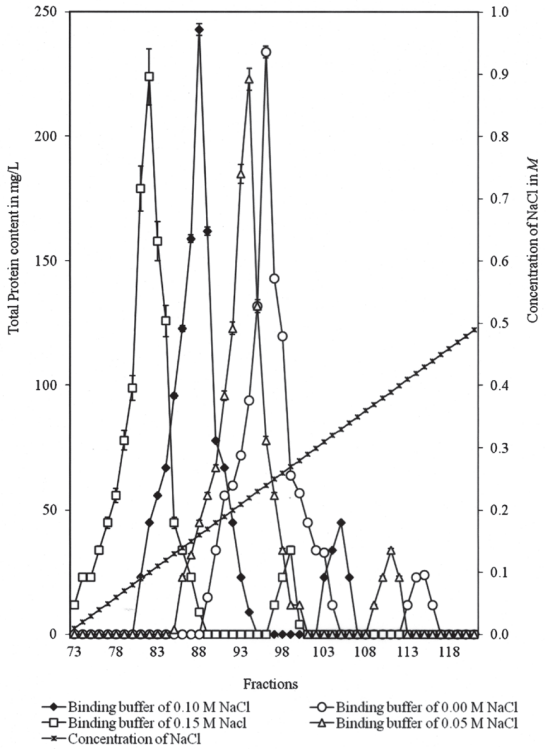

Fig. 5 Elution profile of protein in DEAE-cellulose column at various ionic strengths of binding buffer at pH 8.4.

Chromatogram obtained using binding buffer of ionic strength 0.00, 0.05, 0.10 or 0.15 M NaCl when eluted using the elution buffers of linear ionic gradient from respective initial ionic strength to 0.5 M NaCl in 25 min at the flow rate of 1 mL/min, respectively, showed alkaline phosphatase activity in between 94-104, 90-100, 86-94 and 78-88 fractions. During this period, the time required for the appearance of the first protein fraction reduced by 4, 4, and 8 fractions, respectively, as the molar concentration of the NaCl in the binding buffer increased from 0.00 M NaCl to 0.05 M NaCl, from 0.05 M NaCl to 0.10 M NaCl, and from 0.10 M NaCl to 0.15 M NaCl.

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.

3.3. 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 1mL/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 (Fig. 6). 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

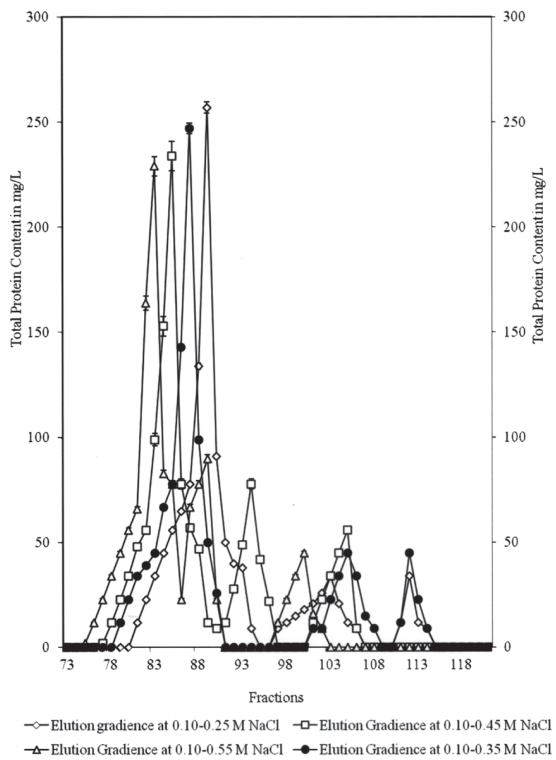

Fig. 6 Elution profile of the proteins in DEAE-cellulose column at various gradient slope in 25 min.

Of the total peaks obtained at linear gradient slope of 0.10-0.25, 0.10-0.35, 0.10-0.45 or 0.10-0.55 M NaCl in 25 min A major protein peak was showing alkaline phosphatase activity 84-94, 82-91, 80-88 and 77-86 fractions. Increasing the gradient slope, in one hand decreases the number of peaks and on the other hand reduces the time required for the first protein peak to appear during elution stage.

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 were 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. 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. 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.

3.4. 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 (Fig. 7). Subsequent increase in 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 (Fig. 8). For eluted protein, increasing the flow rate for a given linear salt gradient 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. 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.

Recovery details of alkaline phosphatase from the hepatopancreatic tissues from the hepatopancreas of Red shrimp are summarized in the Table 1. Even though volume of the samples during the chromatographic separation was increased by around three folds in comparison to the volume of the respective shrimp homogenates loaded to the column, during the entire recovery process volume of the homogenate reduced to less than two third and specific activity was increased by 19 folds while retaining more than half of alkaline phosphatase activity in comparison to the enzyme in the initial tissue 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.

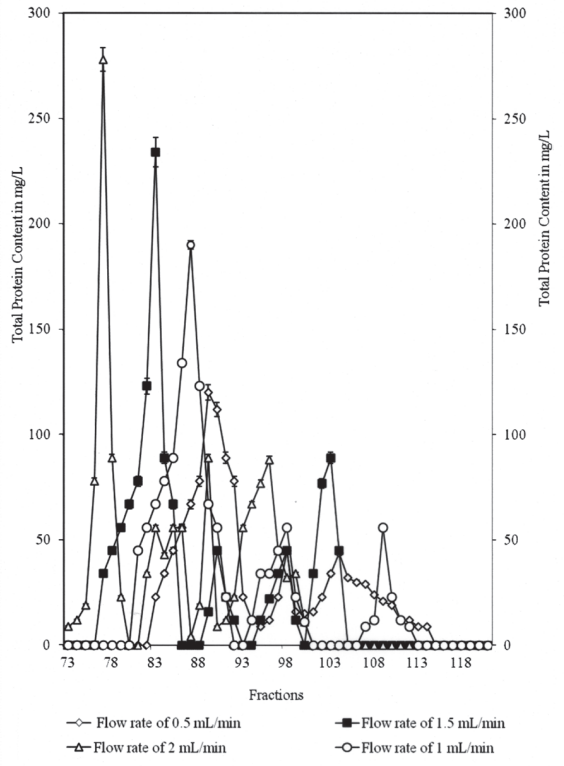

Fig. 7 Elution profile of the proteins in DEAE-cellulose column at various flow rate.

Excluding at very low flow rate, increase in the flow rate of the elution buffer increased the resolution in anion exchange chromatography. However, elution buffer at the flow rate of above 1.5 mL/min produced a major protein peak of alkaline phosphatase that was shouldering the minor peaks of no alkaline phosphatase activity in the elution stage. Binding buffer at a flow rate of 1 mL/min was able to elute unbound proteins as clearly separated protein peaks in chromatogram.

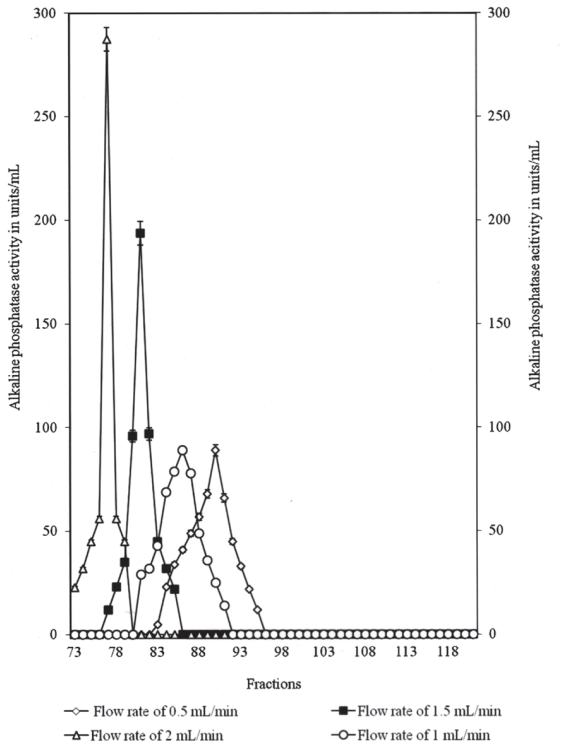

Fig. 8 Elution profile of the proteins with alkaline phosphatase activity in DEAE-cellulose column at various flow rate.

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 activity. Alkaline phosphatase was resolved optimally from other impurities that were confirmed in the chromatogram showing clearly separated single major peaks with alkaline phosphatase from other three minor peaks without any activity.

Table 1 Changes in total volume, specific activity, and yield of the sample during homogenisation, centrifugation, precipitation, reconstitution and dialysis of the clarified tissue homogenate to recover alkaline phosphatase.

	Volume		Specific activity		Yield	
Purification stage	Volume in mL	Change in %	Specific activity in units/mL	Change in fold	Step yield %	Process yield %
Tissue sample g	100.00 ±0.72	1.00 ±0.07				
Homogenisation 3000 rpm/10 min	1069.00±1.22	1069.00±1.22	0.031±0.01	1.00±0.01	100.00	100.00
Centrifugation RCF 1681.1 x g	818.53 ±1.45	76.57±0.01	0.129±0.01	4.14±0.02	94.29±0.09	94.29±0.09
Precipitation 65% saturation	210.57±1.23	25.72±0.01	0.235±0.01	7.55±0.01	98.25±0.06	92.63±0.04
Dialysis for 24 h	396.19±1.54	188.15±0.02	0.238±0.01	7.58±0.02	99.00±0.06	92.86±0.04
DEAE-cellulose Chromatography	2456.35±1.32	620.00±0.01	0.594±0.03	19.05±0.01	55.09±0.56	51.01±0.02

Data presented in the table is the mean of quadruplet readings i.e. Mean ± S.D.

4. Discussion

4.1. Optimum pH and ionic strength of the binding buffer

Alkaline phosphatase activity was registered only in one of the protein peaks of the elution stage but not in any of the peaks eluted in the binding stage using buffer of pH 7.6, 8.0, 8.4, or 8.8. The finding supports the view that alkaline phosphatase bound optimally to the DEAE at this pH and elution profile of the alkaline phosphatase was unaffected by the variation in the pH of the buffer in this range. Previous report suggest that DEAE is a weak ion exchange resin that is positively charged below pH 8.5 (pKa~10.0) and 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[5]. However, reducing the pH of the binding buffer increases the resolution of the alkaline phosphatase from other protein impurities, and also reduces the time gap to elute alkaline phosphatase from the column. The present study shows that elution buffer of pH 8.4 resolves alkaline phosphatase optimally with maximum cumulative alkaline phosphatase activity in comparison of binding buffers of other pH levels. This is supported by the view that working buffer of pH 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[16]. Carefully selected and designed chromatographic conditions can purify alkaline phosphatase to near homogeneity in DEAE-cellulose columns[17].

Eventhough, increase in the ionic strength of the binding buffer to 0.15 M NaCl reduces the time gap for the elution of the alkaline phosphatase from the column without affecting the elution profile of the protein peaks, ionic strength of 0.15 M NaCl was not able to resolve alkaline phosphatase from the protein impurities eluted during the binding stage. Binding buffer with the ionic strength of 0.1 M NaCl at pH 8.4 allows alkaline phosphatase to bind optimally to the DEAE, but as close to the point of release as possible from the resins when eluted using elution buffers of ionic gradience, while removing nearly half of protein impurities in the binding stage itself[18]. At a given condition, both ionic strength and pH of the binding buffer is critical in order to achieve the most effective resolution of the alkaline phosphatase from other protein impurities, because efficient binding behavior of proteins to ion exchange resins is dependent on various factors such as net charge, surface charge distribution, protein hydrophobicity, van der Waals interactions and choice of the adsorbent materials,

that are in turn influenced by pH and ionic strength of the buffer[19].

4.2. Optimising gradient slope and flow rate of the elution buffer

Eventhough ionic gradience of 0.10-0.25 M NaCl and 0.10-0.35 M NaCl in 25 min resolves alkaline phosphatase from other protein impurities, ionic gradience of 0.10-0.35 M NaCl in 25 min efficiently elutes bound proteins that is supported by narrow and symmetrical major protein peak with alkaline phosphatase activity well isolated from protein peaks with no alkaline phosphatase activity. This observation is supported by the report that at a given pH, 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[19]. In our study, increase in the ionic gradience of the elution buffer decreases the resolution of the major protein peak and reduces the time gap to elute alkaline phosphatase from the column. This observation is supported by the previous work stating that 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[19].

One more important parameter in the chromatographic purification is the flow rate of the elution buffer as increase in flow rate from 0.5 mL/min to 2.0 mL/min increases the resolution of alkaline phosphatase from other protein impurities and reduces resolution time. Here, excluding at very low flow rate, increase in flow rate increases the resolution in anion exchange chromatography that is supported by the previous finding that flow rate has a direct bearing on the resolution of the fractions in the chromatogram[20]. In this study, elution buffer at a flow rate of 1.5 mL/min at ionic gradience of 0.10-0.35 M NaCl in 25 min is efficiently elutes alkaline phosphatase shown in the chromatogram showing clearly isolated single major peak with alkaline phosphatase from other three other minor peaks without any alkaline phosphatase activity.

Binding buffer of pH 8.4 and ionic strength 0.1 M NaCl at flow rate of 1 mL/min, 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 efficiently binds alkaline phosphatase to DEAE during stage and elutes alkaline phosphatase from the column during the elution stage. This is supported by the finding that the protein impurities were reduced by half, specific activity was increased by 14 folds and alkaline phosphatase activity was retained by more than half of the enzyme activity in comparison to the initial tissue homogenate. Efficiency of these parameters was also supported by the SDS-PAGE analysis 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 have 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.

5. 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 are present in the eluent.

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