Abstract
Alkaline phosphatase was recovered from hepatopancreas of tiny shrimp using homogenizer at 3,000 rpm for 10 min at 4°C, centrifugate at RCF of 1681.1 x g for 5 min at 4°C, and precipitation at 65% ammonium sulfate saturation level at 0°C. Purification parameters of alkaline phosphate was optimized in DEAE-cellulose columns was done 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 optimally bound 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 eluted the enzyme with optimum resolution.

Key words: Alkaline phosphatase, Tiny shrimp, anion exchange chromatography, enzyme purification, DEAE-cellulose.

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1. Introduction
Enzymes unique physico-chemical properties adapted to various environmental conditions can be recovered from the hepatopancreas of shrimps as this organ is a very good source of commercially important enzymes such as alkaline phosphatase that can be commercially used as replacement enzymes in nutritional disorders (Lee and Chuang, 1991; Nilson et al., 2001). Impurities present in the pancreas are the bottle neck during the purification of alkaline phosphatase for its commercial exploitation because this is an important organ functions in enzymes secretion, food absorption, transport and storage of lipids, glycojen and minerals (Chuang and Yang, 1990). Purification of alkaline phosphatase from other protein impurities of the hepatopancreas requires an important strategy that involves careful selection of chromatographic parameters to exploit their physico-chemical properties by discriminating their surface charges based on their type, density and distribution that in turn affects their degree of interaction on charged chromatography media (Ward and Swiatek, 2009). Charged group within the protein molecule that contributes to the net surface charges possess different pKa values depending on their structure and environment. DEAE-cellulose is one of the frequently used purification techniques of alkaline phosphatase from other impurities with high resolution, 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 (Scott, 2012). Parameters of DEAE-cellulose chromatography such as pH and ionic strength of the binding buffer, and gradient slope and flow rate of the elution buffer can be controlled in order to favor binding or elution of specific molecules and achieve separation of alkaline phosphatase from other protein impurities (Mao and Hearn, 1996). Lots of research was carried out to purify alkaline phosphatase from different sources using DEAE-cellulose chromatography (Junior et al., 2008; Cho-Ngwa, 2007). However, in one hand number and types of protein impurities varies from one enzyme source to other source, and on the other hand no single set of chromatographic operating conditions were standardized in terms of the pH and ionic strength of the binding buffer, and gradient slope and flow rate of the elution buffer to isolate alkaline phosphatase from hepatopancreatic tissue homogenate of Tiny shrimp (Parapeneaopsis stylifera). Present study was carried out to determine the effect of pH and ionic strength of the binding buffer, and gradient slope and flow rate of the elution buffer in the DEAE-cellulose columns to optimize the purification of alkaline phosphatase from other protein impurities.

2. Materials and Methods
2.1 Chemical and 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 (1999). 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°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. 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. The buffers were added with MgCl₂ and of ZnCl₂ to respective final concentration of 0.1 mM. All the binding buffer preparations were filtered and sterilized at 121°C for 20 min.

2.2 Sample collection

Indian white shrimp (*Peneaus indicus*) 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 subsequent experiment. Resins were allowed to settle, and excess buffer was aspirated 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. The column was equilibrated to respective pH at the rate of 1 mL/min, until no protein appear in the eluent (Yeh et al., 1989). Mobile phase was run by two Scigenics Model 4735 peristaltic pump, and flow rate was controlled by Honeywell DC 1040 controller.

2.6 Ion exchange chromatography

A series of four chromatographic columns of size 8x80 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 m eq/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 aspirated 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. The column was equilibrated to respective pH at the rate of 1 mL/min, until no protein appear in the eluent (Yeh et al., 1989). Mobile phase was run by two Scigenics Model 4735 peristaltic pump, and flow rate was controlled by Honeywell DC 1040 controller.

2.6.1 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 0.0-0.5 M NaCl in 25 min at the flow rate of 1 mL/min.

2.6.2 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 0.0-0.5 M NaCl in 25 min at the flow rate of 1 mL/min.

2.6.3 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.

2.6.4 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.

2.7 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 et al. (1951), 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°C 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 (1970).

2.8 Enzyme assay
The procedure used for alkaline phosphatase analysis was based on the method of Bowers and McComb (1975) using disodium paranitrophenyl phosphate (pNPP) as a substrate using p-nitrophenol (pNP) as a standard. Alkaline phosphatase activity is expressed as units/L, is the liberation of 1 mM of pNP 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 pNP 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 and discussion
The most suitable pH and ionic strength of the binding buffer conducive for optimum binding of the hepatopancreatic protein with alkaline phosphatase activity 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.

![Fig. 1 Elution profile of the proteins in DEAE-Cellulose column using binding buffer of pH 7.6.](image)
Fig. 2 Elution profile of the proteins in DEAE-Cellulose column using binding buffer of pH 8.0.

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

Of the total 8, 7, 6, and 5 protein peaks collected, respectively, using buffers of pH 7.6, 8.0, 8.4, and 8.8, four protein peaks with no alkaline phosphatase were recorded in the binding stage and rest of the protein peaks were observed during elution stage (Figure 1-4). 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 four protein peaks eluted during the binding stage did not show any alkaline phosphatase activity, and only a protein peak of alkaline phosphatase was seen in the elution stage along with other minor peaks of protein with alkaline phosphatase activity. 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 (Scott, 2012). 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 (Ahamed et al., 2008). Alkaline phosphatase can be purified to near homogeneity using DEAE-Cellulose chromatography (Wilson and Walker, 1995).

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 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 38.42±1.34% of the total proteins of no alkaline phosphatase activity in the binding stage itself (Figure 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, 0.01 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.
Fig. 5 Elution profile of protein in DEAE-Cellulose column at various ionic strengths of binding buffer at pH 8.4

Fig. 6 Elution profile of the proteins in DEAE-Cellulose column at various gradient slope in 25 min.
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 (Burgess, 2008). 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 in turn influenced by pH and ionic strength of the buffer (Jansen, 2011).

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 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 6). Here, gradience 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 (Jansen, 2011).

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 (Jansen, 2011). 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.

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

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Flow rate of 0.5 mL/min  
Flow rate of 1 mL/min  
Flow rate of 2 mL/min  
Flow rate of 1.5 mL/min
### 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 (Figure 7). 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 (Fig. 8).

![Fig. 8](image)

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

**Table 3.1** 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.

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Volume in mL</th>
<th>Change in %</th>
<th>Specific activity in units/mL</th>
<th>Change in fold</th>
<th>Step yield %</th>
<th>Process yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue sample g</td>
<td>100.00±0.91</td>
<td>1.00 ±0.09</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Homogenisation 3000rpm/10 min</td>
<td>1058.00±0.45</td>
<td>1050.00±0.99</td>
<td>0.048±0.01</td>
<td>1.00±0.01</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Centrifugation RCF 1681.1×g</td>
<td>803.97±1.93</td>
<td>75.99±0.019</td>
<td>0.140±0.01</td>
<td>2.94±0.02</td>
<td>92.31±0.08</td>
<td>92.31±0.08</td>
</tr>
<tr>
<td>Precipitation 65% saturation</td>
<td>210.57±1.33</td>
<td>26.19±0.01</td>
<td>0.208±0.02</td>
<td>4.37±0.04</td>
<td>92.16±0.06</td>
<td>85.08±0.05</td>
</tr>
<tr>
<td>Dialysis For 24 h</td>
<td>378.61±1.23</td>
<td>179.81±0.01</td>
<td>0.207±0.01</td>
<td>4.36±0.02</td>
<td>99.55±0.45</td>
<td>85.09±0.07</td>
</tr>
<tr>
<td>DEAE-Cellulose Chromatography</td>
<td>2309.57±1.13</td>
<td>610.00±0.01</td>
<td>0.723±0.01</td>
<td>15.13±0.03</td>
<td>55.67±0.09</td>
<td>47.36±0.03</td>
</tr>
</tbody>
</table>

Data presented in the table is the mean of quadruplet readings i.e. Mean ± S.D.
flow rate, increase in flow rate increases the resolution in anion exchange chromatography (Hjertén et al., 1988). 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 (Table 3.1). 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.

3. 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.

Reference


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