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Optimization of Chromatographic Criterion to Recover Indian White Shrimp (*Fenneropenaeus indicus*) Alkaline Phosphatase from Hepatopancreas

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Author's contribution

The study was designed, analyzed and discussed by the author. The author takes full responsibility for the whole study including data collation, manuscript drafting and editing.

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ABSTRACT

Commercially important alkaline phosphatase can be recovered from the hepatopancreatic tissue of Indian white shrimp with optimum yield, activity, and purity by carefully designing the criterions of ion exchange chromatographic purification. The tissues were homogenized, clarified, and concentrated. Concentrated homogenate was purified with optimum yield, activity and purity by optimising criterions of chromatography such as pH and ionic strength of the binding buffer, and ionic gradient and the flow rate of the elution buffer in DEAE-cellulose column. The enzyme was optimally bound to the column at pH 8.4 and ionic strength 0.1 *M* NaCl at flow rate of 1mL/min, and eluted with high resolution at ionic gradient of 0.10-0.35 *M* NaCl in 25 min at a flow rate of 1.5mL/min, and hence is the optimum criterions of chromatographic purification.

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1. INTRODUCTION

Hepatopancreatic alkaline phosphatase isolated from shrimps caught at different geographical regions exhibits unique physicochemical characteristics due to its adaptation to diverse oceanic conditions [1-3]. Hepatopancreas is a very good source of proteins and other biopolymers of similar physicochemical characteristics, because it plays as vital organ for secretion of enzymes, absorption of food, and transport and storage of lipids, glycogen and minerals. Such components released from the hepatopancreas may interfere during the subsequent purification process or may retain with the recovered fraction making the process much more complicated [4,5]. Careful exploitation of the physicochemical properties of the protein of interest and impurities of similar physicochemical properties such as type and number of surface charge through careful selection of criterion of chromatography clears the bottleneck of recovery [6,7]. Proteins with unique pKa values possess net surface charge due to the peripheral amino terminal residues that in turn change with the environment. Proteins with slight variation in their pKa values can also be separated from one another by using ion exchange chromatography when criterions of chromatography are carefully selected. Alkaline phosphatase which is negatively charged under biological condition charged because of its pKa value can bind to weak ion exchangers such as DEAE-cellulose that is positively charged under suitable environmental condition [8-10]. Criterions of chromatography such as pH and ionic strength of the binding buffer, and ionic gradient and flow rate of the elution buffer can be carefully set in order to favor binding or elution of specific molecules and achieve separation of protein of interest from other protein impurities [11,12]. Alkaline phosphatase was isolated from different sources using DEAE-cellulose chromatography by various workers [5,13-16]. However, physicochemical properties of the components isolated from various source vary, for which no set of standardized chromatographic criterion is available. The aim of the present study is to optimise the criterions of chromatographic purification such as the pH and ionic strength of the binding buffer, and ionic gradient and flow rate of the elution buffer in the DEAE-cellulose columns in such as way that alkaline phosphatase is isolated from the hepatopancreas of Indian white shrimp (*Fenneropenaeus indicus*) with optimum yield, activity and purity.

2. MATERIALS AND METHADOLOGY

2.1 Isolation of Alkaline Phosphatase

Indian white shrimp (*Fenneropenaeus indicus*) caught near Mangalore, Karnataka (12º2' N, 74º53' E, FAO fishing Area, 51, Indian Ocean Western) between the months of July and December were transported to the laboratory at 4ºC within four to six hours and identified [17]. Samples belonging in the size group of 86-120 mm in length, and weighing around 30-55g were washed and dissected to remove the hepatopancreatic tissues. The tissues were weighed and homogenized (Potter-Elvehjem RH-2 Homogenizer, Rotek Instruments, Kerala, India) at pestle speed of 3,000rpm for 10 min at 4ºC using 0.1 *M* Tris-HCl buffer of pH 8.4 at 1:10 tissue to buffer ratio [18]. The homogenate were clarified at relative centrifugal force (RCF) of 1681.1x*g* for 5 min at 4ºC (C-24BL/CRP24 model centrifuge, Remi Laboratory Instruments, Mumbai, India) [19]. The clarified homogenate were subjected to ammonium sulfate precipitation at 65% saturation level at 0ºC and centrifuged at 15,124.8x*g* for 30 min at 0ºC [20]. 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, and dialyzed using 10 kDa cellophane tubes (Himedia, Mumbai, India) for 24 h[21].

2.2 Purification of Alkaline Phosphatase

DEAE-cellulose (Himedia, Mumbai, India) columns (8 mm internal diameter × 80 mm height) were connected to two Scigenics Model 4735 peristaltic pump through Honeywell DC 1040 flow controller (Scigenics, Chennai) [8,11,22]. Suitable pH of the binding buffer required for optimum binding of the alkaline phosphatase to the column was selected by equilibrating the columns using binding buffer of pH 7.6, 8.0, 8.4, or 8.8 at the flow rate of 1mL/min and observing the flow profile of the protein during biding stage and elution stage at each pH level. Here, elution buffer was run at the flow rate of 1mL/min for 48 min using buffer of respective pH at gradient of 0.0-0.5*M* NaCl. Similarly, suitable ionic strength of the binding buffer required for optimum binding of the enzyme to the column was selected by equilibrating the columns 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 at the flow rate of 1 mL/min and observing the flow profile of the protein during biding stage and elution stage at each ionic strength. Here, elution buffer was run at the flow rate of 1mL/min using the elution buffer but at salt gradient of 0.00-0.50, 0.05-0.53, 0.10-0.55, or 0.15-0.58*M* NaCl, respectively. Suitable ionic strength of the elution buffer required for optimum resolution of the protein peaks were selected amongst the columns equilibrated with binding buffer of pH and ionic strength proven to be optimum in the previous two experiments, but eluted at NaCl gradient of 0.10-0.25, 0.10-0.35, 0.10-0.45 or 0.10-0.55 *M* at flow rate of 1 mL/min. Similarly, suitable flow rate of the elution buffer required for earliest release of the protein peak of alkaline phosphatase from the column were selected amongst the columns equilibrated with binding buffer of pH and ionic proven to be optimum in the previous two experiments, but elution buffer of salt gradient proven to be suitable was run at flow rate of 0.5, 1.0, 1.5, or 2.0mL/min.

2.3 Analysis of the Samples

All the chemicals and reagents (Merck Limited and Himedia Mumbai, India) used were of analytical grade and solutions were prepared using chemicals and reagents according to the current American Chemical Society specifications [23]. Hepatopancreatic samples were collected at different intervals at each unit steps of time as indicated in the text. Each experiment was performed in quadruplicates. The protein content of the samples collected at various stages of the samples were estimated as per the Folin-Ciocalteau method using bovine serum albumin (BSA) as a standard as per Lowry and others [24]. Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) was used to determined homogeneity of the preparation as per the standard protocol of Laemmli [25]. Alkaline phosphatase activity was assayed using disodium paranitrophenyl phosphate (*p*NPP) as a substrate and *p*-nitrophenol (*p*NP) as a standard as per the protocols of Bowers and McComb [26].

2.4 Statistical Analysis

The analysis of alkaline phosphatase 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

Suitable pH and ionic strength of the binding buffer is selected based on its efficiency in binding alkaline phosphatase in the column, which is indicated by elution of the protein fractions without alkaline phosphatase activity in this stage. Similarly, suitable salt gradience and flow rate of the elution buffer was determined based on its efficiency in isolating peaks of alkaline phosphatase from other protein impurities and reducing release of bound proteins from the column.

3.1 Effect of the pH of the Binding Buffer

Chromatogram clearly shows total of 7, 6, 5, and 4 protein peaks when the pH of the buffer was 7.6, 8.0, 8.4, and 8.8, respectively (Fig. 1).

Fractions collected using buffers of different pH showed three protein peaks without any alkaline phosphatase in the binding stage. Variation in the pH of the buffer did not have any effect on the elution pattern of the proteins in the binding stage. Fractions collected during the binding stage shows a major protein peaks of alkaline phosphatase in all the cases. Resolution of alkaline phosphatase from other protein impurities improved with the reducing of pH of the buffer from 8.8 to 7.6 and remained at a significant level of 5%. However, reducing the pH of the buffer reduces the time required for the first appearance of the protein fractions with alkaline phosphatase activity. Here, when a binding buffer of pH 8.8 was used, protein peak of alkaline phosphatase activity was merging with a protein peak without any alkaline phosphatase activity, but at pH 8.4 these two protein peaks were well isolated. Elution buffer of pH 8.4 resolves alkaline phosphatase from other protein impurities with maximum cumulative enzyme activity in comparison to the peaks obtained at other pH levels. Hence, for subsequent work pH of the working buffer was maintained at 8.4.

3.2 Effect of the Ionic Strength of the Binding Buffer

Binding buffers of ionic strength 0.00, 0.05, 0.01 and 0.15 *M* NaCl did not significantly (p>0.05) affect the elution profile of the proteins in the binding stage (Fig. 2). However, the binding buffers ionic strength 0.00, 0.05, 0.01 and 0.15 *M* NaCl eluted, respectively, at salt gradient of 0.00-0.50, 0.05-0.53, 0.10-0.55, or 0.15-0.58 *M* NaCl significantly (p<0.05) effected the elution profile during the elution stage. During the elution stage, time required for the appearance of the first major peak of alkaline phosphatase activity significantly (p<0.05) decreased with increase in the ionic strength of the binding buffer. However, resolution of the alkaline phosphatase from other protein impurities significantly (p<0.05) gets affected at 0.15 *M* NaCl, where last protein peak of the binding stage was merging with the major peak of alkaline phosphatase activity. Binding buffer with 0.1 *M* NaCl is suitable ionic strength of the binding buffer at pH 8.4 because shrimp homogenates lost 42.41±1.34% of the other protein impurities at binding stage itself favourably effecting the lag time and resolution of the protein peaks of the elution stage. Hence, for the entire subsequent work binding buffer with 0.1 *M* NaCl at pH 8.4 was used.

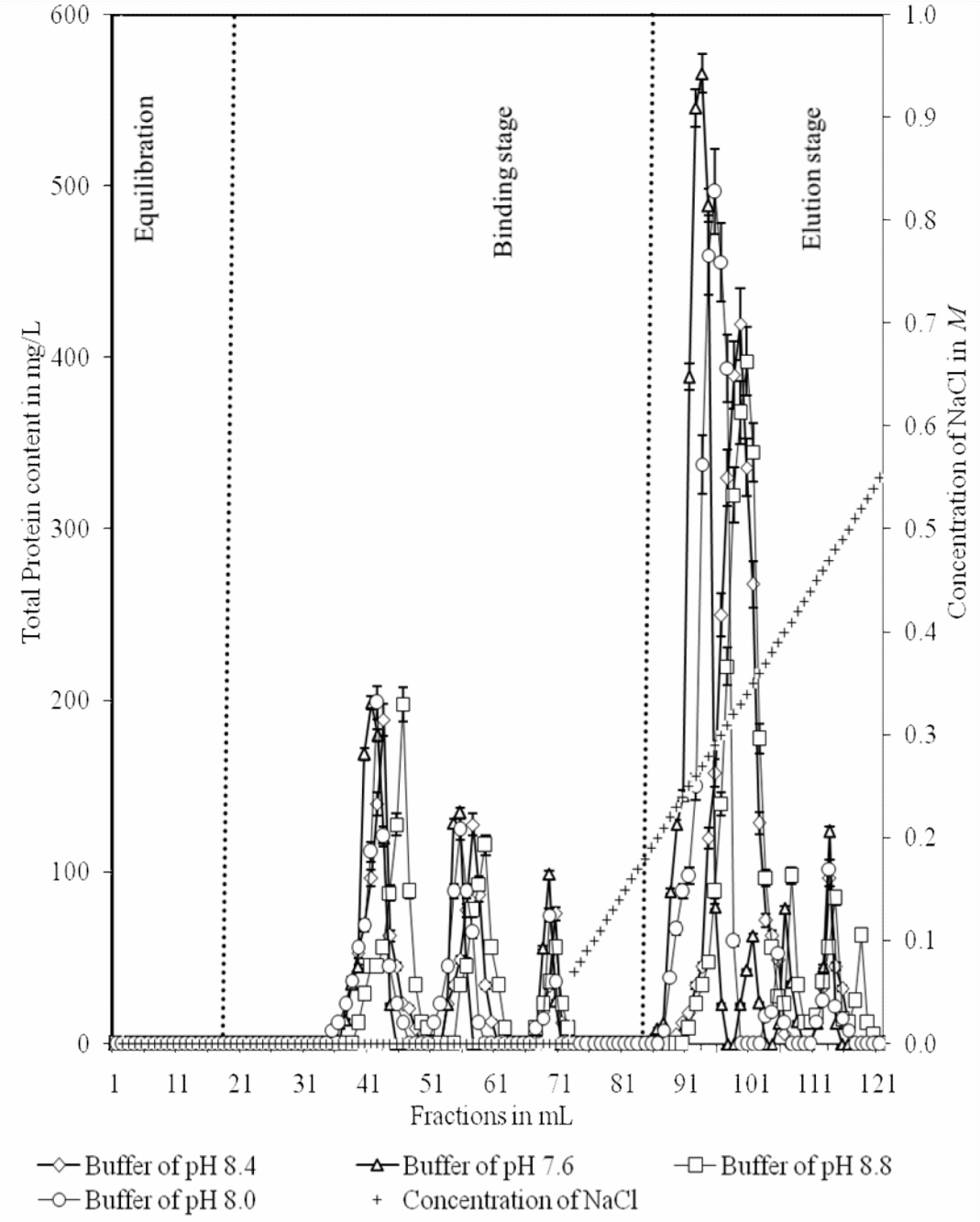

Fig. 1 Elution profile of the Indian white shrimp protein in DEAE-Cellulose column using binding buffers of different pH.

3.3 Effect of Salt Gradience of the Elution Buffer

Elution buffer of salt gradient of neither 0.10-0.55 *M* NaCl nor 0.10-0.45*M* NaCl was able to clearly resolve a major protein peak of alkaline phosphatase from a neighboring two peaks of protein impurities (Fig. 3). Elution buffer at salt gradient of 0.10-0.25*M* NaCl and 0.10-0.35 *M* NaCl was able to clearly resolve alkaline phosphatase peak from two protein impurities. Increasing the salt gradient of the elution buffer deteriorates the resolution of the alkaline phosphatase from other protein impurities and improves the lag time to elute bound alkaline phosphatase during elution stage. However, in any case protein peaks of the binding stages did not merge with the protein peaks of elution stage. Elution buffer at gradient slope of 0.10-0.35 *M* NaCl was able to produce well isolated, narrow and symmetrical major protein peak with alkaline phosphatase activity in the elution stage. Hence for subsequent work gradient slope of 0.10-0.35 *M* NaCl was used.

3.4 Effect of Flow Rate of the Elution Buffer

Elution buffer eluted at 0.5 mL/min was not able to release the alkaline phosphatase optimally as at this rate protein a peak of alkaline phosphatase activity were merging with the two peaks of protein impurities (Fig. 4). Elution buffer run at 1.0 mL/min produced a sharp major peak clearly isolated from its neighboring minor peak. Elution buffer at a flow rate of 1.5 mL/min was able to clearly resolve alkaline phosphatase from other three minor peaks of protein impurities (Fig. 5). During the elution stage, effect of increasing the flow rate of the elution buffer at ionic gradient 0.10-0.35 *M* NaCl on reducing the lag time of the appearance of the protein peak with alkaline phosphatase activity was performed and significant effect as indicated by low *p*-value was recorded. Excluding at very low flow rate such as 0.5 mL/min, increase in flow rate increases the resolution in anion exchange chromatography. However, elution buffer above flow rate of 1.5mL/min was not able to resolve alkaline phosphatase from other protein impurities.

Changes in the volume, specific activity and yield of alkaline phosphatase from the hepatopancreatic tissues of Indian white shrimp are summarized in the Table 1. Volume of the dialyzed homogenate loaded to the DEAE-Cellulose column increased by around three folds. However, during the entire recovery process volume of the homogenate reduce by nearly half. Specific activity of the homogenate was increased by 14 folds during the entire process of enzyme recovery. During the chromatography specific activity was increased nearly by three folds. Entire recovery process is able to recover more than half of the alkaline phosphatase from the homogenate, and chromatographic at given criterion is able to retain about 60% of the alkaline phosphatase. Pooled protein fractions of alkaline phosphatase was analyzed using SDS-PAGE showed the presence of a major band and no minor bands were observed indicating homogeneity (Fig. 6).

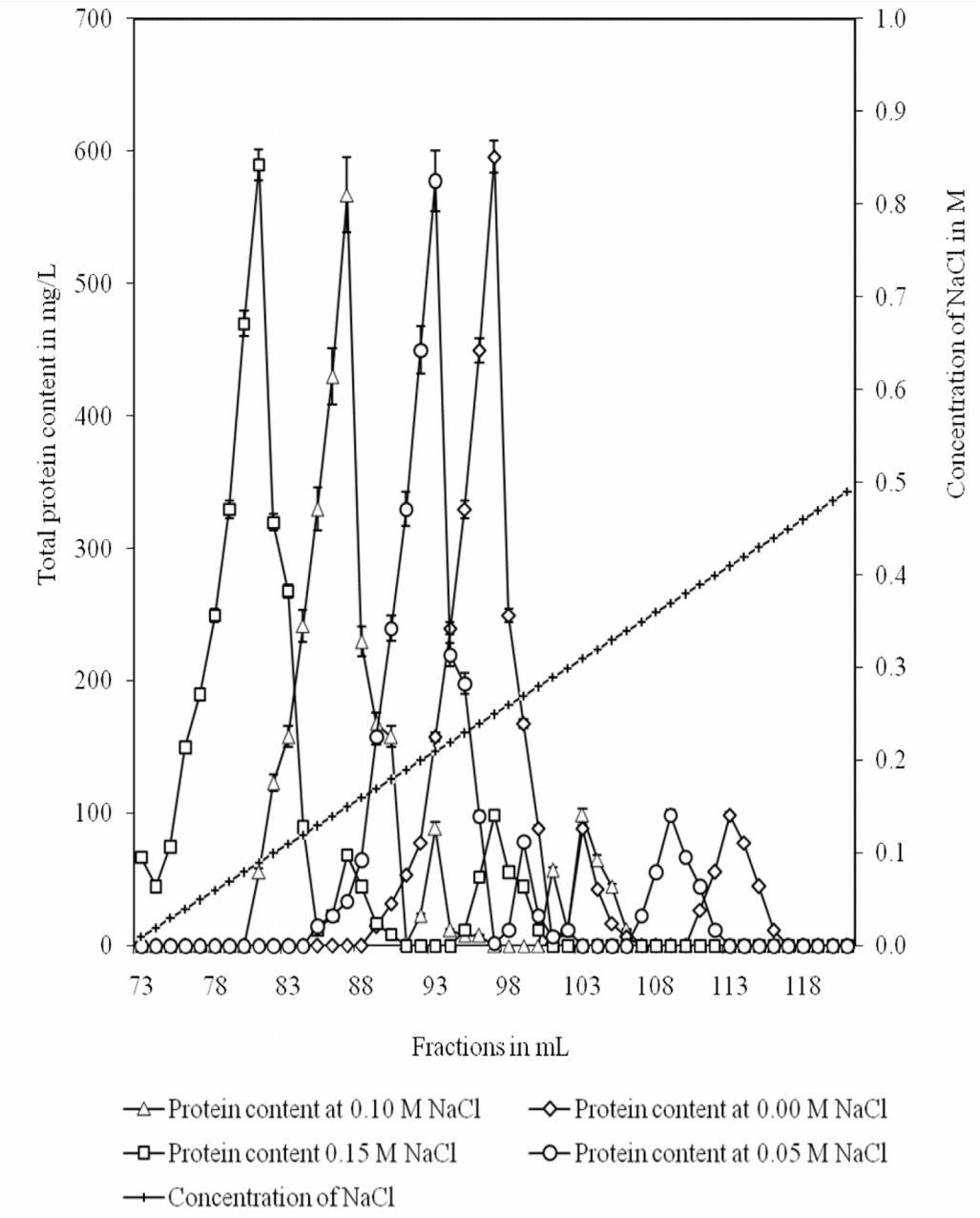

Fig. 2 Elution profile of protein in DEAE-Cellulose column at various ionic strengths of binding buffer at pH 8.4

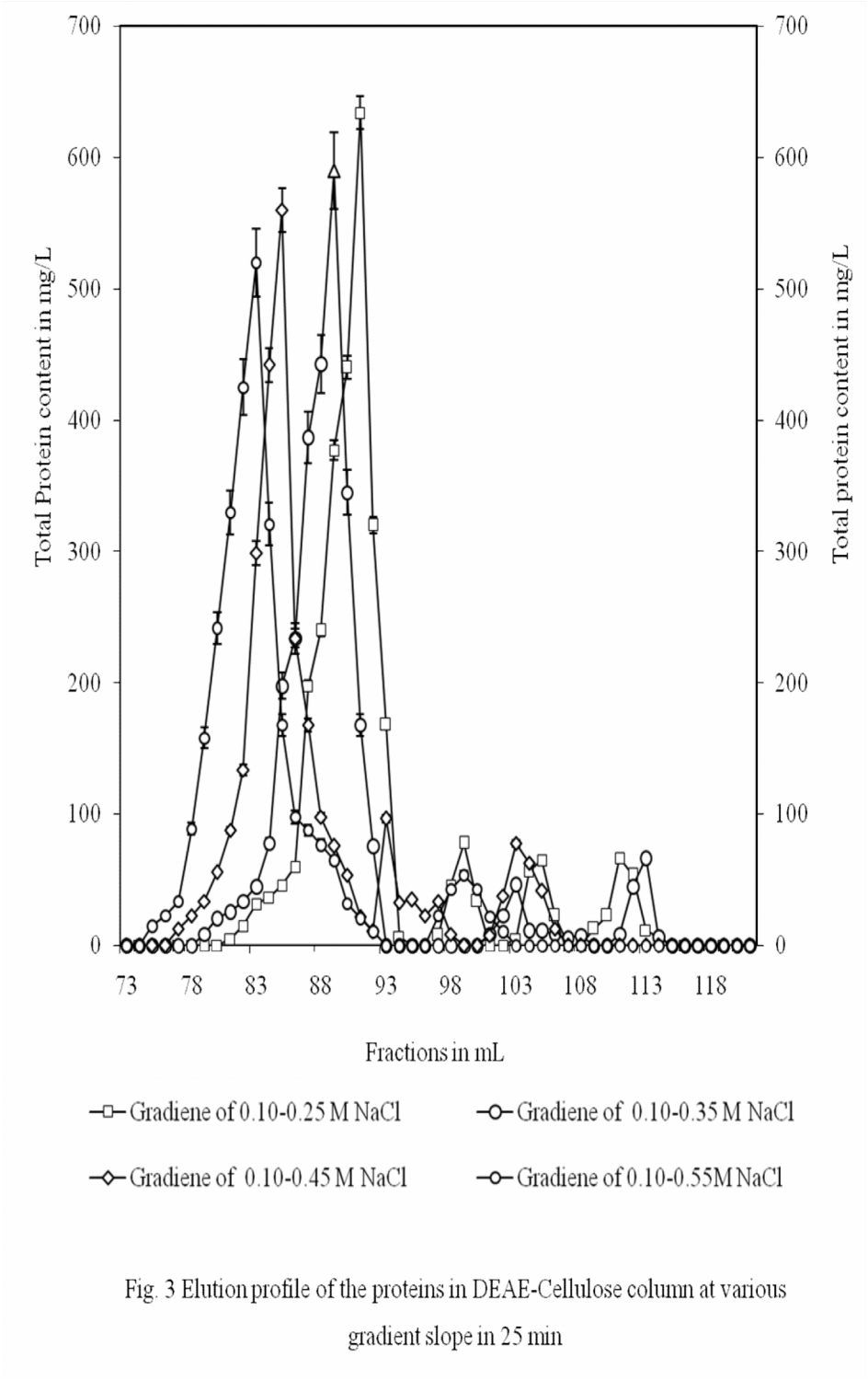

Fig. 3 Elution profile of the proteins in DEAE-Cellulose column at various gradient slope in 25 min

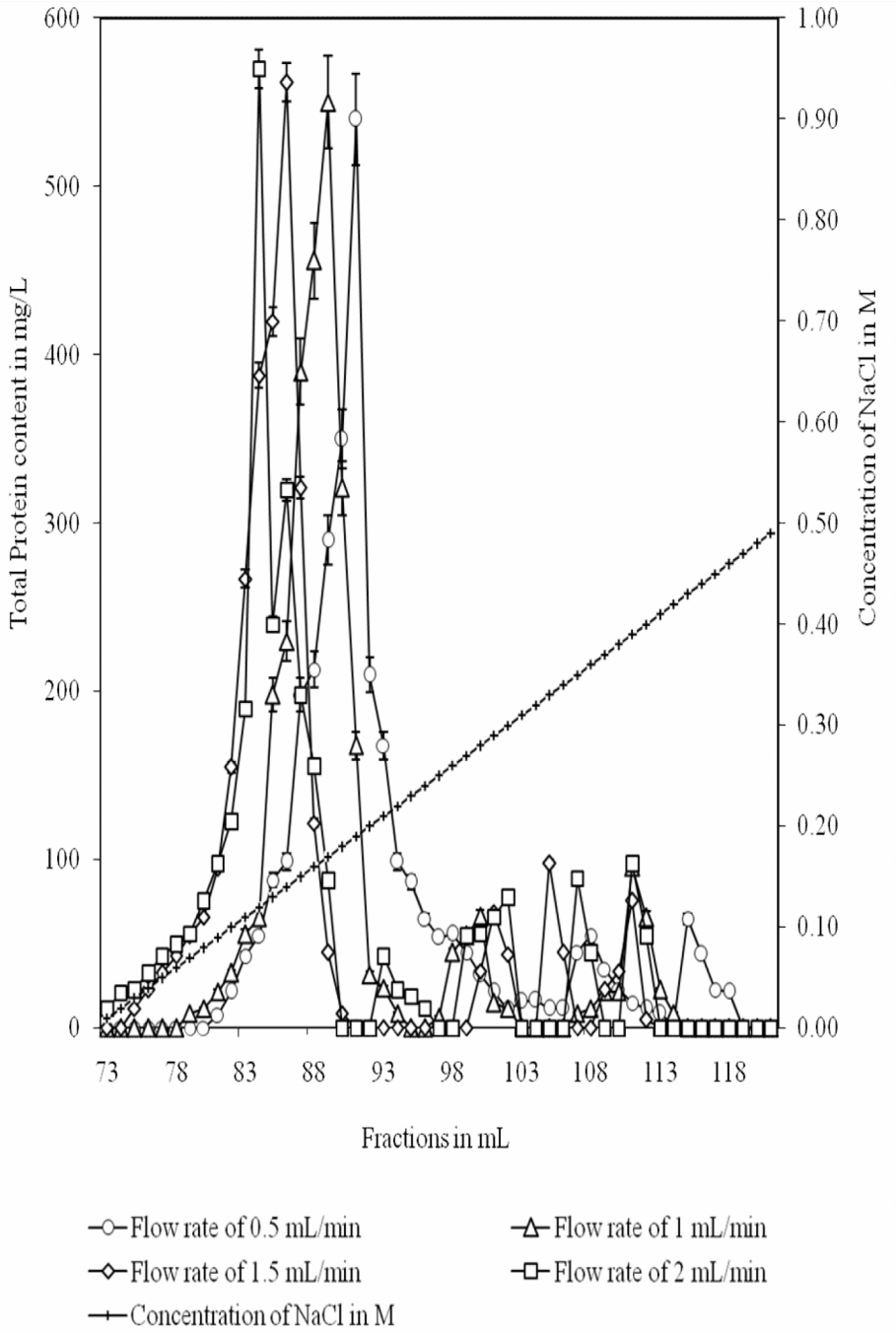

Fig. 4 Elution profile of the proteins in DEAE-Cellulose column at various flow rate

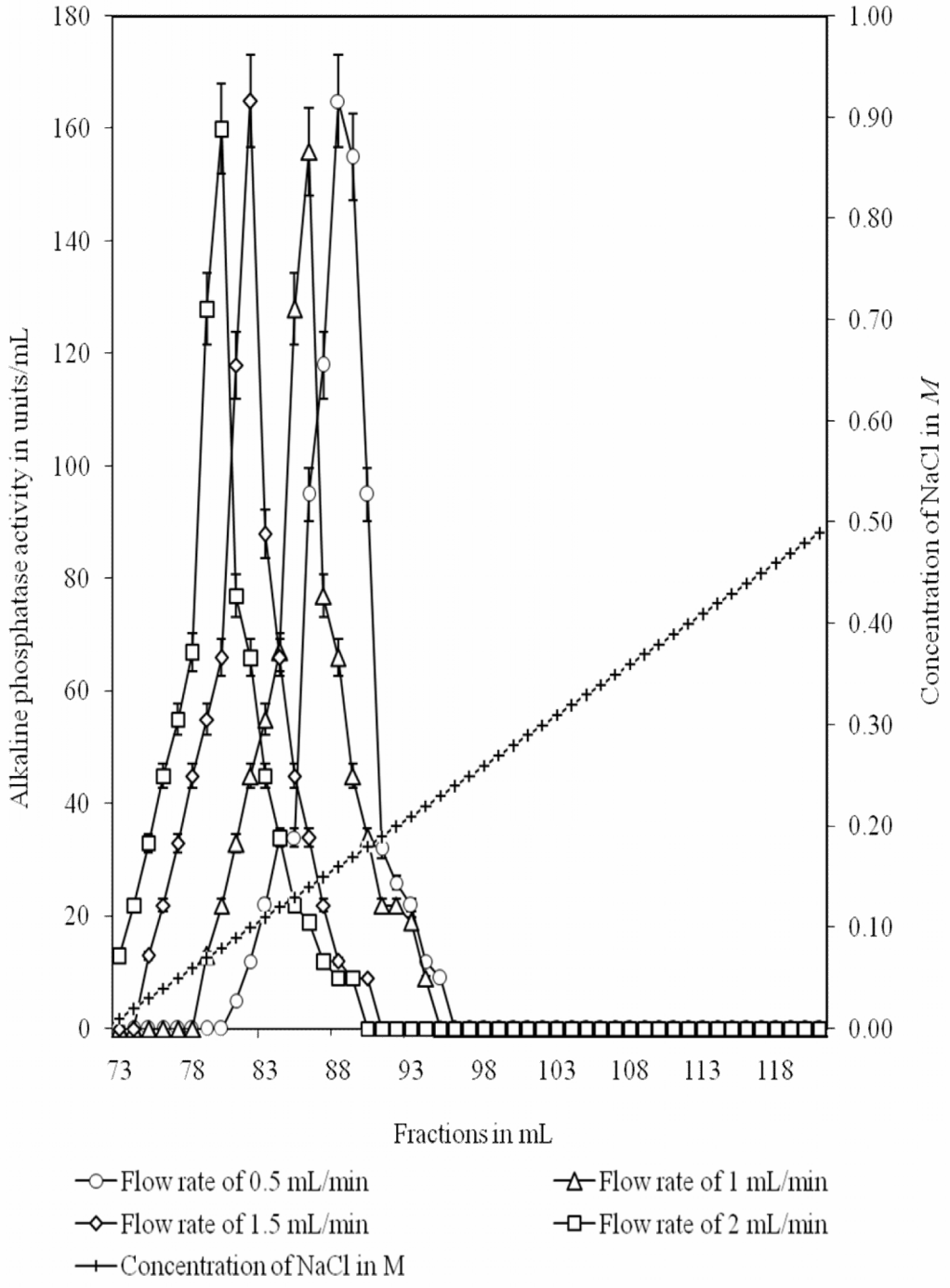

Fig. 5 Elution profile of the proteins in DEAE-Cellulose column at various flow rate

Table 1. Details of alkaline phosphatase recovery from the hepatopancreatic tissues of Indian white shrimp

Purification stage	Volume		Specific activity		Yield	
	Volume mL	Change %	Specific activity units/mL	Change folds	Step yield %	Process yield %
Hepatopancreas	100.00±0.82	1.00±0.01	-	-	-	-
Homogenisation	1060.00±0.79	1060.0±0.79	0.029±0.01	1.00±0.01	100.00	100.00
Clarification	812.64±1.12	76.57±0.01	0.0795±0.01	2.70±0.02	90.28±0.07	90.28±0.07
Concentration	210.59±1.32	25.94±0.01	0.1475±0.05	5.01±0.03	96.75±0.09	87.34±0.03
Dialysis	421.87±0.99	200.30±0.04	0.148±0.10	5.02±0.02	99.60±0.40	87.19±0.08
Chromatography	2531.20±2.22	600.00±0.06	0.414±0.01	14.05±0.02	61.03±0.11	53.29±0.03

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

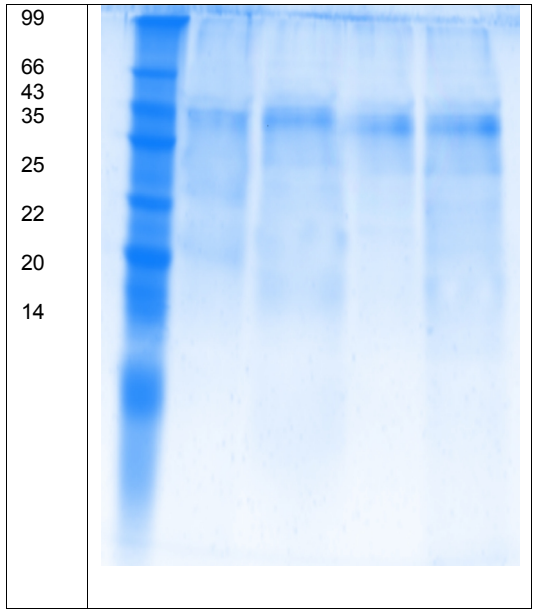

Fig. 6. SDS-PAGE Analysis of the pooled protein fractions of alkaline phosphatase activity

4. DISCUSSION

Binding of the alkaline phosphatase to DEAE-Columns were not effected by the variation of pH of the binding of the buffer from 7.6 to 8.8. This finding is supported by the fact that weak ion exchange resins such as DEAE with pKa value of 10.0 is positively charged below pH 8.5 can binds to negatively charged shrimp alkaline phosphatase 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(8-10). Binding buffer at lower pH range used improves the resolution of the alkaline phosphatase and reduces time gap to release the bound proteins from the column. Binding buffer of pH of 8.4 is suitable as it favours optimum binding of the proteins to the columns and releases protein fractions of maximum cumulative alkaline phosphatase activity when eluted using elution buffer of ionic gradience of 0.0-0.5*M* NaCl at respective pH. This is because the buffer of pH between 7.5 and 8.5 affects the protonation of histidine that in turn helps in discriminating protein of interest and protein impurities by anion-exchange resins [27]. Hence carefully selecting the criterion of the DEAE-Cellulose Chromatography can resolve alkaline phosphatase to near homogeneity [28]. Loss of more than one third of the protein fractions without any alkaline phosphatase activity during the elution stage of the process using binding buffer of ionic strength of 0.1 *M* NaCl at pH 8.4 is the clear indication of the efficient binding of alkaline phosphatase to the DEAE-Cellulose column [29]. Where as, increase in the ionic strength more than this level reduces the durations to release bound proteins from the column and decreases the resolution of the resolution of the bound protein when eluted. Efficiency at which proteins bind to the protein depends on net charge, surface charge distribution, polarity of the proteins, van der Waals interactions between components and characteristics of the ion exchange resins that are in turn influenced by pH and ionic strength of the buffer [30].

Similarly ionic gradient of the elution buffer is also very important for improving the resolution and reducing the time required to release bound proteins. Bound protein when eluted using buffer of ionic gradient 0.10-0.35 *M* NaCl fractions of proteins with alkaline phosphatase efficiently resolved from fractions of protein impurities. When the results were plotted narrow and symmetrical major protein peak of alkaline phosphatase emerged first followed by clearly isolated peaks of protein impurities. This finding is supported by the view that proteins with the lowest net charge at a given pH is the first component to be eluted from the column as ionic strength increases, and the proteins with the highest charge are to be eluted last because it is most strongly retained [30]. Ionic gradient beyond 0.10-0.35 *M* NaCl deteriorated the resolution and below this increased the retention time of the bound proteins in the column. With increase in the ionic gradient, Na^+ or Cl^- of buffer compete with the bound proteins for charges on the surface of the ion exchange resins resulting in releasing of one or more of the bound protein species begin to release and percolate down the column[30]. Hence for subsequent work elution buffer of ionic gradient of 0.10-0.35 *M* NaCl was used. One more important parameter in the chromatographic purification is the flow rate of the elution buffer. Clearly isolated single major peak of alkaline phosphatase activity from other three other minor peaks without any alkaline phosphatase activity in the elution stage of the chromatogram produced by running the elution buffer at 1.5 mL/min depicts the efficiency of this rate. Above and below this rate resolution of the alkaline phosphatase from other protein impurities gets effects, the findings are supported by the previous finding that flow rate has a direct bearing on the resolution of the fractions in the chromatogram [31]. Hence, suitable chromatographic criterion such as pH and ionic strength of the binding buffer and ionic gradient and flow rate of the elution buffer is crucial for efficiency of the ion exchange chromatography. Efficiency of the chromatographic criterion

on the resolution of the alkaline phosphatase from other proteins was also supported by the SDS-PAGE analysis indicating homogeneity.

5. CONCLUSION

Alkaline phosphatase can be efficiently bound to the DEAE-cellulose column in such a way that little change in the ionic gradient of the elution buffer is sufficient to release bound protein from the column with optimum yield using binding buffer of pH of 8.4 and ionic strength of 0.10 *M* NaCl at flow rate of 1mL/min. The bound proteins can efficiently be released from the column in such a way that well isolated sharp protein peaks of maximum activity is clearly separated from peaks of protein impurities when eluted using elution buffer of ionic gradient at 0.10-0.35 *M* NaCl at flow rate of 1.5 mL/min. Hence, criterions of ion exchange chromatography such as 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 favor optimum binding of the shrimp alkaline phosphatase to the DEAE-cellulose in such as way that little change in ionic gradient and flow rate is sufficient to release alkaline phosphatase from with high resolution, yield and activity.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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