

<Original Article>

High-throughput primary clarification of Tiny shrimp (*Parapenaeopsis styliifera*) hepatopancreatic tissue homogenate during the recovery of crude alkaline Phosphatase

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Summary Removal of insoluble from hepatopancreatic homogenate of Tiny shrimp (*Parapenaeopsis styliifera*) was done by at relative centrifugal force (RCF) of 67.2, 1681.1, 6724.3, 15124.8, or 26897.4 \times g for 5, 10, 15, or 20 min at 4°C to optimize primary clarification. Resident time of than 5 min at relative centrifugal force of 1681.1 \times g or at the centrifugal force of more than 1681.1 \times g for 5, 10, 15, or 20 min was able to remove 95.69 \pm 0.17% of the total solids, 88.12 \pm 0.48% of soluble protein, and 87.52 \pm 0.99% of the lipids from the respective initial homogenate, but hydrodynamic shear denatured more than half of the alkaline phosphatase even at the temperature of 4°C. The force of 1681.1 \times g for 5 min efficiently retained 92.31 \pm 1.52% of the enzyme, clarified 88.61 \pm 0.32% of the total solids, 68.56 \pm 0.88% of soluble protein, and 66.99 \pm 0.36% of the lipids from the respective initial homogenate.

Key words: Alkaline phosphatase, Shrimps, Insoluble, Centrifugation, Clarification, Hepatopancreas

1. Introduction

Tiny shrimp (*Parapenaeopsis styliifera*) regularly harvested all along coastal Karnataka during fishing season for production of value added seafood products that inturn leaving behind large amount of hepatopancreatic wastes, which is a rich source of alkaline phosphatase. Alkaline phosphatase (EC 3.1.3.1) are non-specific phosphomonoesterase that are found widely in species ranging from bacteria to man, and purified alkaline phosphatase are commercially exploited in medicine, diagnostics, dairy and leather processing industries. Marine organisms are the potential source of enzymes with unique novel proper-

ties because marine environment contains a large pool of diversified species adapted to a variety of habitat conditions that can be exploited commercially after purification. Highly efficient clarification of the hepatopancreatic tissue homogenate is very important at the initial stage of enzyme purification itself as the presence of insoluble in the improperly clarified fluid may subsequently interfere with enzyme recovery process resulting decreased enzyme yield, reduced purification fold, increased recovery unit steps increased products cost and wasted effort¹. Centrifugation is one of the primary choices of method to clarify tissue homogenates to isolate alkaline phosphatase in comparison of other process alterna-

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tives such as filtration when the product of interest is in the medium of tissue homogenate². However, undesirable insoluble such as cell debris, organelles and lipids of the tissue homogenates shows variation in quantity, ratio and physico-chemical properties and removal of these undesirable components is a significant bottle-neck in primary clarification³⁻⁵. Hence, it is very important to formulate a strategy to design a primary clarification of the homogenate by careful selection of centrifugal force and resident time in centrifuge to ensure optimum yield, product consistency and reproducibility⁶. Optimisation of these parameters are the need of the hour because reduction in relative centrifugal force and centrifugation time results in partial isolation of insoluble from the tissue homogenate, and increase in relative centrifugal force and centrifugation time may increase the sedimentation rate^{7,8}. If not properly designed the primary clarification method may result in the complete or partial loss of the enzyme from the purification stream due to hydrodynamic shear force of centrifugation^{9,10}. This may adversely affect purification strategy and cost effectiveness, and hence this issue should be considered with special concern. Properly performed enzyme purification by careful selection of each of these centrifugation parameters based on laboratory test values will pave the way for the optimum productivity and purity¹¹. Even though centrifugation as a tool to remove insolubels and extract proteins from of biological tissue homogenates is widely investigated¹²⁻¹⁴, no single set of standard operating conditions to isolate enzymes from the tissue homogenates in terms of centrifugation speed and centrifuge time have been defined to achieve optimum activity, yield and purity. Hence, we have investigated the effectiveness of varied relative centrifugal force and centrifugation duration on the removal of insoluble from the hepatopancreatic tissue homogenates of Tiny shrimp to isolate alkaline phosphatase optimally using a set of standard operating conditions with optimum yield, purity and activity.

2. Materials and method

2.1 Chemicals

Solutions were prepared using analytical grade

chemicals and reagents obtained from Merck Limited (Mumbai, India) according to the current American Chemical Society specifications¹⁵. Buffer used for the homogenisation of hepatopancreatic tissues of shrimps was 0.1 M Tris-HCl buffer of pH 8.4. The homogenisation buffer was prepared by adding 12.111 g of the free Tris base to 900 mL of deionized water. This mixture was then titrated with 1 M HCl solution to pH 8.4, and volume was made up to 1,000 mL. Resulting mixture was incorporated with MgCl₂ and ZnCl₂ to respective final concentration of 0.1 M. However, 2-amino-2-methyl-1-propanol (AMP) buffer of pH 10.3 was used as an assay buffer thought the experiment. The assay buffer was prepared by dissolving 78 g of AMP in 500 mL of deionized water. To this 200 mL of 1 M HCl is added. Subsequently, volume of the mixture was made up to 1,000 mL using deionized water in 1,000 mL volumetric flask. All the buffer preparations were filtered using 15 mm Whatman Grade-1 cellulose filter paper and sterilized in moist heat at 121 °C for 20 min¹⁶.

2.2 Sample collection

Tiny shrimp 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 was collected, identified and used for the present study¹⁷. The time elapsed between catching and landing may not exceed over four to six hours. The material was kept in an insulated container after adequately icing them in the proportion of 1:1 shrimp to ice, and transported to the laboratory within two hours. Freshly caught Tiny shrimps with the size range of 53-110 mm in length and weighing around 23-47 g were washed, dissected to remove the hepatopancreas, and weighed. Hepatopancreatic tissues were packed in plastic bags, labeled, frozen at -40 °C, and stored at -20 °C in a refrigerator (JHBio, Chennai, India) until further use.

2.3 Homogenization

The hepatopancreas along with attached tissues were thawed at room temperature of about 28 °C, weighed and homogenized using a Potter-Elvehjem homogenizer (RH-2 Homogenizer, Rotek Instruments,

Kerala, India) with a sample holding tank mounted in a cooling jacket maintained at 4°C. The samples were homogenized in the homogenizer at a homogenization speed of 3,000 rpm for 10 min at the temperature of 4°C using 0.1 M Tris-HCl buffer of pH 8.4 at 1:10 tissue to buffer ratio¹⁸.

2.4 Centrifugation

Microprocessor controlled low volume high speed refrigerated centrifuge of model C-24BL/CRP24 (Remi Laboratory Instruments, Mumbai, India) with programming facility for temperature, speed, and time in table top design was used for the present work. The centrifuge was fitted with R-248 model 24x1.5 mL fixed-angle rotor. Total volume used in this experiment was 1 mL. The distance from rotor center to the middle of the protein layer, allowing corrections for rotor stretch, radial shift of the meniscus, and protein layer was calculated to be 6.02 cm. The crude homogenate with highest protein content were centrifuged at relative centrifugal force (RCF) of 67.2, 1681.1, 6724.3, 15124.8 or 26897.4×g for 5, 10, 15, or 20 min at 4°C in the centrifuge. After centrifugation, each infranatant collected was estimated for total solid content, protein content, fat content and alkaline phosphatase activity. Supernatant layer and pellets were reconstituted using 0.1 M Tris-HCl buffer of pH 8.4 at 1:10 pellets to buffer ratio and was estimated for total solid content, protein content, fat content and alkaline phosphatase activity.

2.5 Proximate analysis

Samples were drawn at different intervals and force of centrifugation was performed in quadruplicates. Moisture content and solid content of the samples were estimated as per the guidelines of Food and Agriculture Organization of the United Nations¹⁹, and expressed as percentage moisture. Known weight of hepatopancreatic tissues of Tiny shrimp, or known volume of the hepatopancreatic tissue homogenates before and after centrifugation were collected, and were kept in hot air oven at 110°C for 16 h, and loss of weight after heating is used to calculate total moisture content and total solid content in the sample. The protein content was estimated as per the Folin-

Ciocalteu method of Lowry et al.²⁰, using bovine serum albumin (BSA) as a standard. Total protein content of the hepatopancreatic tissues were done by incubating 0.4 mg of tissues with 0.5 mL of 4 M NaOH at 100°C for 5 min, and the resulting homogenate was cooled and assayed for total protein by Folin-Ciocalteu method. Similarly, protein content in the hepatopancreatic tissue homogenate at the end of homogenization or in three phases after centrifugation was estimated using Folin-Ciocalteu method. The total lipids were quantitatively estimated by Sulpho-phosphovanillin method of Barnes and Blackstock²¹. Here, 10 mg each of the hepatopancreatic tissues or homogenates from each stage taken in clean dry test tubes were homogenized in chloroform:methanol mixture (2:1, v/v CH₂Cl:CH₃OH) and kept overnight at 4°C in tightly stoppered test tubes for complete extraction. The resulting mixtures were mixed well and centrifuged at 3,000 rpm for 15 min. The supernatant containing the lipids decanted to dry test tubes. Each 0.5 mL of supernatant was taken in a separate test tube and dried in vacuum over silica gel in desiccators. To the dried samples containing lipid, 0.5 mL of concentrated sulfuric acid was added and shaken well. The tubes were then heated at 100°C in a boiling water bath for 10 min after plugging with nonabsorbent cotton wool, and rapidly cooled to room temperature under running tap water. Subsequently, 2.5 mL of phosphovanillin reagent was added to 0.1 mL of this acid digest, and mixed well in a cyclomixer. The pink-red colour developed during 30 min were read at 520 nm in UV-spectrophotometer (Systronics, Mumbai) along with the standard cholesterol (8 mg cholesterol in 10 mL 2:1 v/v CH₂Cl:CH₃OH mixture) and reagent blanks. Here, Phosphovanillin reagent was prepared by mixing 2 g vanillin, 800 mL orthophosphoric acid, and 200 mL double distilled water.

2.6 Enzyme assay

Alkaline phosphatase was assayed as per the method of Bomers and McComb²². The substrate was prepared by dissolving 83.5 mg of disodium parani-trophenyl phosphate (pNPP) in 1.0 mL of 1.5 mM magnesium chloride solution and stored at 4°C. This

solution was colorless and its absorbance was measured at 410 nm < 0.800. A stock solution of 10.8 mM of *p*NP was prepared by dissolving 150 mg of *p*NP in about 80 mL of 0.25 M NaOH solution and stored at room temperature of about 28°C in amber colored bottle. A working solution of 54 mM of *p*NP was freshly prepared by pipetting 0.5 mL of *p*NP stock solution in 100 mL volumetric flask and the volume was made up to the mark using 0.25 M NaOH solution. Enzyme assay incorporates AMP buffer. About 1.4 mL of buffer was mixed with the solution and incubated at 37°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). Colorless *p*NPP gets hydrolyzed by alkaline phosphatase at a given buffer pH 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. Here, 0.05 mL of tissue homogenate was mixed with reagent and incubated for 15 min and the total volume was made up to 5.55 mL. However, the total volume in the case of each standard was 5.0 mL. Hence, *p*NP in mM or alkaline phosphatase activity in units/L in the tissue homogenate = $(\text{Test absorbance} \times 0.027 \times 5.55 \times 1,000) / (\text{Standard absorbance} \times 15 \times 5.0 \times 0.05)$. Alkaline phosphatase activity in units/L is the liberation of 1 mM 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.

2.7 Statistical analysis

The analysis of cell disruption 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 \pm 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 and discussion

Effectiveness of various centrifugal force and centrifugation duration to remove insoluble from the Tiny shrimp homogenate was done by estimating total solids, protein, lipid and alkaline phosphatase activity of supernatant, infranatant, and pellet obtained through combination of these operational parameters²³.

3.1 Effectiveness of different centrifugal force and time on solids removal

Total solid content in the homogenates of Tiny shrimp hepatopancreas was estimated as $26.67 \pm 0.89\%$. During centrifugation at RCF of $67.2 \times g$, almost $66.30 \pm 0.12\%$ of the total solids of the homogenate were clarified before 5 min of centrifugation, even though this force was able to clarify $70.99 \pm 0.54\%$ of the total solids by 20 min of centrifugation. Takagi et al. reported that RCF of $67 \times g$ was able to recover 91% of the tissues by 5 min⁸. When the RCF was increased to $1681.1 \times g$, this force was able to clarify $88.61 \pm 0.32\%$ of the total solids by 5 min of centrifugation, and $94.79 \pm 0.09\%$ of the total solids by 10 min of centrifugation from the tissue homogenates. Erasmus et al. reported that centrifugation of hepatopancreatic tissue homogenates at RCF of $1089 \times g$ was able to remove all the cell debris by 15 min of centrifugation²⁴. Nevertheless, no significant ($p > 0.05$) difference in the solid content was registered amongst the homogenates produced at 10, 15 and 20 min of centrifugation at $1681.1 \times g$, or at 5, 10, 15 and 20 min of centrifugation at RCF of 6724.3, 15124.8, or $26897.4 \times g$, as estimated by one-way ANOVA with *post hoc* Tukey's test (Fig. 1). Here, centrifugation period of more than 5 min at RCF of $1681.1 \times g$ or centrifugal force more than $1681.1 \times g$ for 5, 10, 15, or 20 min at 4°C was able clarify $95.69 \pm 0.17\%$ of the total solids from the respective initial homogenate, retaining only $4.31 \pm 0.17\%$ of solids of the respective tissue homogenate produced.

Reduced centrifugal force and centrifugation time would contribute partial isolation of insoluble from the tissue homogenate, and increasing RCF and centrifugation time up to certain limit may increase the sedimentation rate^{8,25}.

3.2 Effectiveness of different centrifugal force and time on protein isolation

Here, 5850.00 ± 57.73 mg/L of the protein present in the homogenate underwent fractionation between solid and liquid phases during the centrifugation of the homogenate at different centrifugal force and centrifugation time. Even up to 20 min of centrifugation of the homogenate at RCF of $67.2 \times g$, this force was able to fractionate only $19.94 \pm 0.92\%$ of the total soluble protein of the homogenate in to pellets. However, RCF of $1681.1 \times g$ was able to fractionate $66.99 \pm 0.88\%$ of the total soluble protein by 5 min of the centrifugation, and $85.62 \pm 0.98\%$ of the protein by 10 min of centrifugation. One-way ANOVA with *post hoc* Tukey's test was not able to establish any signif-

icant ($p > 0.05$) difference in the protein content amongst the pellets collected at the end of 10, 15, and 20 min of centrifugation at RCF of $1681.1 \times g$, or at RCF of 6724.3 , 15124.8 , or $26897.4 \times g$, for 5, 10, 15, or 20 min (Fig. 2). All along these period at a give force, we have not registered any protein accumulation ($p > 0.05$) in the supernatant.

3.4 Effectiveness of different centrifugal force and time on Lipid removal

Of the total 3630 ± 22.29 mg/L of lipid present in the initial homogenates underwent floatation to form a fully developed layer on top of the medium during centrifugation of the homogenate at different centrifugal force and time interval, and no lipid accumulation was registered in the pellets²⁵. Even at the end of 20 min of centrifugation at RCF of $67.2 \times g$, this force was able fractionate only $15.94 \pm 0.12\%$ of the lipids of the initial homogenate to the supernatant. When the RCF was increased to $1681.1 \times g$, this force was able to reduce $66.99 \pm 0.36\%$ of lipid

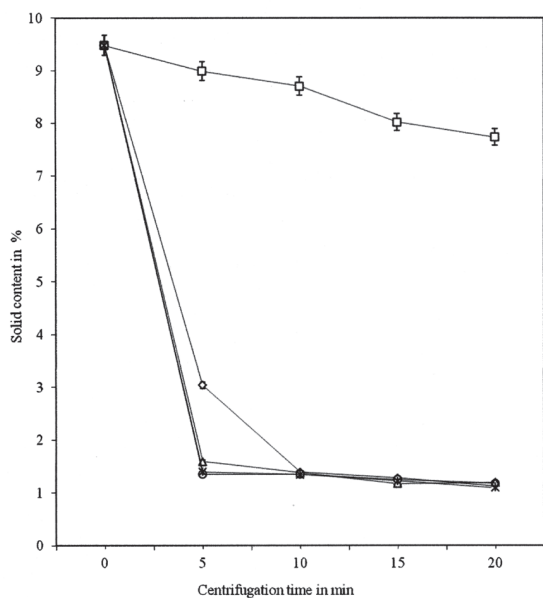


Fig. 1 Fractionation of solids of the homogenate at different centrifugal force.

- Infranant at $67.2 \times g$
- ◇— Infranant at $1681.1 \times g$
- △— Infranant at $6724.3 \times g$
- *— Infranant at $15124.8 \times g$
- Infranant at $26897.4 \times g$

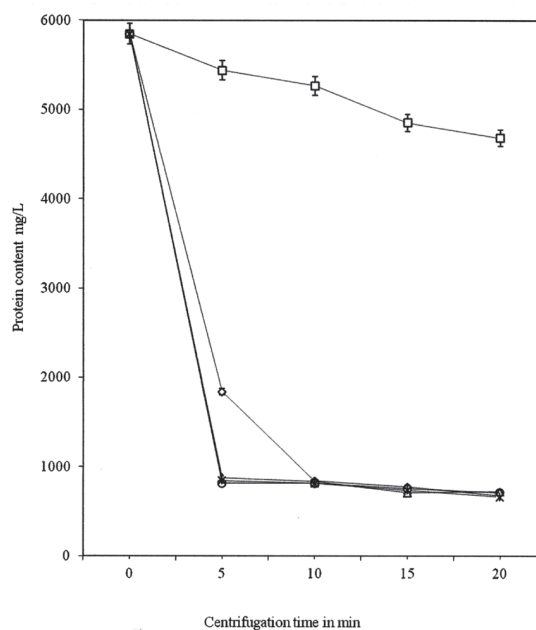


Fig. 2 Soluble protein fraction of the homogenate at different centrifugal force for different time interval.

- Infranant at $67.2 \times g$
- ◇— Infranant at $1681.1 \times g$
- △— Infranant at $6724.3 \times g$
- *— Infranant at $15124.8 \times g$
- Infranant at $26897.4 \times g$

from the homogenate by 5 min of centrifugation, and $84.91 \pm 0.83\%$ of the lipid from the homogenate at the end of 10 min of centrifugation. However, neither subsequent increase in the centrifugation period even up to 20 min at RCF of $1681.1 \times g$, nor subsequent increasing the RCF of beyond $1681.1 \times g$ up to 20 min was able to change ($p > 0.005$) any lipid content in the homogenate or the supernatant, as estimated by one-way ANOVA with *post hoc* Tukey's test (Fig. 3). Optimum centrifugation parameters are those forces and time that efficiently removes the lipid into supernatant, retains enzyme of interest in the infranatant, and removes solids into pellets²³.

3.5 Effectiveness of different centrifugal force and time on enzyme recovery

During the entire 20 min of centrifugation at RCF of $67.2 \times g$, the homogenate lost only $9.57 \pm 1.20\%$ alkaline phosphatase (Fig. 4). Even when the RCF was increased to $1681.1 \times g$, this force was able to retain $92.31 \pm 1.52\%$ of the alkaline phosphatase of

initial homogenates at the end of 5 min of centrifugation. According to Nagahashi and Hirike low centrifugal force is efficient in maintain optimum enzyme activity and increase in speed and time does not have any effect on the activity of the enzymes²⁶. Nevertheless, when the resident time in the centrifuged was increased to 10 min at RCF of to $1681.1 \times g$, alkaline phosphatase activity in the homogenate was only $44.64 \pm 1.23\%$ of the activity seen in the respective initial homogenates. Proteins in the biological fluids (i.e., especially enzymes) are subjected to fluid forces during centrifugation and resulting hydrodynamic shear force may cause damage to the low molecular weight proteins, resulting in denaturation and inactivation of protein¹⁰. In our study, centrifugation of the homogenates at and beyond 10 min at RCF of to $1681.1 \times g$ or beyond RCF of to $1681.1 \times g$ even at 5 min resulted in significant ($p < 0.05$) loss in the activity of the alkaline phosphatase. Properly designed purification strategy with carefully selected unit process based on laboratory test values is critical to improve

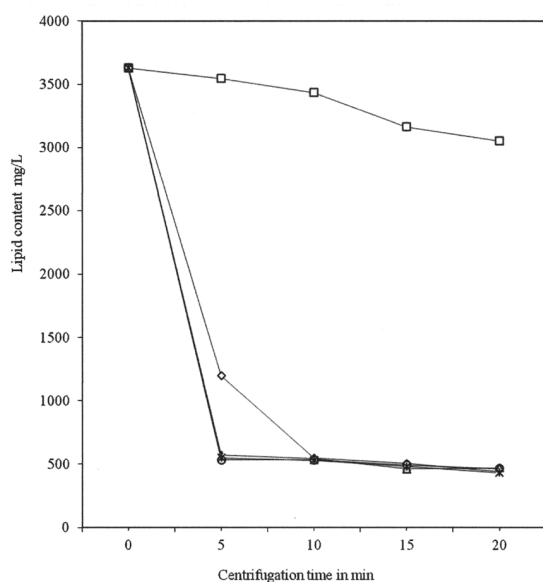


Fig. 3 Lipid fractionation of the homogenate at different centrifugal force and time.

- Infranatant at $67.2 \times g$
- ◇ Infranatant at $1681.1 \times g$
- △ Infranatant at $6724.3 \times g$
- * Infranatant at $15124.8 \times g$
- Infranatant at $26897.4 \times g$

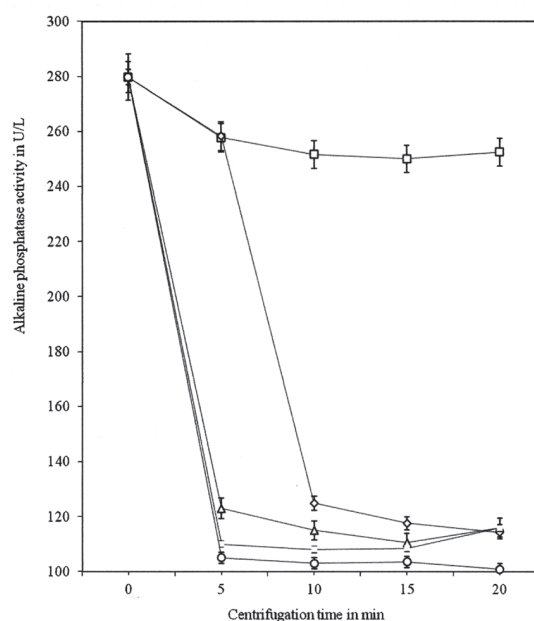


Fig. 4 Alkaline phosphatase activity of the homogenate at different centrifugal force and time.

- Infranatant at $67.2 \times g$
- ◇ Infranatant at $1681.1 \times g$
- △ Infranatant at $6724.3 \times g$
- * Infranatant at $15124.8 \times g$
- Infranatant at $26897.4 \times g$

enzyme productivity⁵.

During the centrifugation of homogenate at RCF of $67.2 \times g$ for 5, 10, 15, or 20 min, alkaline phosphatase activity per mg of protein of the infranatants remained at a level of 0.050 ± 0.013 units/mg (Fig. 5). When the RCF of centrifugation was increased to $1681.1 \times g$, the specific activity of the infranatant increased by 2.94 ± 0.01 folds at the end of 5 min of centrifugation compared to its respective homogenate, and the specific activity registered during this period was 0.14 ± 0.01 units/mg. Even after registering the same level of alkaline phosphatase activity in all the infranatants produced either at $67.2 \times g$ for 5, 10, 15, or 20 min, or at RCF of $1681.1 \times g$ for 5 min, specific activity of the infranatant obtained after centrifugation at RCF of $1681.1 \times g$ for 5 min was increased by more than two folds compared to the initial homogenate. Her, beyond 5 min at RCF of $1681.1 \times g$ or beyond RCF of $1681.1 \times g$ for 5, 10, 15 or 20 min the incremental specific activity of the tissue homogenates remained at a constant level of

3.37 ± 0.30 folds compared to its respective homogenate. Beyond 5 min of centrifugation at RCF of $1681.1 \times g$ or beyond RCF of $1681.1 \times g$ for 5, 10, 15 or 20 min the specific activity of the tissue homogenates increased significantly ($p < 0.05$) in comparison to homogenates obtained at RCF of $1681.1 \times g$ for 5 min. However, centrifugation of the homogenates at and beyond 10 min resulted in significant ($p < 0.05$) loss in the activity of the alkaline phosphatase. In our study, RCF of $1681.1 \times g$ for 5 min was able to attain optimum alkaline phosphatase activity in the homogenates. Wide variation in the physico-chemical properties and proportion of these components in biological fluids is a significant bottleneck in primary clarification, and properly selecting the g-force and resident time of the centrifugation affect the efficiency of the centrifuge^{5, 6}. Optimum yield of the enzyme achieved at RCF of $1681.1 \times g$ for 5 min at $4^\circ C$, and increase in centrifugal force beyond this level was efficient in reducing the total protein content from the homogenate but the homogenate lost its alkaline phosphatase activity^{9, 10}.

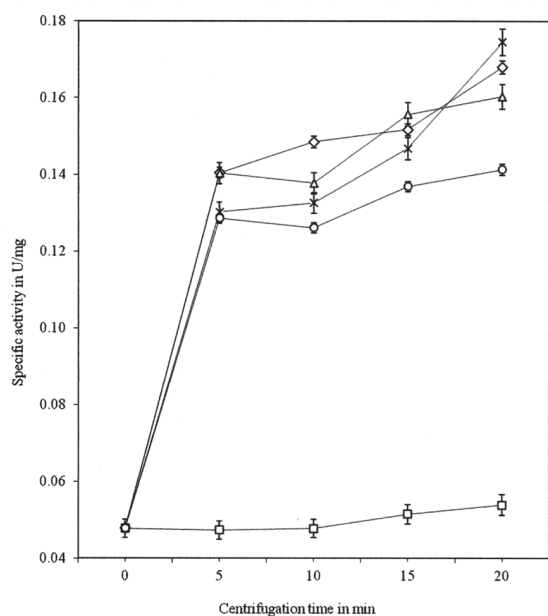


Fig. 5 Specific activity the homogenates of different centrifugal force for different time interval.

- Infranatant at $67.2 \times g$
- ◇— Infranatant at $1681.1 \times g$
- △— Infranatant at $6724.3 \times g$
- *— Infranatant at $15124.8 \times g$
- Infranatant at $26897.4 \times g$

4. Conclusion

Relative centrifugal force of $1681.1 \times g$ for the resident time of 5 min in the centrifuge at $4^\circ C$ is a high-throughput clarification of the hepatopancreatic tissue homogenate of Tiny shrimp. The Force below $1681.1 \times g$ at any given resident time results in the incomplete removal of lipids and cell debris from the homogenate, and the centrifugal force above $1681.1 \times g$ at any given time or resident time above 5 min at $1681.1 \times g$ results in partial loss of the enzyme from the purification stream due to hydrodynamic shear force of centrifugation. This issue should be considered with special concern, as it may adversely affect purification strategy and cost effectiveness.

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