〈Original Article〉

Effective primary clarification techniques to remove of insolubles from the proteins of hyaluronidase activity in the hepatopancreatic tissue homogenate of Brown shrimp, *Metapenaeus monoceros*

Krishna Prasad Nooralabettu

Summary Clarification parameters required removing insolubles from homogenates of hepatopancreas of *Metapenaeus monoceros*; commonly known as Brown shrimp was optimized in terms of its effectiveness at which proteins of hyaluronidase was retained in homogenate during clarification. Optimum resident time was 5 min at relative centrifugal force of 1681.1×g, or optimum relative centrifugal force was 1681.1×g for 5 min, at which 92.06±1.44% of the proteins with hyaluronidase activity was retained in the homogenate, while removing 83.33±1.01% of the total solids, 66.07±1.23% of soluble protein, and 96.29±1.66% of the lipids. Whereas, resident time at relative centrifugal force of 1681.1×g for more than 5 min, or centrifugal force for 5 min at more than relative centrifugal force of 1681.1×g was able to remove 96.75±2.80% of the total solids, 96.95±1.45% of soluble protein, and 98.46±1.23% of the lipids, but more than half of the proteins with hyaluronidase activity was lost due to hydrodynamic sheer force even at 4°C.

Key words: Hyaluronidase, Shrimp, Brown, Insoluble, Centrifugation, Clarification, Hepatopancreas

1. Introduction

Shrimp processing factories produce accumulate nearly half of the total catch as shrimps are processed and exported mostly in headless or peeled form[1]. Hence shrimp processing factories in India generates more than a lakh ton of solid waste per year[2]. Indian seafood export expected to cross 6 billion US$ in the fiscal year 2018 by expecting 11,34,948 ton, according to the report of Marine Product Export Development Authority (MPEDA), Cochin[3]. Converting huge quantity of the hepatopnaceatic wastes of shrimp processing factories in to commercially important products reduces environmental burdon, decreased public health risks, generates ancillary industries, increases profit margin and improves socioeconomic status of fisherman[4]. Hepatopancreas of *Metapenaeus monoceros*, commonly known as Brown shrimp is involved in the production and logistics of digestive enzymes, and storage of minerals, glycogen and lipids[5].

Professor and Head, Department of Biotechnology, P.A. College of Engineering, Nadupadavu, Mangalore, Karnataka, India, Pin-574153.
E-mail: hod_biotech@pace.edu.com, Phone: 00-91-9448529048, Fax: 00-91-8242284704.

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Hepatopancreas is a good source of enzymes such hyaluronoglucosidase (EC 3.2.1.35), commonly known as hyaluronidase[6]. Hyaluronidase of unique properties can be recovered from marine shrimps adapted to extreme habitat conditions with optimum yield activity and purity, if proper strategies were adopted at each unit steps carefully. This is because insolubles such as tissue debris, organelles and fat content released during the cell disruption process might interfere with the subsequent purification of hyaluronidase[5]. These insolubles can be effectively clarified at optimum conditions of centrifugation, as it is an effective clarification technique compared to other methods such as sedimentation, filtration, and agglutination[7]. Variation in number and types of insolubles in the tissue homogenate complicates the clarification process significantly[8]. By carefully developing a strategy to design a clarification technique by carefully optimizing the parameters of centrifugation such as centrifugal force and centrifugation time for a given homogenate one can pave the way for the recovery of the hyaluronidase with optimum yield, activity and purity[9]. In one hand, less than optimum relative centrifugal force or short exposure time to centrifugal force might partially clarify the tissue homogenate[10]. And on the other hand, high relative centrifugal force or prolonged exposure to centrifugal force might denature the enzymes due to hydrodynamic sheer force of centrifugation even at low temperature[11]. Success of enzyme purification depends on the careful selection of parameters of centrifugation based on the laboratory experiments[12]. Eventhough number of efforts was made to standardize the clarification parameters no efforts were made to clarify the hepatopancreatic tissue homogenates during the recovery of hyaluronidase from shrimp[13-15]. Hence this attempt optimally standardized the clarification parameters such as relative centrifugal force and resident time in centrifuge to remove insoluble of hepatopancreatic tissues of shrimp from the homogenated containing proteins of hyaluronidase activity.

2. Materials and methods

1. Chemicals and reagents

Analytical grade chemicals and reagents manufactured and supplied by Merck Limited (Mumbai, India) were prepared as per the current American Chemical Society specifications[16]. Homogenization buffer, 0.5 mol/L sodium acetate solution was prepared by mixing 4.1 g of sodium acetate anhydrous using deionised water in volumetric flask, made the volume up to 1 L, and adjusted the pH to 5.5 by adding 0.05 mol/L acetic acid. 0.05 mol/L acetic acid solution was prepared by mixing 2.85 mL of glacial acetic acid with deionised water in volumetric flask and made the volume to 1 L. Assay buffer, 0.3 mol/L sodium phosphate of pH 5.35 at 37°C was prepared by slowly dissolving 40.2 g sodium sulfate mono basic anhydrous (NaH_2PO_4) in 400 mL of ultra pure water and then warming up the solution in hot plate and the pH of the mixture was adjusted to 5.35 at 37°C using 1 mol/L NaOH or 1 mol/L HCl. Hyaluronic acid solution was prepared by mixing 30 mg of hyaluronic acid in 100 mL of sodium phosphate assay buffer, heating and mixing the solution to 95°C without boiling for 30 min, cooling the mixture up to 37°C in water bath, and a adjusting the pH to 5.35 at 37°C using 1 mol/L NaOH or 1 mol/L HCl after adjusting the volume 100 mL. Enzyme diluents was prepared by mixing 4.23 mL of 0.2 mol/L sodium phosphate monobasic, 5.77 mL of 0.2 mol/L sodium phosphate dibasic, 1.54 mL of 5.0 mol/L sodium chloride solution, and 10 mg of bovine serum albumin, then the volume was made up to 100 mL using ultrapure water and pH was adjusted to 7.0 at 37°C using 1 mol/L NaOH or 1 mol/L HCl. Acid albumin solution was prepared by mixing 2.4 mL of 1.0 mol/L sodium acetate solution of pH 4.8, 0.45 mL of acetic acid and 100 mg bovine serum albumin, and volume was made up to 100 mL using ultra pure water, and then the pH was adjusted to 3.75 using solution made up of 1:1 ration of $HCl:H_2O$ at 25°C. All buffer solutions were filtered and sterilized at 121°C for 20 min.

2. Sample collection

Shrimps harvested using trawl net in the month of August from the Arabian sea was collected from "Bundar Area", landing centre and transported to the Department of Biotechnology, P. A. College of Engineering, Mangalore within 2 hours at 1:1 ice to shrimp ration in an insulated container. The minimum period between harvesting and landing at Mangalore may not cross more than 6 h. *Metapenaeus monoceros*, Fabricius, 1798[17], locally referred as brown shrimp was identified based on the characteristics detailed in FAO shrimp identification manual[18]. Samples were washed in chilled waster, hepatopancreas along with the connected tissues were transferred to plastic bags, frozen at –40°C, and stored at –20°C in a deep freezer after properly labeling.

3. Homogenization

Known quantity of frozen hepatopancreas were thawed at 28°C and chopped into a size of 1 mm using scissor. These samples were homogenized in Potter-Elvehjem homogenizer (Rotek Instruments, Kerala) with a clearance of 0.1-0.2 mm between Teflon pestle and glass mortar with ground interior surface at the ratio of 1:10 tissue to homogenization buffer. Speed of the electric motor was 3,000 rpm, time of the exposure was 10 min, temperature of the sample was 4°C, homogenization buffer was 0.5 mol/L sodium acetate of pH to 5.5, and number of passes through the pestle and mortar was 15 passes/min.

4. Centrifugation

1 mL of the tissue homogenate was clarified at relative centrifugal force (RCF) of 67.2, 1681.1, 6724.3, 15124.8 or 26897.4×gfor 5, 10, 15, or 20 min at 4°C in the programmable refrigerated centrifuge of model C-24BL/CRP24 fitted with R-248 model 24x1.5 mL fixed-angle rotor (Remi Laboratory Instruments, Mumbai, India). The distance from the middle of the protein layer to rotor centre was taken as 6.02 cm by considering corrections for radial shift of the meniscus and rotor stretch during centrifugation. Effectiveness of parameters of centrifugation was done by estimating the total solids, protein content, fat content, and hyaluronidase in the infranatant or the reconstituted supernatant and pellets using homogenization buffer at 1:10 pellets to buffer ratio.

5. Proximate analysis

Known quantity of homogenates were taken before and after centrifugation, and dried in hot air oven at 110°C for 16 h, and difference of weight in these samples were used to estimate the total solids[19]. Protein content in the samples were determined as per the Folin-Ciocalteu method of Lowry and others, and values were expressed as mg/L, using bovine serum albumin (BSA) as a standard using UV-spectrophotometer (Systronics, Mumbai)[20]. Lipid content of the samples were quantified by Sulpho-phosphovanillin method of Barnes and Blackstock using UV-spectrophotometer (Systronics, Mumbai) and values were expressed as mg/L[21]. Hyaluronidase activity was assayed by turbidimetric method and values were expressed as percentage of transmittance at 600 nm and light path of 1 cm[22]. Here, 0.5 mL of the sample was mixed with 0.5 mL of enzyme diluents and immediately mixed in vortex (Rotech, Cochin) thoroughly for 10 min at 37°C. To this mixture 1 mL of hyaluronic acid solution was added with an interval of 10 min and immediately mixed in vortex (Rotech, Cochin) and incubated at 37°C for exactly 45 min. After 45 min of incubation, 0.5 mL of the test sample and blank sample was mixed thoroughly with 2.5 mL of acidic albumin solution and incubated exactly for 10 min at 37°C. Transmission through the samples was estimated at 600 nm using spectrophotometer. To plot standard calibration curve of absorbance against the concentration of hyaluronidase, aliquot amount of 0.3% hyaluronidase viz. 0.0, 0.1, 0.2, 0.3, 0.4, 0.5 mL in different test tubes were made up to 0.5 mL using phosphate buffer and then 2.5 mL of acidic albumin was added. Turbidity was measured at 600 nm exactly after 10 min of incubation of each test tube. Tissue homogenates at different dilution using enzyme diluents were testes so as to get the results within 130 to 170% transmission. Hyaluronidase

activity was expressed in unit/mL of sample = [(% of transmission of test sample - % of transmission of test blank)(Dilution factor of the enzyme)]/[(Extinction coefficient of 15)(mL of the enzyme solution)].

6. Statistical analysis

Samples were estimated in quadruplicate, obtained results were treated through analysis of variance (ANOVA), analyzed by Tukey's test using the software Statistica 6.0 (Statsoft, Tulsa, OK, USA), and the values were expresses as± standard deviations and analyzed at a significance level of 95% ($p< 0.05$).

4. Results and discussion

Optimization of the clarification conditions of the hepatopancreatic tissue homogenate of Brown shrimp was performed by analyzing the fractionation of solid content, soluble protein, lipids and proteins with hyaluronidase in supernatant, infranatant, and pellets at different relative centrifugal force and centrifugation time. Optimization of combination of operation parameters of centrifugation is crucial for the effectiveness of the clarification method[23]. Total solid content of the hepatopancreas of Brown shrimp was 26.33±0.12% on wet weight of the tissues. Hepatopancreatic tissue of red shrimp is rich in lipid reserves that constitute 12.38±0.36% of wet weight of the body tissues. Hepatopancreatic tissue homogenate was made up of 13.96±0.12% of total protein. Total solid content of the hepatopancreatic tissue homogenate produced at speed of 3,000 rpm, for 10 min using sodium acetate buffer at 1:10 tissue to buffer ration was 25806.67±23.23 mg/L of the homogenate. Released protein content of the homogenate was 7815.73±12.13 mg/L and released lipid content of the homogenate was 5445.73±14.93 mg/L of the homogenate.

1. Effect of centrifugal force and time on the removal of solids

When the homogenate was centrifuged at RCF of 67.2×g, solid content of the infranatant was reduced to 39.66±1.22% at the end of 20 min of exposure (Fig. 1). However, we were not able to establish any significant difference in the solid contents of the homogenates removed after 5, 10, 15 or 20 min at RCF of 67.2×g, as established by One-way ANOVA with *post hoc* Tukey's test. Here, RCF of 67.2×g was able to clarify 60.33±1.18% of the solids of the infranatant at the end of 20 min, however 58.41±1.04% of the solids were clarified before 5 min of centrifugation. Findings of this experiment is in line with the published research of Takagi and others stating that RCF of 67×g achieves less than 91.00% clarification of the homogenate in 5 min[24]. However, when the RCF was increased to 1681.1×g clarification of the homogenate was 83.33±1.01% in 5 min, and beyond 5 min at RCF of 1681.1×g clarification of the homogenate remained at 98.50±1.01%. Here, One-way ANOVA with *post hoc* Tukey's test was not able to establish any significant difference ($p>0.05$) in the solid content of the homogenates removed after 10, 15, and 20 min of centrifugation at RCF of 1681.1×g. On analyzing the variation in solid content in samples removed after 20 min of centrifugation at RCF of 67.2×g, and 5 and 10 min of centrifugation at RCF of 1681.1×g with p-values establishes significance for the regression model for both centrifugal force and the resident time in the centrifuge on the clarification of the homogenate. Here, One-way ANOVA with *post hoc* Tukey's test establish over all significance effect of interaction terms on resident time at various centrifugal force and clarification of the homogenate at 5% level of significance. Resident time of 20 min at RCF of 67×g, and 5 and 10 min at RCF of 1681.1×g and corresponding p-values establish that, for independent parameters, speed or time had significant effects on clarification of tissue homogenate of Brown shrimp. Our experiment indicates that RCF of 67×g up to 20 min was partially able to remove insolubles from the homogenates, which is in line with the previous work stating that reduced force and resident time in the centrifuge would partially clarify the tissue homogenate and increasing the force and time might increase the sedimentation rate[10,24]. Study by Erasmus and others suggested that

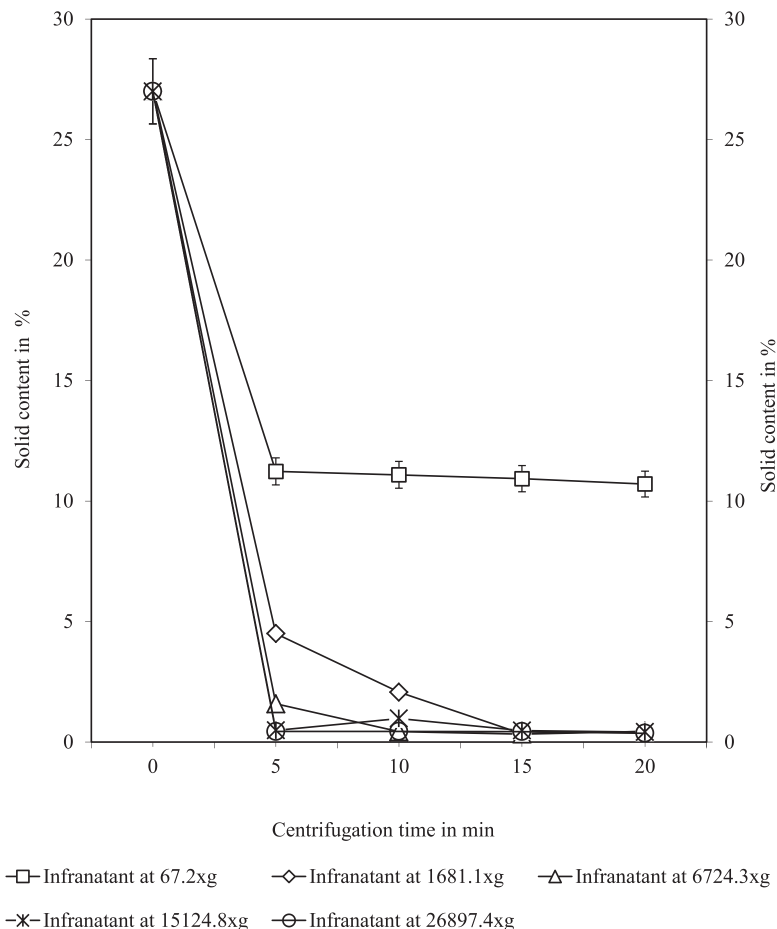

Fig. 1 Changes in solid content in the infranatant at different centrifugal force and time

centrifugation at RCF of 1089×g for 15 min was able to remove all the cell debris from the hepatopancreatic tissue homogenates[25]. These above results establish that resident time and centrifugal force are the key factors effects the clarification owing to the lowest *p*-value among the others at time of 20 min at RCF of 67×g, and 5 and 10 min at RCF of 1681.1×g. However, resident time of 10, 15 or 20 min of centrifugation at 1681.1×g, or resident time of 5, 10, 15 and 20 min of centrifugation at RCF of 6724.3, 15124.8, or 26897.4×g was not significantly ($p<0.05$) able to further improve the clarification of the homogenate. Hence RCF of 1681.1×g and resident time of 10 min resulted in maximum clarification.

2. Protein fractionation at different centrifugal force and time

Total moisture content of the hepatopancreas of Brown shrimp was 73.66±0.97% on wet weight of the tissues (Fig. 2). Hepatopancreas of brown shrimp contains 13.96±0.12% of total protein on wet weight basis and protein released into the medium at 1:10 ratio of tissue to buffer during homogenization was 7815.73±12.13 mg/L. Earlier work on Pacific white shrimp, *Litopenaeus vannamei* reported that hepatopancreas had moisture content of 71.90±0.5%, protein content of 13.4±0.3%[26]. During centrifugation at RCF of 67.2×g, loss of protein from the infranatant was 10.84±0.95% at the end of 20 min of centrifugation. Here, One-way ANOVA with *post hoc* Tukey's test was able to establish significant ($p<0.05$) variation in the protein contents in the infranatant collected at 5, 10, 15 or 20 min of clarifi-

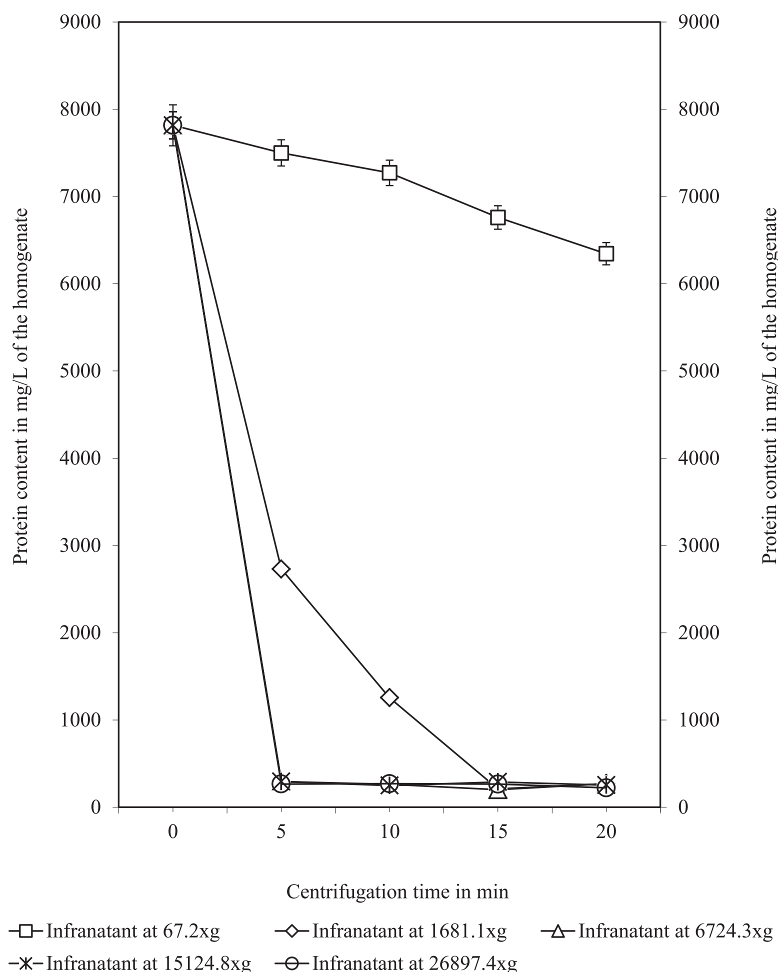

Fig. 2 Changes in protein content in the infranatant at different centrifugal force and time.

cation at RCF of 67.2×g.

On increasing the RCF to 1681.1×g, loss of the protein from the homogenate was 66.07±1.23% in 5 min, and the protein loss at above 10 min was 96.70±1.33% at RCF of 1681.1×g. Here, One-way ANOVA with *post hoc* Tukey's test was not able to establish any significant difference (p>0.05) in the accumulation of protein content in the pellets removed after 10, 15, and 20 min of centrifugation at RCF of 1681.1×g. Protein content in the infranatant reduced significantly during 20 min of centrifugation at RCF of 67.2×g, with p-values establishes significance for the regression model for both centrifugal force and the resident time in the centrifuge on the clarification of the homogenate. Analysis of changes protein content in the infranatanat removed at 5 min and 10 min at RCF of 1681.1×g by One-way ANOVA with *post hoc* Tukey's test establishes over all significance effect of interaction terms on resident time at various centrifugal force and clarification of the homogenate at 5% level of significance. Changes in protein content in the infranatant samples removed after 5, 10, 15 and 20 min above RCF of 1681.1×g or beyond 5 min at RCF of 1681.1×g were analyzed, and corresponding p-values establish that, for independent parameters, speed or time had no significant effects on change in protein content in tissue homogenate of Brown shrimp.

3. Lipid Fractionation at different centrifugal force and time

Total lipid content of the hepatopancreas of Brown shrimp 12.38±0.36% on wet weight basis and lipid released into the medium at 1:10 ratio of tissue to buffer during homogenization was

5445.73±14.93 mg/L (Fig. 3). Findings of the current work is in line with the previous research work on Pacific white shrimp, *Litopenaeus vannamei* stated that hepatopancreas lipid content of 10.7±0.02%[26]. At RCF of 67.2×g for 20 min, fractionation of lipid to the supernatant from the infranatant was 5.79±0.03%. We were not able to establish significant (p>0.05) change in the lipid contents of the infranatant collected after 5, 10, 15 or 20 min of centrifugation at RCF of 67.2×g. On increasing the RCF to 1681.1×g, fractionation of lipids to the supernatant from the infranatant was 96.29±1.66% at and above 5 min. We were not able to establish any significant difference (p>0.05) in the lipid content in the supernatant removed after 5, 10, 15, and 20 min of centrifugation at RCF of 1681.1×g. Lipid content in the infranatant reduced significantly as the RCF increased from 67.2×g to 1681.1×g, with p-values establishes significance for the regression model for centrifugal force at a given resident. Changes in the lipid content in the infranatant on increasing the resident time from 5 min to 20 min did not reduced significantly neither at RCF of 67.2×g nor at 1681.1×g, with p-values did not establishes significance for the regression model for resident time at given centrifugal force. Analysis of changes in lipid content in the infranatanat removed at 5, 10, 15, and 20 min of centrifugation at RCF of 1681.1, 6724.3, 15124.8 or 26897.4×g by One-way ANOVA with *post hoc* Tukey's test did not establishes any significance effect of interaction terms on resident time at various centrifugal force at 5% level of significance. Resident time of 5 min at RCF of 1681.1×g is the optimum parameter in effectively removing lipids from the homogenate with is in line with the previous findings[23].

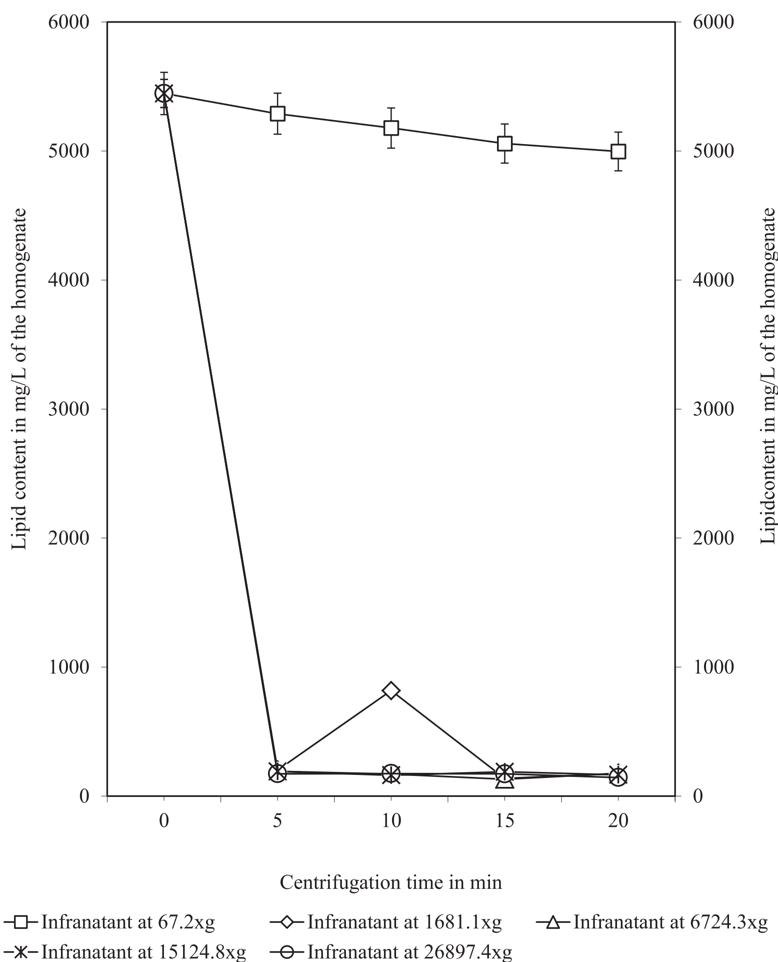

Fig. 3 Changes in lipid content in the infranatant at different centrifugal force and time.

4. Fractionation of the enzyme activity at different centrifugal force and time

During entire 20 min of centrifugation at RCF of 67.2×g, the infranatant lost only 6.70±1.23% enzyme activity(Fig. 4 and 5). Even 5 min of resident time at RCF of 1681.1×g was able to retain 92.03±1.44% of the activity of the homogenate. However RCF of 1681.1×g was alone was able to maintain the specific activity of the hyaluronidase at optimum level. Findings of the current work clearly suggest that lower centrifugal force is effective in retaining hyaluronidase activity even for prolonged centrifugation at 4°C, the findings is in line with the research of [27]. On increasing the resident time beyond 5 min at RCF of 1681.1×g or increasing the centrifugal force from 1681.1×g to 6724.3, 15124.8 or 26897.4×g reduced the hyaluronidase activity by half. At very high centrifugal force tissue homogenates are subjected to fluidic forces resulting in the denaturation of the enzymes due to hydrodynamic sheer force[11]. Productivity of the given centrifuge for a tissue homogenate in clarification can be optimized when purification of the enzymes were made based on the correct laboratory test results[12]. Inappropriate resident time at a given centrifugal force and vice versa is the bottleneck in the effective clarification of the biological fluids[11]. Appropriate *g*-force and resident time of the centrifugation are the two important factors when these bottle necks during the centrifugation are appropriately handled[9]. These two factors has to the handled very carefully during removal of insolubles from the tissue homogenate at the early stage as these insoluble might interfere with the subsequent purification steps.

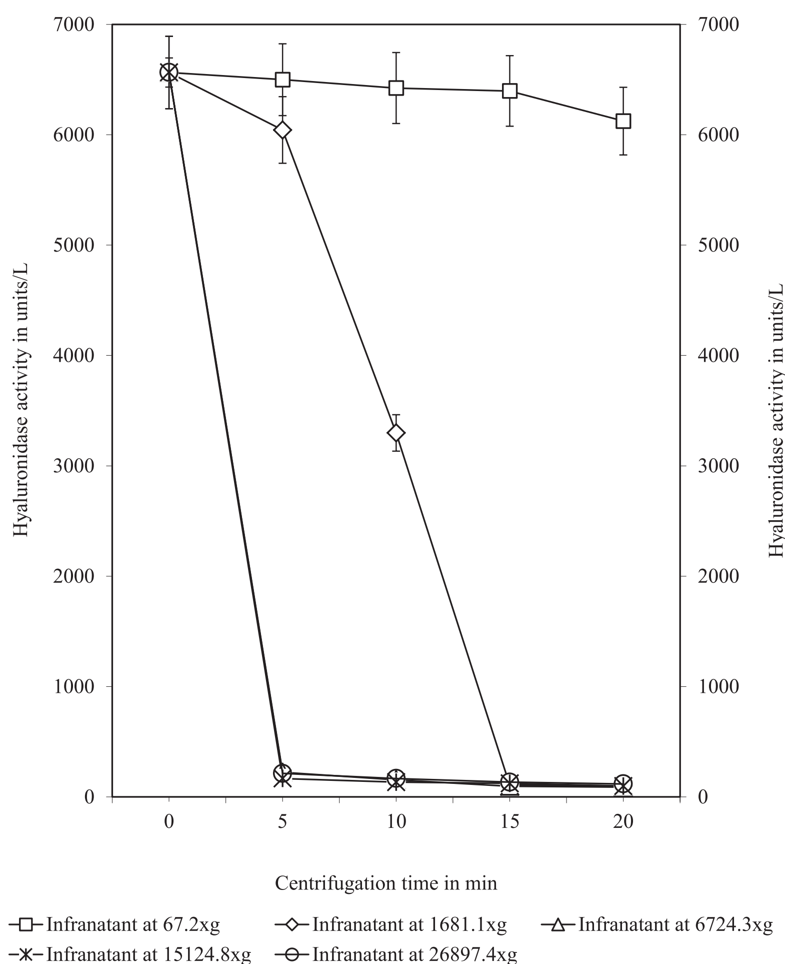

Fig. 4 Changes in hyaluronidase activity in the infranatant at different centrifugal force and time.

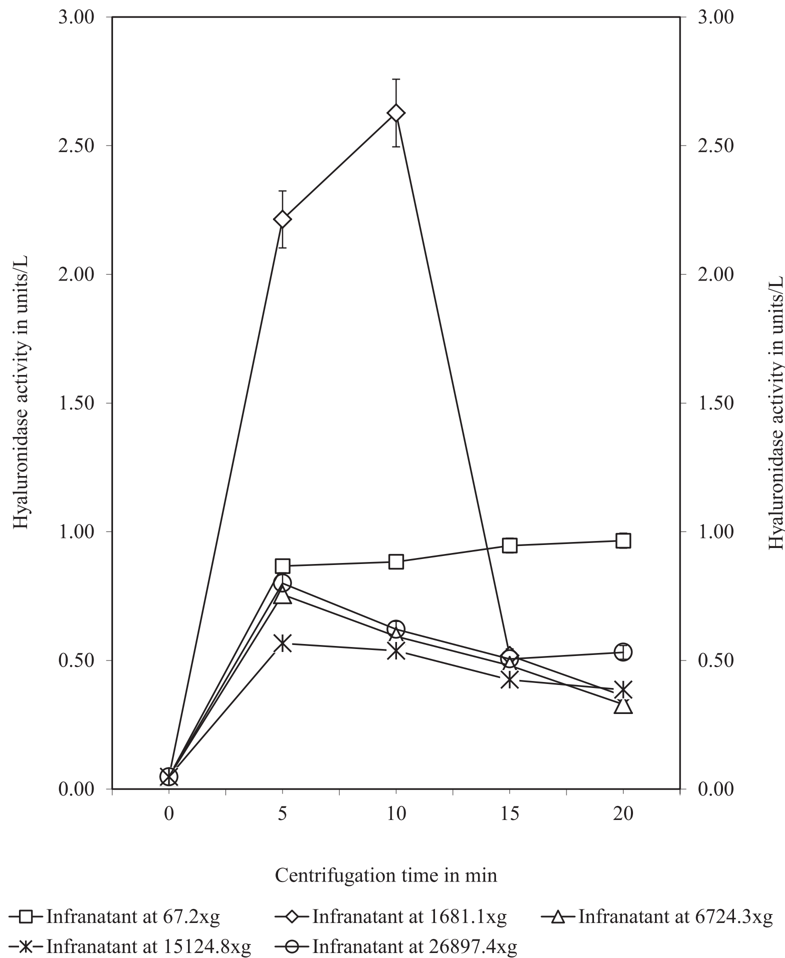

Fig. 5 Changes in Specific activity in the infranatant at different centrifugal force and time.

5. Conclusion

Centrifugal force and resident time are the two crucial factors that affect the effective removal of insolubles from the hepatopancreatic tissue homogenates of shrimps while retaining very high hyaluronidase activity in the infranatant to be used for subsequent purification. Optimum clarification of the tissue homogenates are achieved at RCF of 1681.1×g for 5 min when four fifthe of the solids are removed, one third of the protein impurities were removed, nine tenth of the lipids were fractionated as supernatant, while retaing one ninthe of the hyaluronidase actvity in the hepatopancreatic samples of brown shrimp. Low centrifugal force was inefficient in removing insolubes from the homogenate and high centrifugal force and resident time adverly effected the hualuronidase activity of the homogenate even at low temperature. Hence resident time in the centrifuge and the centrifugal forcer are the two important factor of clarification of biological tissue homogenates.

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