Effective disruption method of shrimp hepatopancreatic tissues to release hyalauronidase

Smitha Blessie Rosario, Krishna Prasad Nooralabettu*

Abstract— Tissues of hepatopancreas of shrimps were homogenized Potter-Elvehjem homogenizer at 15 passes per min in 20 mM Sodium acetate buffer of pH 5.5 and temperature 4°C. Effectiveness of the homogenization was estimated using total protein released and the hyalauronidase activity of the homogenate. Hyaluronidase optimally released at an operating speed of 3000 rpm for 10 min. Operating speed beyond 3000 rpm at given time reduced the activity of hyaluronidase or increase in exposure time beyond 10 min at 3000 rpm caused sheer damage to the enzyme. Pestle speed beyond 3000 rpm did not improve protein yield, but adversly affected the hyalauronidase activity.

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Index Terms— : Hyaluronidase, Shrimps, cell disruption, homogenization, homogenizer.

1 INTRODUCTION

The hepatopancreas is a crucial organ of shrimps in the production, secretion and transportation of hydrolytic enzymes, accumulation of lipids, glycogen and number of minerals[1]. Hyalauronidase can be effectively released from the hepatopancreas provided parameters of homogenization is carefully taken into consideration, because releasing every component of the cells increases the types and number of impurities that makes the subsequent enzyme purification steps cumbersome. B-cells of hepatopancreas release hyalauronidase from time to time after the food intake by the shrimp, careful selection of hepatopancreas, associated tissues and drip is important to improve yield of the enzyme[2]. One needs to establish a strategy to design a cell disruption conditions and select an appropriate cell disruption method to release hyalauronidase effectively with optimum yield and activity. Of the various mechanical and non-mechanical methods, mechanical methods of cell disruption that relay on the shearing and grinding forces are efficient methods[3]. Potter-Elvehjem Glass-pestle Teflon-mortar homogenizer is effectively releases the protein from soft tissues like hepatopancreas of shrimps as the cells does not contain cell wall[4]. Potter-Elvehjem is effective homogenizer because, effectiveness at which homogenization method releases protein affected directly by the mechanical properties of the cell membrane, location of the enzyme, speed of mortar in pestle stoke mortar in through, and the type of the mechanical cell disrupter[5][6][7][8]. Hence, optimising parameters of cell disruption paves the way for the efficient release of commercially important hyalauronidase from the hepatopancreas of shrimps, *Parapenaeopsis stylifera, Penaeus indicus, Solenocera choprai* and *Penaeus monodon*.

- *First author Smitha Blessie Rosario is currently pursuing Doctoral degree program in Biotechnology in Visvesvarayya Technological University, Belgum, India, PH-+0919113558866. E-Mail: smitharosario@yahoo.com*
- *Corresponding author Krishna Prasad Nooralabettu is currently working as Professor and Head, Department of Biotechnology, P. A. College of Engineering, Mangalore, India, PH-+09194485290 48. E-Mail: hod_biotech@pace.edu.in*

2 MATERIALS AND METHODS

2.1 Chemicals

Hepatopancreatic tissues of the shrimps were homogenised using 0.5 M Sodium acetate buffer of pH 5.5 as per ACS[9]. Sodium acetate buffer was used for the homogenisation of the sample. Sodium acetate buffer was constituted in volumetric flask by dissolving 4.1 g of sodium acetate anhydrous in 1 L deionised water. The pH of the homogenizing buffer was adjusted to 5.5 using 0.05 M acetic acid. The acetic acid solution was prepared in 1 L volumetric flak by taking 2.85 mL of glacial acetic acid and making the volume using deionised water. Activity of hyaluronidase in the resulting homogenate was assayed using 0.3 M Sodium Phosphate of pH 5.35 as assay buffer at 37°C. The assay buffer, 0.3 M Sodium Phosphate was prepared by adding 40.2 g sodium sulfate mono basic anhydrous (NaH_2PO_4) to 400 mL of sterile distilled water slowly while warming up the solution on hot plate[10]. The pH of the buffer was adjusted to 5.35 using 1 M NaOH at 37°C. Enzyme diluents was prepared using 20 mM Sodium phosphate with 77 mM Sodium chloride and 0.01 (w/v) Bovine Serum Albumin, of pH 3.58 at 37°C. The 0.3% (w/v) hyaluronic acid solution was prepared by dissolving hyaluronic acid in the Assay buffer and heated to 95°C and then mixed properly upto 20 min to dissolve, and then the preparation was cooled to 37°C. Acid albumin reagent was prepared by adding 24 mM Sodium Acetate with 79 mM Acetic Acid and 0.1% (w/v) Bovine Serum Albumin at pH 3.75 and 37°C at the ratio of 1:1 of HCl:H_2O. All the buffer preparations were filtered and sterilized at 121°C for 20 min.

2.2 Sample Collection

Hepatopancreas for the current work was collected from the shrimps landed in Mangalore "Bunder Area" in the be-

tween July and December month. Shrimps were caught using trawl nets from the Arabian Sea and transported to the Department of Biotechnology, P. A. College of Engineering, Mangalore in insulated container iced at 1:1 ice to shrimp ratio within 2 h. The duration between catching the shrimp and docking at Mangalore may not exceed over 4-6 h. Shrimps were washed, segregated, graded and identified as *Parapenaeopsis stylifera* (Tiny), *Penaeus indicus* (White), *Solenocera choprai* (Red) and *Penaeus* M*onodon* (Black tiger)[11][12][13]. Shrimps were beheaded, hepatopancreatic tissues dissected along with the connecting tissues and drip, packed in plastic bags along with the drip, labeled appropriately, frozen in quick freezers at –40°C, and stored at –20°C in a deep freezer until further use.

2.3 Homogenisation

The frozen hepatopancreas with attached tissues and drip were thawed at 28°C, selected and weighed before the processing. The tissues were homogenized in Potter-Elvehjem homogenizer (Rotek Instruments, Kerala,). Potter-Elvehjem homogenizer is made up of Teflon pestle tight fitted into glass mortar with a gap of about 0.1-0.2 mm. Hepatopancreatic tissues were chopped into a size of 1 mm in cross section using scissor, and transferred to glass-pestle along with at 8 fold volume of homogenisation buffer. The pestle was run using electric motor and speed was controlled using speed controller. Glass mortar containing tissues and buffer were raised and lowered at a constant speed of 15 passes/min. Tissues were homogenised as it moved through a narrow gap of the rough teflon-mortar and ground inner wall of the glass-pestle. Temperature of the tissues during homogenisation was maintained by keeping the holding tank in cooling jacket at 4°C. Effect of homogenisation speed on the protein release was performed at a speed of 1000, 2000, 3000, 4000, 5000 or 6000 rpm for 10 min. Effect of duration of homogenisation on protein release was performed for intervals of 5, 10, 15, 20, 25, and 30 min at 3000 rpm. The pestle head speed was controlled. Effectiveness of the homogenisation parameter on the yield of protein and the hyaluronidase was estimated using total released protein and the hyaluronidase activity in the homogenate.

2.4 Enzyme assay

Activity of hyaluronidase was performed using turbidometic assay that estimates the percentage of transmittance at 600 nm. One unit of hyaluronidase activity in the homogenate causes a change in A_{600} of 0.33 per min at 37°C in 2 mL reaction mixture in 42 min. First, 2 mL of reaction mixture was prepared containing 160 mM of sodium phosphate, 39 mM of sodium chloride, 0.005% (w/v) of bovine serum albumin, 0.015% (w/v) of hyaluronic acid, and appropriate amount of sample. Here 1 mL of the sample was mixed with 1 mL of the hyaluronic acid solution. The reaction mixture was swirled and equilibrated to 37°C and pH 5.55 for 10 min. After 45 min of reaction, 0.5 mL aliquot of reaction mix was added to 2.5 mL of acidic albumin, mixed well by inversion, exactly after 10 min at room temperature, turbidity was measured at 600 nm. Turbidity decreases with decrease in hyaluronic acid after the enzyme action. Blank was maintained. Calibration curve of hyaluronic acid quantification was done using stock solution of 0.3% of hyaluronic acid. Aliquot amount of (0.0, 0.1, 0.2, 0.3, 0.4, 0.5 mL) of 0.3% of hyaluronic acid was pipetted out in different test tubes and volume was made upto 0.5 mL using phosphate buffer and into each test tube 2.5ml of acidic albumin was added. Reaction mixture was incubated at room temperature for 10 min. Turbidity was measured at 600 nm and calibration curve was plotted to converts the absorbance to concentration of the substance.

2.5 Statistical analysis

Proximate analysis of the samples was performed in quadruplicate and results obtained were analysed and treated by analysis of variance (ANOVA). And this was followed by Tukey's test, using the software Statistica 6.0 (Statsoft, Tulsa, OK, USA). The final values were expresses as± standard deviations and all analyses were performed considering a confidence level of 95% ($p < 0.05$). Protein was estimated by Lawry's method[14].

3 RESULTS

3.1 Effect of motor speed on protein release

Effectiveness of the speed of the motor of Potter-Elvehjem homogenizer on optimally releasing protein from tissues was performed at speed of 1000, 2000, 3000, 4000, 5000 or 6000 rpm for 10 min at the ratio of 1:8 hepatopancreas to 0.5 M Sodium acetate buffer of pH 5.5. Changes in the released protein from the hepatopancreatic tissue homogenate of shrimps at different homogenization speed are illustrated in the figures 1-4. Total released protein and the hyaluronidase activity of the homogenate was performed to determine the effectiveness.

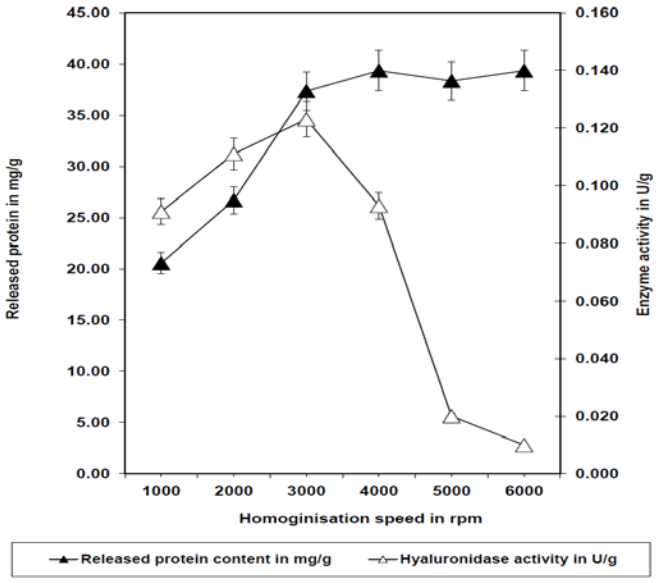

Fig. 1 Hyaluronidase and protein release from the hepatopancreatic tissues of Tiny shrimp at different homogenisation speed for 10 min

Increase in the speed of the pestle from 1000 rpm to 2000 rpm resulted in 1.30, 1.24, 1.45, and 1.42 folds increase in the release of protein from the hepatopancreatic tissues of Tiny, White, Red and Black Tiger shrimps, respectively.

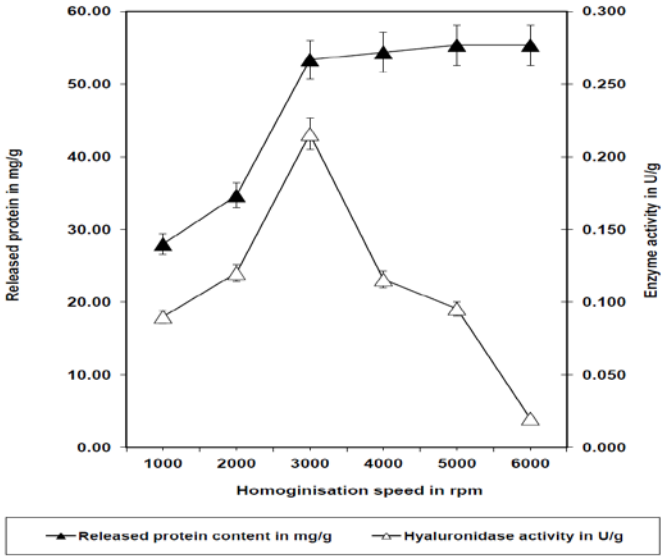

Fig. 2 Hyaluronidase and protein release from the hepatopancreatic tissues of White shrimp at different homogenisation speed for 10 min

When the speed of the motor was increased to 3000 rpm, protein released to the medium was 1.82, 1.91, 1.88, and 1.93 folds in comparison to the protein released at 1000 rpm for 10 min, respectively, in hepatopancreatic tissues of Tiny, White, Red and Black Tiger shrimps at the end of 10 min. It is very interesting to note here that when the hepatopancreatic tissues were passed through narrow clearance between rough Teflon pestle and ground surface of glass mortar at constant jerk of 15 passes/min, significant (p<0.05) rise in the quantity of protein released from the tissues were registered on increasing the motor speed from 1000 rpm to 3000 rpm.

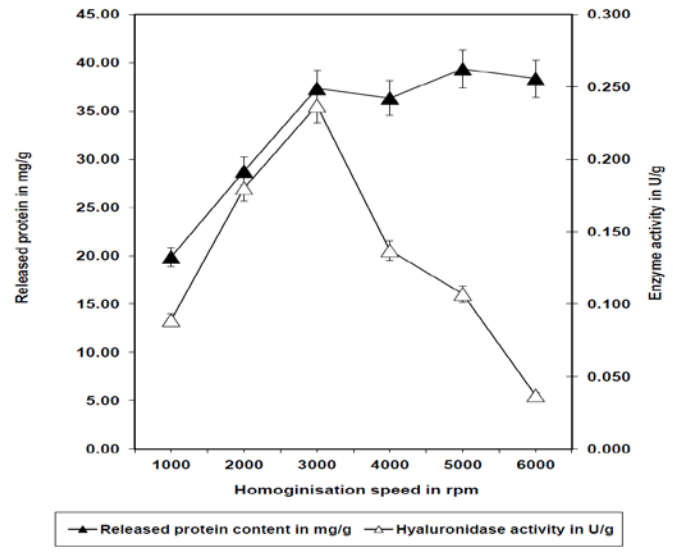

Fig. 3 Hyaluronidase and protein release from the hepatopancreatic tissues of Red shrimp at different homogenisation speed for 10 min

We have not registered any significant difference in the quaintly of the protein released from the hepatopancreatic tissues of Tiny, White, Red and Black Tiger shrimps on increasing the speed of motor beyond 3000 rpm even at the end of 10 min of homogenization. Motor speed of 3000 rpm with 150 passes in 10 min at 4°C through narrow gap between rough Teflon pestle and ground surface of glass mortar was able to effectively rupture hepatopancreatic tissues of Tiny, White, Red and Black Tiger shrimps to the medium.

3.2 Effect of motor speed on enzyme activity

Effectiveness of the motor head speed of the homogenizer in maintaining the activity of hyaluronidase was conducted at speed of 1000, 2000, 3000, 4000, 5000 or 6000 rpm for 10 min at the ratio of 1:8 hepatopancreas to homogenization buffer. Here, homogenization was carried out in 0.5 M Sodium acetate buffer of pH 5.5 and assay of hyaluronidase was performed using 0.3 M Sodium Phosphate of pH 5.35.

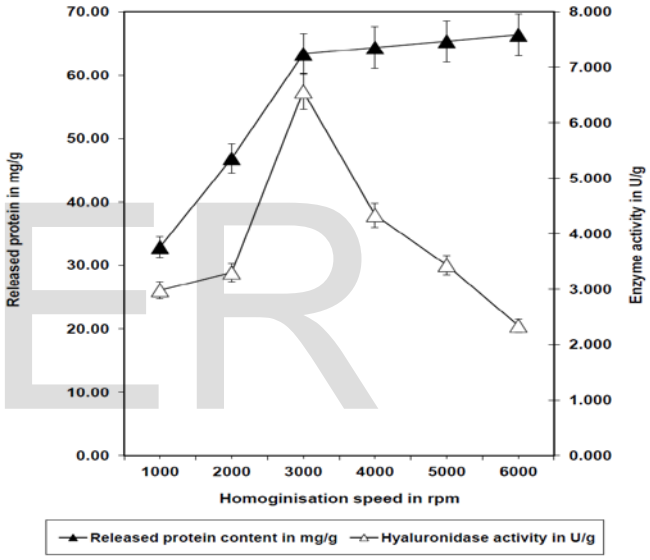

Fig. 4 Hyaluronidase and protein release from the hepatopancreatic tissues of Black tiger shrimp at different homogenisation speed for 10 min

Changes in the hyaluronidase activity of the hepatopancreatic tissue homogenate of shrimps at different homogenization speed are illustrated in the figure 1-4. During homogenization, activity of the hyaluronidase in the homogenates of hepatopancreatic tissues of Tiny, White, Red and Black Tiger shrimps increased by 1.22, 1.33, 2.02, and 1.10 folds when the speed of the motor increased for 1000 rpm to 2000 rpm, respectively. On increasing the speed of the motor to 3000 rpm resulted in homogenates of hepatopancreatic tissues of Tiny, White, Red and Black Tiger shrimps with the hyaluronidase activity that is 1.35, 2.39, 2.66, and 2.20 folds more that the activity observed in homogenates obtained at 1000 rpm at the end of 10 min.

Increase in speed of the motor beyond 3000 rpm did not significantly (p>0.05) change protein content of the homogenate in comparison to the protein content of the homogenate at 3000 rpm. It is very interesting to note here that we have registered significant (p<0.05) difference in the hyaluronidase

activity between the samples homogenized at 3000 rpm for 10 min and samples homogenized at 4000, 5000 or 6000 rpm for 10 min.

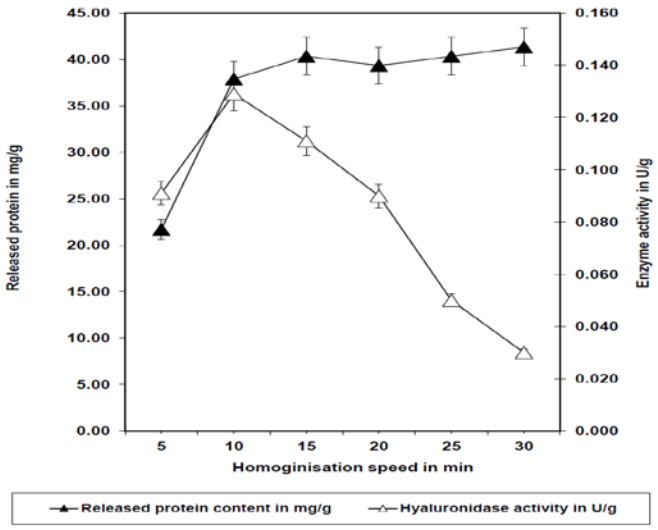

Fig. 5 Hyaluronidase and protein release from the hepatopancreatic tissues of Tiny shrimp at different homogenisation at 3000 rpm for different time intervals

Beyond motor speed of 3000 rpm activity of hyaluronidase affected adversely even when the homogenate maintained at 4°C. Homogenization speed of less than 3000 rpm was not effective in releasing all the protein and proteins with hyaluronidase activity and beyond 3000 rpm adversely affected the enzyme activity without further improving the protein yield. In this experiment, effectiveness of the homogenization was determined by taking both the protein released to the medium and the hyaluronidase activity of the homogenate.

3.3 Effect of time on homogenization on protein release

Effectiveness duration homogenization time through narrow clearance between rough Teflon-pestle and ground surface of glass mortar at a fixed speed of 3000 rpm for 5, 10, 15, 20, 25 and 30 min at 1:8 tissue to buffer ratio on the protein release was determined (Fig. 5-8).

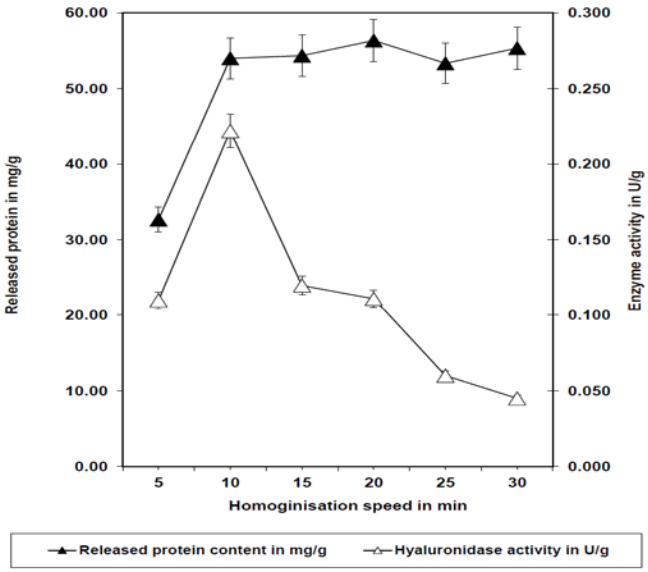

Fig. 6 Hyaluronidase and protein release from the hepatopancreatic tissues of White shrimp at different homogenisation at 3000 rpm for different time intervals

At constant jerk through the homogenizer at 3000 rpm, increase in exposure time from 5 min to 10 min significantly (p<0.05) increased the protein from the tissues. Beyond 10 min of exposure at 3000 rpm no significant effect (p>0.05) on the release of the protein from the tissues were registered even up to 30 min.

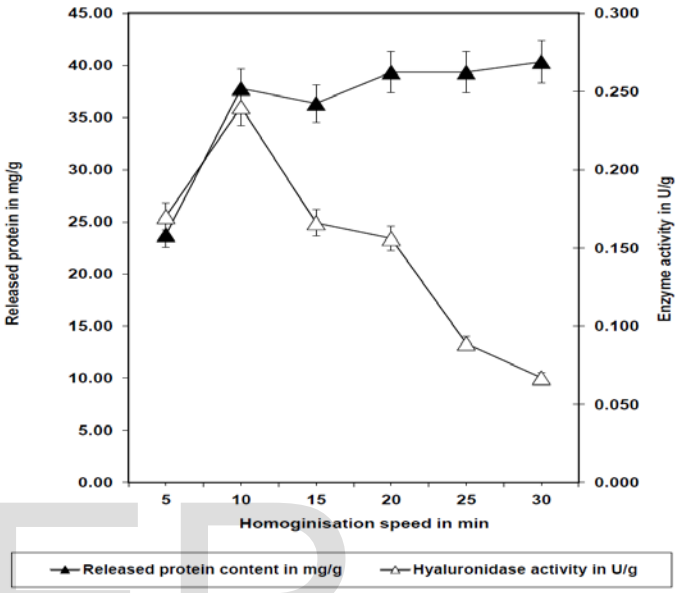

Fig. 7 Hyaluronidase and protein release from the hepatopancreatic tissues of Red shrimp at different homogenisation at 3000 rpm for different time intervals

Similarly, on increasing exposure time from 5 min to 10 min significantly (p<0.05) increased the hyaluronidase activity of the tissue homogenates. When exposure of the tissues to the homogenizer increased from 10 min to 15, 20, 25 or 30 min, activity of the hyaluronidase in hepatopancreatic tissue homogenates reduced significantly, even when the temperature of the homogenate maintained at 4°C. Constant jerk of 15 passes/ min through narrow clearance between rough Teflon-pestle and ground surface of glass-mortar at a fixed motor speed of 3000 rpm for 10 min using 0.5 M Sodium acetate buffer of pH 5.5 and temperature 4°C at 1:8 tissue to buffer ratio optimally released proteins with optimum hyaluronidase activity. Protein released from the hepatopancreatic tissue homogenates of Tiny, White, Red and Black Tiger shrimps increased, respectively, by 1.75, 1.65, 1.59, and 1.10 folds at motor speed of 3000 rpm for 10 min in comparison to the protein content of the homogenates at 1000 rpm for 10 min.

3.4 Effect of time of homogenisation on enzyme activity

Effectiveness of duration of jerk of 15 passes/min through narrow clearance between rough Teflon-pestle and ground surface of glass mortar at a fixed speed of 3000 rpm for 5, 10, 15, 20, 25, or 30 min at 1:8 tissues to buffer ratio on the Hyaluronidase activity was performed. Hyaluronidase activity of the hepatopancreatic tissue homogenates of Tiny, White,

Red and Black Tiger shrimps increased, respectively, by 1.41, 1.02, 1.41, and 2.12 folds at motor speed of 3000 rpm for 10 min in comparison to the activities of the homogenates at 1000 rpm for 10 min. Eventhough increase in time exposure from 5 min to 10 min at 3000 rpm significantly increased the release of protein (p<0.05) in comparison to the protein content of the samples exposed for 5 min at 3000 rpm, beyond 10 min no significant (p>0.05) change in the protein release was registered.

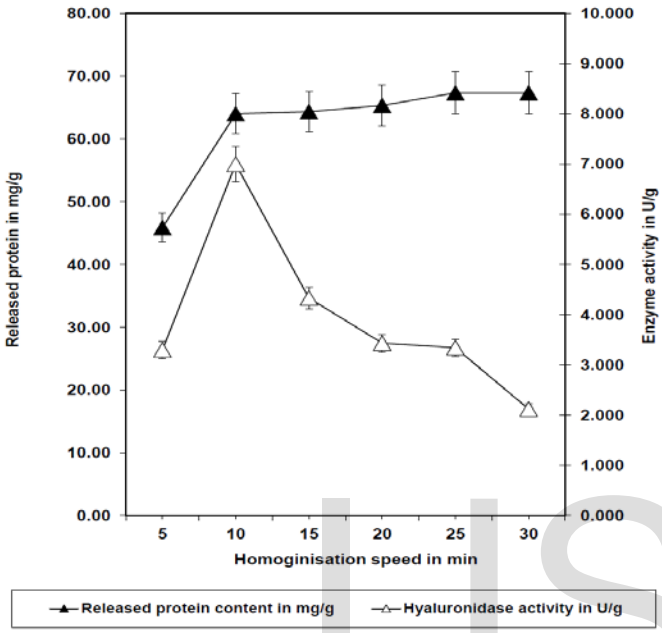

Fig. 8 Hyaluronidase and protein release from the hepatopancreatic tissues of Black Tiger shrimp at different homogenisation at 3000 rpm for different time intervals

Here, we were not able to register any significant (p>0.05) difference in the released protein contents of the samples homogenized at 3000 rpm for 10, 15, 20, 25 and 30 min, but were able to establish a significant (p<0.05) difference in the released protein in samples homogenized at 3000 rpm for 5 min and samples homogenized at 3000 rpm for 10, 15, 20, 25 or 30 min. Hyaluronidase activity of the homogenate at the end of 30 min of homogenisation was only 23, 20, 28, and 30% of the activity registered in the in Tiny, White, Red and Black Tiger samples removed at the end of 10 min, respectively. In our study, at constant jerk of 15 passes/min through narrow clearance between rough Teflon-pestle and ground surface of glass mortar at a fixed speed of 3000 rpm for 10 min using 0.5 M Sodium acetate buffer of pH 5.5 and temperature 4^{o}C at 1:8 tissue to buffer ratio was optimum condition for homogenisation to release hyaluronidase from the hepatopancreas, and beyond which the activity adversely gets affected.

4 DISCUSSION

Release of hyaluronidase from hepatopancreatic tissues of shrimps is very important for achieving purification strategies like yield, activity and purity as these may affect the subsequent purification steps and loss yield, activity and purity affect the final product[15]. The protein released during the initial stages of homogenisation follows the first order reaction kinetics and rate of release of the protein is directly proportional to the applied force input per volume of the tissues up to 3000 rpm for 10 min[16].

Effectiveness of the homogenization depend on the factors such as cell type, pestle speed, sample volume, cell concentration, exposure time, recovery extent, and final quality of the homogenate[17]. There are published results showing the non linear release of proteins as we have registered no changes in protein content beyond 3000 rpm for 10 min[18]. Very high pressure might degrade the protein and at this speed protein might result in rupture and loss of activity[19]. Multiple pumping of hepatopancreatic tissues through narrow clearance between rough Teflon pestle and ground surface of glass mortar at constant jerk of 15 passes/min exerted continuous extensive fluid forces on protein during release[20]. Hydrodynamic shear force of homogenization process beyond 3000 rpm might have damaged the freely suspended hyaluronidase enzyme in the hepatopancreatic tissue homogenates of shrimps might denature or inactivate enzymes[21].

In this experiment effectiveness of the homogenization was determined by taking both the protein released to the medium and the hyaluronidase activity of the homogenate. This is because during the initial stage of enzyme purification such as cell disruption yield of the process is quantified in terms of the protein released by the process rather than the specific activity of the enzyme[22].

Eventhough increase in time exposure from 5 min to 10min at 3000 rpm significantly increased the release of protein (p<0.05) in comparison to the protein content of the samples exposed for 5 min at 3000 rpm, beyond 10 min no significant (p>0.05) change in the protein release was registered. This is because hepatopancreatic tissues do not have tough cellular organization and absence of rigid cell wall making it more prone to disruption process using Potter-Elvehjem homogenizer in comparison to cells with tough cell wall [23]. Reason for no increase in released protein in the homogenate produced beyond 10 min of exposure at 3000 rpm might be due to the maximum possible release of proteins from the tissues. This true as we were not able to register any significant (p>0.05) difference in the released protein contents of the samples homogenized at 3000 rpm for 10, 15, 20, 25 and 30 min, but were able to establish a significant (p<0.05) difference in the released protein in samples homogenized at 3000 rpm for 5 min and samples homogenized at 3000 rpm for 10, 15, 20, 25 or 30 min.

Some of the previous repost suggest that most of the free enzymes are susceptible to high pressure homogenizer to significant levels, which holds true at and below 3000 rpm for 10 min[24] However, activity of hyaluronidase affected to significant level (p<0.05) above 3000 rpm for 10 min or above 10 min at 3000 rpm as indicated by ANOVA with *post hoc* Tukey's test, the results is in conformation with some other reported works suggest that prolonged homogenisation adversely affects the activity of enzyme[3][25]. These findings are in confirmation with the previous work suggesting that pressure of Microfluidizer has greater impact on the amino peptidase loss in *Lactobacillus casei* than the number of passes through homogenizer[26].

In our study, at constant jerk of 15 passes/min through narrow clearance between rough Teflon-pestle and ground surface of glass mortar at a fixed speed of 3000 rpm for 10 min using 0.5 M Sodium acetate buffer of pH 5.5 and temperature 4°C at 1:8 tissue to buffer ratio was optimum condition for homogenisation to release hyaluronidase from the hepatopancreas, and beyond which the activity adversely gets affected.

5 CONCLUSION

Speed of homogenisation and duration of exposure in Potter-Elvehjem homogenizer plays a major role in optimally releasing protein from hepatopancreatic tissues of Tiny, White, Red and Black Tiger shrimps and maintaining optimum hyaluronidase activity in tissue homogenate at constant jerk of 15 passes/min, at temperature of 4°C, in 0.5 M Sodium acetate buffer of pH 5.5 and at 1:8 tissue to buffer ratio. Adverse homogenisation conditions such as increased speed of homogenisation and more exposure of tissues in Potter-Elvehjem homogenizer adversely affects the activity of the hyaluronidase activity due to hydrodynamic sheer effects. Insufficient homogenisation speed and less exposure to homogenisation process may affect the yield of the protein of the homogenate. In our study optimum recovery of the hyaluronidase was achieved at constant jerk of 15 passes/min through narrow clearance between rough Teflon-pestle and ground surface of glass mortar at a fixed speed of 3000 rpm for 10 min using 0.5 M Sodium acetate buffer of pH 5.5 and temperature 4°C at 1:8 tissues to buffer ratio.

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