Effect of pH and components of buffer on the hyaluronidase activity in hepatopancreatic tissues of shrimps during homogenization

Smitha Blessie Rosario, Krishna Prasad Nooralabettu*

Abstract— Components and pH of the homogenization buffer and assay buffer plays a major role in the activity of the hyaluronidase. Hepatopancreatic tissues of shrimps were disrupted at 3000 rpm/10 min using various homogenization buffers like 0.5 M Sodium acetate buffer of pH 5.5, 2 M KCl solution of pH 5.35, Deionised water of pH 7.4, 0.1 M Tris-HCl buffer of pH 8.4 and 0.1 M Glycine-NaOH buffer of pH 9.0. Hyaluronidase activity of each of the resulting homogenates was performed using acidic hyaluronic acid as a substrate in either respective homogenization buffer or 0.3 M Sodium Phosphate of pH 5.35 as assay buffer. Tissues homogenized using 0.5 M Sodium acetate buffer of pH 5.5 as a homogenization buffer exhibited optimum hyaluronidase activity when assayed using 0.3 M Sodium Phosphate of pH 5.35 in comparison to the samples homogenized using buffers of higher pH.

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Index Terms— : Hyaluronidase, Shrimps, cell disruption, homogenization, buffer, assay, pH, hepatopancreas.

1 INTRODUCTION

Hyaluronidase is a is referred to group of dissimilar enzymes that degrade high-molecular-weight glycosaminoglycan of extracellural matter known as hyaluronic acid or hyaluronam. Of these enzymes some degrades additional glycosaminoglycan such as albeit at slower rate. Eukaryotic hyaluronidase exhibit both hydrolytic and transglycosic activity, and bacterial hyaluronidase exhibit β-eliminating activity[1]. Purified hyaluronidase has commercial angle where it can be used to degrade hyaluronic acid that plays crucial role in rapid cell proliferation and tumerigenisis, as this enzyme controls the logistics and deposition of hyaluronic acid. Since, hyaluronic acid is crucial for embryogenesis, repair, wound healing, and regeneration, hyaluronidase is often found in very lower concentration, but at high often unstable activity[2]. Lack of sensitivity of most of the available assay methods and instability of the hyaluronidase made it as one of the most neglected enzyme[3]. This makes the purification of hyaluronidase cumbersome and requires careful consideration of factors that might adversely affect the activity making its purification much more complicated. Hyaluronidase activity gets affected adversely by important interdependent factors such as pH and components of the medium. Acetate buffer is reported to have inhibitory effect on the hyaluronidase activity of embryonic chic brain extract, in comparison to formate buffer even at same pH[4]. Since hyaluronidase inhibitors are ubiquitous and reported to inhibit hyaluronidase at low concentrations, it is very important to compare and establish an optimum buffer to assay and purify such as unstable but commercially important enzyme. Formulating suitable components of buffer of non inhibitory in nature at suitable pH both for both assay and homogenisation of shrimp hepatopancreas is crucial for isolating it with optimum activity, purity and yield. Present study aimed at comparing the effect of components and pH of homogenization buffer and assay buffer on the hyaluronidase activity during homogenisation of hepatopancreatic tissue of shrimps, *Parapenaeopsis stylifera* (Tiny), *Penaeus indicus* (White), *Solenocera choprai* (Red) and *Penaeus monodon* (Black tiger).

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2 MATERIALS AND METHODS

2.1 Chemicals

Buffers used for the disruption of hepatopancreatic tissues of Tiny, White, Red and Black tiger were prepared as per ACS[5]. Various buffers such as 0.5 M Sodium acetate buffer of pH 5.5, 2 M KCl solution of pH 7.00, Deionised water of pH 7.4, 0.1 M Tris-HCl buffer of pH 8.4 and 0.1 M Glycine-NaOH buffer of pH 9.0 was used for the homogenisation of the sample. The homogenates so obtained were assayed either using respective homogenisation buffer as assay buffer or using 0.3 M Sodium Phosphate of pH 5.35 as assay buffer. Homogenization buffer of pH 5.5, 0.5 M Sodium acetate buffer was constituted in 1L volumetric by adding 4.1 g of sodium acetate anhydrous using deionised water. The pH of the Sodium acetate buffer was adjusted to 5.5 by adding 0.05 M acetic acid. Here, 0.05 M Acetic acid was constituted in volumetric flask by taking 2.85 mL of glacial acetic acid and adjusting the volume to 1L using deionised water. Homogenization buffer of pH 7.0, 2 M Potassium chloride (KCl) solution was constituted by mix-

ing 149.1 g of potassium chloride in 1 L of deionised water in volumetric flask. The pH the KCl was brought to 7 by adding 0.1 M NaOH solution.

Homogenisation buffer of pH 8.4, 0.1 M Tris-HCl buffer was constituted by mixing 12.111 g of the free Tris base in 900 mL of deionised water. The pH of the buffer was adjusted to 8.4 by using 1 M HCl solution. Here, the volume of the Tris-HCl buffer was made up to 1L. Homogenization buffer of pH 9.0, 0.1 M Glycine-NaOH buffer was constituted in 1 L volumetric flask with 7.5 g of glycine and making the volume to 1 L using deionised water after adjusting the pH to 9.5 using concentrated NaOH solution when the volume in the flask was 900 mL.

The assay buffer, 0.3 M Sodium Phosphate was prepared by slowly mixing 40.2 g sodium sulfate mono basic anhydrous (NaH_2PO_4) in 400 mL of deionised water while warming up the solution on hot plate[6]. The pH of the buffer was brought to 5.35 using 1 M NaOH at 37°C.

Enzyme diluents was constituted 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 constituted by mixing hyaluronic acid in the Assay buffer, then heating to 95°C, mixed thoroughly upto 20 min, and cooling to 37°C.

Acid albumin reagent was constituted by mixing 24 mM Sodium Acetate with 79 mM Acetic Acid and 0.1% (w/v) Bovine Serum Albumin of pH 3.75 at 37°C, where the ratio of HCl:H_2O is 1:1. The entire buffer were filtered carefully and sterilized at 121°C in moist heat for 20 min. All the chemicals and reagents used were of analytical grade and were obtained from Merck Limited (Mumbai, India).

2.2 Sample Collection

Hepatopancreas for the current work was collected from the *Parapenaeopsis stylifera* (Tiny), *Penaeus indicus*(White), *Solenocera choprai* (Red) and *Penaeus monodon* (Black tiger)were caught using trawl nets from the Arabian Sea landed in "Bunder Area" of Mangalore in the month of July. Shrimps were transported to the Department of Biotechnology, P. A. College of Engineering, Mangalore in an insulated container iced at 1:1 ice to shrimp ratio within 2 h. The total time gap between the catching the shrimp and landing at Mangalore landing centre 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 monodon* (Black tiger)[7][8][9]. Hepatopancreatic tissues were dissected from the thorax along with the connecting tissues, and the tissues along with the drip were packed in plastic bags and frozen in quick freezers at –40°C after labeling. Frozen samples were stored at –20°C in a deep freezer until further use.

2.3 Homogenisation

The frozen hepatopancreas along with the attached tissues and drip were thawed at 28°C, segregated and weighed. These samples were disrupted in Potter-Elvehjem homogenizer (Rotek Instruments, Kerala,) to release proteins and activity of the released proteins were estimated for hyaluronidase activity at a motor speed of 3000 rpm for 10 min at 4°C. Homogenisation of hepatopancreatic tissue of Tiny, White, Red and Black tiger was carryout using buffers like 0.5 M Sodium acetate buffer of pH 5.5, 2 M KCl solution of pH 5.35, Deionised water of pH 7.4, 0.1 M Tris-HCl buffer of pH 8.4, or 0.1 M Glycine-NaOH buffer of pH 9.0. Samples were assayed for hyaluronic acid [6] and Total protein[10]

2.4 Enzyme assay

Hyaluronidase activity of the homogenate thus obtained were performed using respective homogenization buffer as Assay buffer instead of sodium phosphate buffer or using 0.3 M Sodium Phosphate as an assay buffer. Hyaluronidase activity of the homogenates prepared in sublots using different homogenization buffers were 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.

Initially, 1 mL of the sample is mixed with 1 mL of the hyaluronic acid solution. First, 2 mL of reaction mixture was constituted using 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 homogenate. The reaction mixture was mixed properly for 10 min at 37°C and pH 5.55. After 45 min of incubation, 0.5 mL aliquot of reaction mixture was again mixed thoroughly with 2.5 mL of acidic albumin and incubated at 10 min at room temperature. Turbidity was measured at 600 nm exactly after 10 min of incubation at room temperature. Turbidity decreases with decrease in hyaluronic acid after the enzyme action. Blank was maintained.

Calibration curve of hyaluronic acid quantification was plotted using stock solution of 0.3% of hyaluronic acid. Aliquot amount, 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 2.5 mL 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

Estimation of the protein content and hyaluronidase was performed in quadruplicate and results obtained were analysed and treated by analysis of variance (ANOVA). Tukey's test was performed 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).

3 RESULTS

3.1 Effect of pH and component of buffer on the activity hyaluronidase of Tiny

Total protein released in the tissue homogenates of Tiny shrimp was 36.99±0.78 mg/g using respective buffer (Fig. 1).

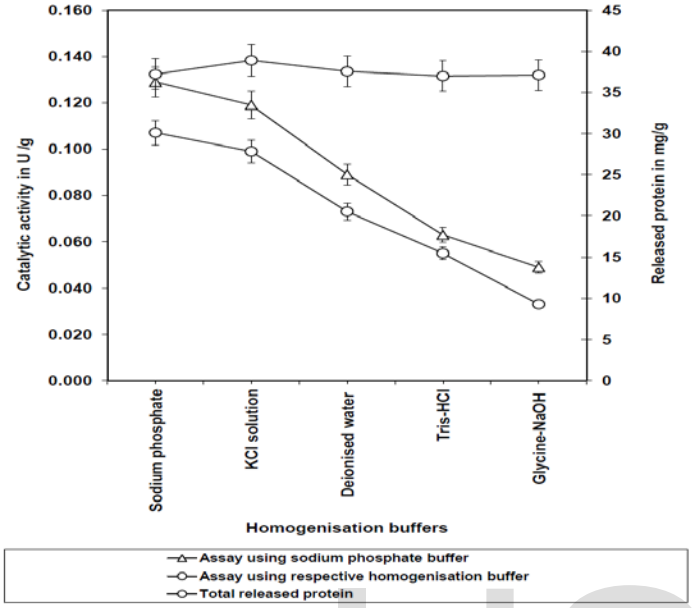

Fig. 1 Hyaluronidase activity of the released protein from hepatopancreatic tissues of *Parapenaeopsis stylifera* (Tiny) using different buffers.

ANOVA with *post hoc* Tukey's test was not able to establish any difference in the released protein from the tissues using any of the different homogenisation buffers used. Proteins of tissue homogenates in 0.5 M Sodium acetate buffer showed maximum hyaluronidase activity when assayed using 0.3 M Sodium phosphate buffer, as indicated by ANOVA with *post hoc* Tukey's test. Hyaluronidase activity was 0.107±0.007, 0.099±0.009, 0.073±0.007, 0.055±0.004 and 0.033±0.005 U/g when both homogenised and assayed using 0.5 M Sodium acetate buffer of pH 5.5, 2 M KCl solution of pH 7.00, Deionised water of pH 7.4, 0.1 M Tris-HCl buffer of pH 8.4 and 0.1 M Glycine-NaOH buffer of pH 9.0, respectively. When only assay buffer was changed from respective homogenization buffer to 0.3 M Sodium phosphate buffer, hyaluronidase activity, respectively, was 0.129±0.007, 0.119±0.009, 0.089±0.007, 0.063±0.004, and 0.049±0.005 U/g of homogenate. When the assay buffer was changed from respective homogenization buffer to 0.3 M Sodium phosphate buffer, hyaluronidase activity increased significantly (p<0.01), as indicated by ANOVA with *post hoc* Tukey's test. No significant difference was observed in hylauronidase when the homogenated were homogenated and assayed using 0.3 M Sodium phosphate buffer. Here, increase in pH significantly (p<0.01) decreases the activity of the hyaluronidase. Maximum activity was registered at pH 5.5, but activity was significant even at pH 9.0.

3.2 Effect of pH and component of buffer on the activity hyaluronidase of white shrimp

In white shrimp, protein released from the tissue homogenates was 53.32±1.24 mg/g (Fig. 2). The homogenates prepared using 0.5 M Sodium acetate buffer of pH 5.5, 2 M KCl solution of pH 7.00, Deionised water of pH 7.4, 0.1 M Tris-HCl buffer of pH 8.4 and 0.1 M Glycine-NaOH buffer of pH 9.0 did not show any difference in protein content, as indicated by ANOVA with *post hoc* Tukey's test.Tissue homogenates in 0.5 M Sodium acetate buffer showed maximum hyaluronidase activity of 0.184±0.004 U/g when assayed using 0.3 M Sodium phosphate buffer. Hyaluronidase activity in homogenate with 2 M KCl solution, Deionised water, 0.1 M Tris-HCl buffer and 0.1 M Glycine-NaOH buffer was 0.166±0.003, 0.119±0.004, 0.106±0.007 and 0.066±0.006 U/g when both homogenized and assayed using these buffers, respectively. When homogenates prepared using 0.5 M Sodium acetate buffer, 2 M KCl solution, Deionised water of, 0.1 M Tris-HCl buffer and 0.1 M Glycine-NaOH buffer was assayed using 0.3 M Sodium phosphate buffer, hyaluronidase activity, respectively, was 0.222±0.003, 0.200±0.003, 0.142±0.004, 0.122±0.008 and 0.092±0.005. Hyaluronidase activity increased significantly (p<0.01), when the assay buffer was changed from respective homogenization buffer to 0.3 M Sodium phosphate buffer. However, we were not able to establish any significant (p>0.01) difference in hylauronidase when homogenated and assayed using 0.3 M Sodium phosphate buffer. Increase in pH of the buffers significantly Optimum hyaluronidase activity was registered at pH 5.5, but activity was significant even at pH 9.0.

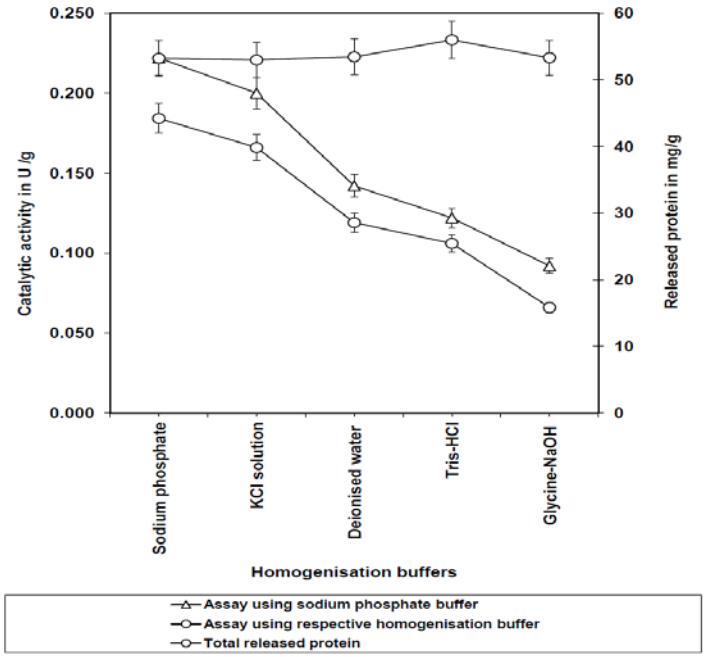

Fig. 2 Hyaluronidase activity of the released protein from hepatopancreatic tissues of *Penaeus indicus* (White) using different buffers.

3.3 Effect of pH and component of buffer on the activity hyaluronidase of brown shrimp

Released protein in the tissue homogenates of brown shrimp was 38.54±0.93 mg/g using 0.5 M Sodium acetate buffer of pH 5.5, 2 M KCl solution of pH 7.00, Deionised water of pH 7.4, 0.1 M Tris-HCl buffer of pH 8.4 and 0.1 M Glycine-NaOH buffer of pH 9.0 (Fig. 3).

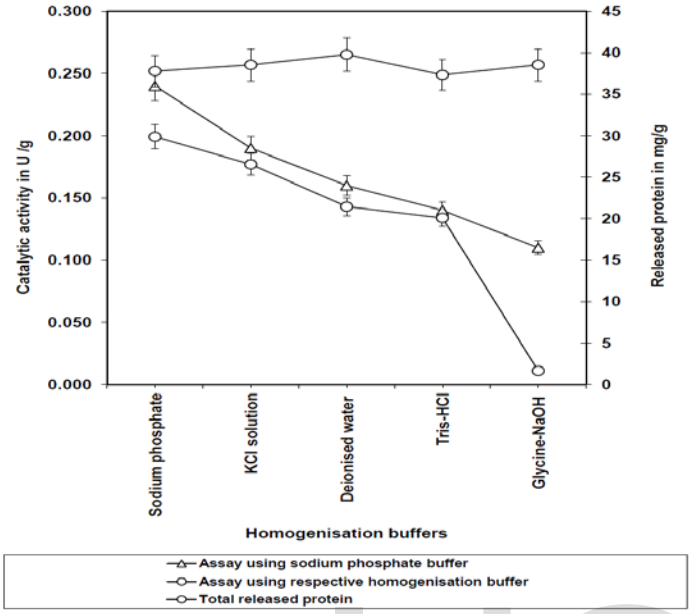

Fig. 3 Hyaluronidase activity of the released protein from hepatopancreatic tissues of *Solenocera choprai* (Red) using different buffers.

We were not able to establish any significant (p<0.05) difference in the released protein from the tissues using any of the homogenisation buffers. Tissue homogenates in 0.5 M Sodium acetate buffer showed optimum hyaluronidase activity on assaying using 0.3 M Sodium phosphate buffer. Catalytic activity was 0.199±0.004, 0.177±0.005, 0.143±0.006, 0.134±0.003 and 0.011±0.006 U/g when both homogenised and assayed using 0.5 M Sodium acetate buffer of pH 5.5, 2 M KCl solution of pH 7.00, Deionised water of pH 7.4, 0.1 M Tris-HCl buffer of pH 8.4 and 0.1 M Glycine-NaOH buffer of pH 9.0, respectively. On changing the assay buffer from respective homogenization buffer to 0.3 M Sodium phosphate buffer, hyaluronidase activity, respectively, was 0.240±0.004, 0.190±0.005, 0.160±0.006, 0.140±0.003 and 0.110±0.006 U/g of homogenate. Changing respective homogenization buffer to 0.3 M Sodium phosphate buffer, enzyme activity increased significantly (p<0.05), as indicated by ANOVA with *post hoc* Tukey's test. We have not registered any significant (p>0.05) difference in hylauronidase in samples homogenated and assayed using 0.3 M Sodium phosphate buffer. Increase in pH significantly (p<0.01) decreases the activity of the hyaluronidase and optimum activity was registered at pH 5.5.

3.4 Effect of pH and component of buffer on the activity hyaluronidase of red shrimp

Protein released from the tissues during homogenisation of red shrimp was 63.56±1.19 mg/g using different buffers (Fig. 4).

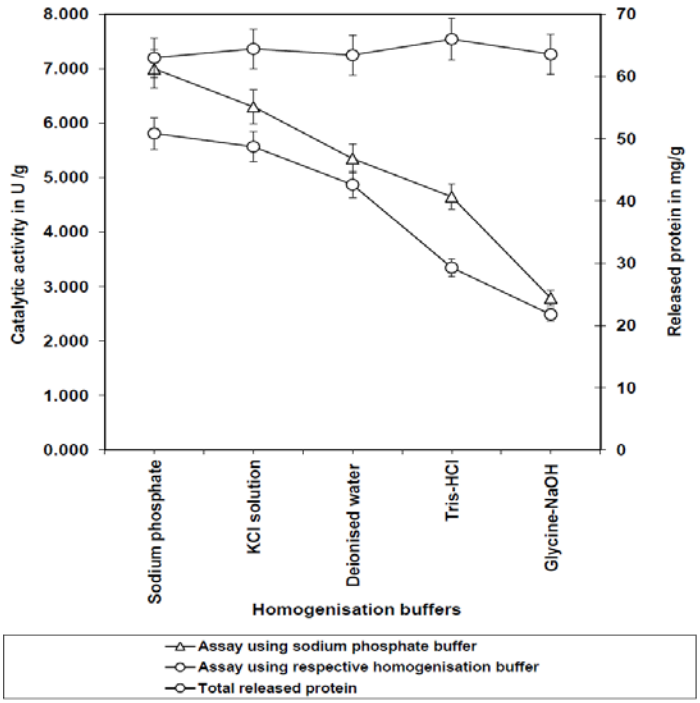

Fig. 4 Hyaluronidase activity of the released protein from hepatopancreatic tissues of *Penaeus monodon* (Black tiger) using different buffers.

Tissue homogenates in 0.5 M Sodium acetate buffer showed maximum hyaluronidase activity when assayed using 0.3 M Sodium phosphate buffer, as indicated by ANOVA with *post hoc* Tukey's test. Hyaluronidase activity was 5.809±0.006, 5.567±0.004, 4.870±0.006, 3.345±0.003 and 2.487±0.007 U/g when both homogenised and assayed using Sodium acetate buffer, KCl solution, Deionised water, Tris-HCl buffer and Glycine-NaOH buffer, respectively. When only assay buffer was changed from respective homogenization buffer to 0.3 M Sodium phosphate buffer, hyaluronidase activity, respectively, was 6.999±0.005, 6.299±0.002, 5.349±0.005, 4.650±0.007 and 2.789±0.004 U/g of homogenate. Here, as the assay buffer was changed from respective homogenization buffer to 0.3 M Sodium phosphate buffer, hyaluronidase activity increased significantly (p<0.01), as indicated by ANOVA with *post hoc* Tukey's test. ANOVA with *post hoc* Tukey's test was not able to establish significant difference in hylauronidase activity when homogenated and assayed using 0.3 M Sodium phosphate buffer. Increase in pH significantly (p<0.01) decreases the activity of the hyaluronidase. We have registered optimum activity at pH 5.5, however activity was significant even at pH 9.0.

4 DISCUSSION

Hepatopancreatic tissue of shrimps, *Parapenaeopsis stylifera, Penaeus indicus, Solenocera choprai* and *Penaeus monodon* were homogenized using buffers such as 0.5 M Sodium acetate buffer of pH 5.5, 2 M KCl solution of pH 7.00, Deionised water of pH 7.4, 0.1 M Tris-HCl buffer of pH 8.4, and 0.1 M Glycine-NaOH buffer of pH 9.0. Suitable working buffer amongst 0.5

M Sodium acetate buffer of pH 5.5, 2 M KCl solution of pH 7.00, Deionised water of pH 7.4, 0.1 M Tris-HCl buffer of pH 8.4, and 0.1 M Glycine-NaOH buffer of pH 9.0 were selected by homogenizing hepatopancreatic tissue of *Parapenaeopsis stylifera, Penaeus indicus, Solenocera choprai* and *Penaeus monodon* in different lots using any one of these buffers at 1:8 tissue to buffer ratio at 3000 rpm/10 min and at 4°C. Effectiveness of the buffer in optimally releasing proteins and on the activity of hyaluronidase was performed by assessing total released protein and activity of the homogenate for hyaluronidase. Since yield of protein is one of the important factors during cell disruption we have taken released protein as one of the important factor along with the activity of the hyaluronidase[11]. Hyaluronidase is referred to group of dissimilar enzymes that degrade high-molecular-weight glycosaminoglycan of extracellural matter known as hyaluronic acid or hyaluronam[1]. Hyaluronidase is an unstable enzyme with low concentrations but at high activity in the tissues[2]. Most of the hyaluronidase assay methods available are not very sensitive due to the very unstable nature of the enzyme[3]. Hence instability of the enzyme in the medium and sensitivity of the hyaluronidase to various effects plays major role in the outcome of the hyaluronidase assay. Hence assay requires careful consideration of factors that might adversely affect the activity. Hyaluronidase activity gets affected adversely by important interrelated factors such as pH of the buffer and components of the medium. Hyaluronidase activity gets affected at low ratio of hyaluronidase to hyaluronic acid and low concentrations NaCl because of the competition of Bovine serum albumin with the enzyme for its substrate and forming electrostatic interaction at pH ranging between 3.0 and 5.25, recovering the catalytic activity. At this pH, BSA is positively charged and hyaluronic acid is negatively charged, and higher the pH values the higher the optimum BSA concentration. Hence maximum pH was reported was at 4 and significant activity up to 9[12]. Components of the medium are known to affect the hyaluronidase activity. Previous research suggests that even at same pH acetate buffer inhibits the hyaluronidase in embryonic chic brain extract, in comparison to formate buffer[4]. Hyaluronidase is known as unstable and exits at low concentrations. Hyaluronidase inhibitors are ubiquitous and known to inhibit hyaluronidase at low concentrations. Hence, it is crucial to compare the effect of various buffers on the enzyme and establish a suitable buffer for homogenization and assay. Suitable components of buffer that are non inhibitory in nature at suitable pH is crucial for isolating the enzyme with optimum activity, purity and yield. In the present study, homogenate in 0.5 M Sodium acetate buffer of pH 5.5 as a homogenization buffer exhibited optimum hyaluronidase activity when assayed using 0.3 M Sodium Phosphate of pH 5.35 in comparison to the samples homogenized using buffers of higher pH.

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