Academy of Romanian Scientists *Volume 5, No. 1, 2016, pp. 108-118*
Annals Series on Biological Sciences

PH INFLUENCE ON DIFFERENT BOVINE TESTICULAR HYALURONIDASE DETERMINATION ASSAYS

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Abstract. Hyaluronidases from testes are enzymes that degrade hyaluronic acid and are essential for fertilization. A soluble fragment from the bovine testicular hyaluronidase PH-20 (BTH), was extensively characterized. However, many studies in literature show variable data regarding the optimum pH conditions for BTH enzymatic activity. The objective of this study was to evaluate the optimum pH in different conditions in parallel assays. The methods employed here were based on the colorimetric technique that determines the amount of N-acetyl-D-glucosamine released after the enzymatic reaction and on zymography that evaluates the degradation of the substrate copolymerized within the polyacrylamide gel matrix. Our results show surprising differential behavior of the enzyme under the abovementioned different assays.

Key words: BTH, enzyme activity, pH**,** zymography.

Introduction

Hyaluronidases were first identified in extracts from mammalian testes. These enzymatic activities were demonstrated to be of type endo-*N*-acetylhexosaminidases that hydrolyse hyaluronan (HA), a glycosaminoglycan of a several million of Da, to tetrasaccharides as the main end product [1,2].

Testicular hyaluronidases (PH-20) are membrane-bound proteins that present a glycosyl-phosphatidyl-inositol (GPI) anchor that allows them little freedom, keeping them associated with the cell surface. This enzyme was demonstrated to have an optimum pH in the neutral range owing to the fact that it should be active in physiological conditions in order to perform the fecundation [3].

Additionally, this study showed that BTH is a soluble fragment of PH-20, which lacks the GPI anchor, and has different optimum of pH, at 4.0, even though it has the same enzymatic catalytic site [3].

Other studies on the effect of pH and ionic strength upon the activity of purified BTH showed that the pH optimum for the hydrolysis of HA occurs at 5.2 in the presence of NaCl, and at 6.0 in the absence of NaCl. Hydrolytic activity towards HA hexasaccharide is favored at pH values below 5.2 whereas transglycosylation activity is favored at higher pH [4]. In another study, the optimal pH value for hydrolase activity is about 4-5, whereas pH 7 is optimal for transglycosylation reaction. In the presence of NaCl, transglycosylase activity is partially inhibited and nearly completely inhibited at concentration higher than 0.5 M [5].

A variety of methods has been developed over the years to measure the hyaluronidase activity: HA substrate-based zymography [6], turbidity assay [7] and colorimetric methods using p-dimethylaminobenzaldehyde (p-DMAB) [8,9,10].

Depending on the BTH preparation, the used substrate, the hyaluronidase assay and the incubation conditions, different pH optima were identified: pH value of 3.7[11], pH value of 5.2 [4] and pH value of 7.5[12].

Due to all these differences in optimum pH obtained by numerous assays conditions, we set to clarify if the optimum pH for BTH varies with the assay. For this, we used in parallel zymography and colorimetric methods to compare the same conditions, having different readouts. Our results open new perspectives for the potential influence of the assay on the enzymatic activity of BTH.

Materials and methods

Materials: Hyaluronic acid (HA) from rooster comb and hyaluronidase type IV-S (EC 3.2.1.35) from bovine testes (BTH) were from Sigma Aldrich and the prestained molecular weight protein marker was from BioRad. All the other reagents used in this study were of the purest grade available from Sigma Aldrich.
Zymography: Zymography is an electrophoretic technique, commonly based on sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE), which contains a substrate copolymerized within the polyacrylamide gel matrix, for the detection of an enzymatic activity. HA (the substrate) at a concentration of 0.17 mg/ml was copolymerized in 10 % polyacrylamide gel. The enzyme (BTH) was solubilized in PBS buffer according the product preparation instructions and diluted with 2x sample buffer containing 0.5 M Tris, pH 6.8, 5 % SDS, 20 % glycerol and 0.1 % bromophenol blue. Migration of the gels was done at 100V for 1.5-2h, in non-reducing conditions. Gels were washed for one hour on ice, with 5 % Triton X-100 to remove the SDS and then were developed for 3 h at 37°C in different buffers. After development, gels were stained with 0.5 % Alcian blue in

20 % ethanol-10 % acetic acid for one hour and then destained in a solution containing 20 % ethanol and 10 % acetic acid. Staining of the gels reveals sites of proteolysis as two translucent bands on a blue background at a molecular weight of approximately 70 and 50 kDa. The bands of hyaluronidase activity were imaged using ChemiDoc MP (BioRad) and quantified using ImageJ software image analyzer. The hyaluronidase enzymatic activity was expressed as arbitrary densitometric units, and plotted in Excel or Prism GraphPad.

When comparisons between various pH of the same buffer, or of different buffers were needed, the same samples were loaded on the gel and the gel was cut in equal pieces. Each piece of gel was incubated as required. After developing, all the pieces of gel were stained in the same solution in order to maintain the same background color.

The colorimetric assay: We used a modified colorimetric assay which is based on the method described in Reissig et al. [8]. The colorimetric assay (Morgan - Elson reaction) assesses the reaction of N-acetyl-D-glucosamine (NAG) at the reducing ends of HA and its fragments with p-dimethylaminobenzaldehyde (p-DMAB) resulting in a red colored product. Briefly, mixtures of 150 mM NaCl in 50 mM acetate buffer, pH=4, and different concentrations of BTH were incubated for 0.5 h at 37°C. The substrate solution (1.25 mg/ml HA in reaction buffer) was added and the enzymatic reaction develops for another 0.5 h. The reaction was stopped by cooling on ice. The next step was addition of alkaline borate solution (0.8 M potassium tetraborate, pH=9.8) and the reaction mixture was boiled for exactly 4 mins at 100°C in a water bath. After rapid cooling on ice, p-DMAB solution is added (0.1 g/ml of p-DMAB in glacial acetic acid which also contains 12.5 % (v/v) 10N HCl; the solution was diluted with 9 volumes of glacial acetic acid before use) and the mixture was incubated at 37°C for 20 min; the change in absorbance was read at 585 nm. Enzymatic activity was expressed as reaction rate comprising of μg of NAG released/min.

The buffers used for these experiments were:

- **formate buffer** (0.1M sodium formate, 0.150M NaCl at pH=3.7, 5.5 and 7.2, pH was adjusted with formic acid, if necessary);
- **acetate buffer** (0.1M sodium acetate, 0.150M NaCl at pH=3.5, 4.0, 5.6, 6.0 and 7.0, pH was adjusted with acetic acid, if necessary)
- **phosphate buffer saline (PBS)** (0.137M NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8 mM KH_2PO_4 at pH=4.0, 5.5 and 7.4, pH was adjusted with HCl if necessary).

Results

Enzymatic activity of BTH can be tested by zymography

In previous studies BTH was shown to have an optimum pH at 4.0 [13], 3.7[11], 5.2 [4], 7.4 [14] and 7.5 [12]. To clarify this discrepancy, we set to evaluate by zymography the enzymatic activity of BTH in two extreme conditions: formate buffer at pH=3.7 (Fig.1a and c) and PBS at pH=7.4 (Fig.1b and d).

We tested the reproducibility and robustness of the method by loading several samples of enzyme in different concentrations. Comparing the zymograms, in both conditions there are two lysis bands at approximatively 70 kDa (band1) and 50 kDa (band2), respectively (Fig. 1. a, b, c and d). In Fig.1a we show that loading four or five different samples of BTH that have the same concentration (5.7, 11.5 or 22.9 Units - U) results in little variation on the enzymatic activity as it is measured by quantification of the bands (the graphs on the right). By developing the gels in PBS buffer it seems that there is a slight increase in the variability of the BTH samples, especially when quantifying the upper band (band1) (Fig.1b).

In order to determine the linearity of the method were verified several concentrations of enzyme in the same conditions as described above. The curves obtained based on the densitometric analysis of most bands show linear correlation coefficients around 0.9 which demonstrate a good correlation between enzymatic activity and enzyme concentration (Fig. 1c and d).

In short, the method is robust and it shows great linearity between the enzyme activity and the number of units loaded on each lane.

An unexpected finding was that the bands obtained after developing the gels in PBS buffer were more intense than those obtained in formate buffer, even though gels were kept in the same temperature and humidity conditions, and also for the same amount of time. Based on this we asked if we will obtain similar profiles of enzymatic activity in different buffers and at different pH values.

By zymography, BTH functions better at a higher pH

We tested several conditions that implied different buffers with variable pH. In Fig. 2a we can observe that the higher pH of formate buffer induces a greater discoloration of the gel, suggesting a better activity for the enzyme. From the quantification (the graph underneath), it seems that pH 5.4 represents the optimum for BTH in this buffer, which correlates with the data from literature that states that the optimum pH for this enzyme is situated between 4.0 and 6.5 [14].

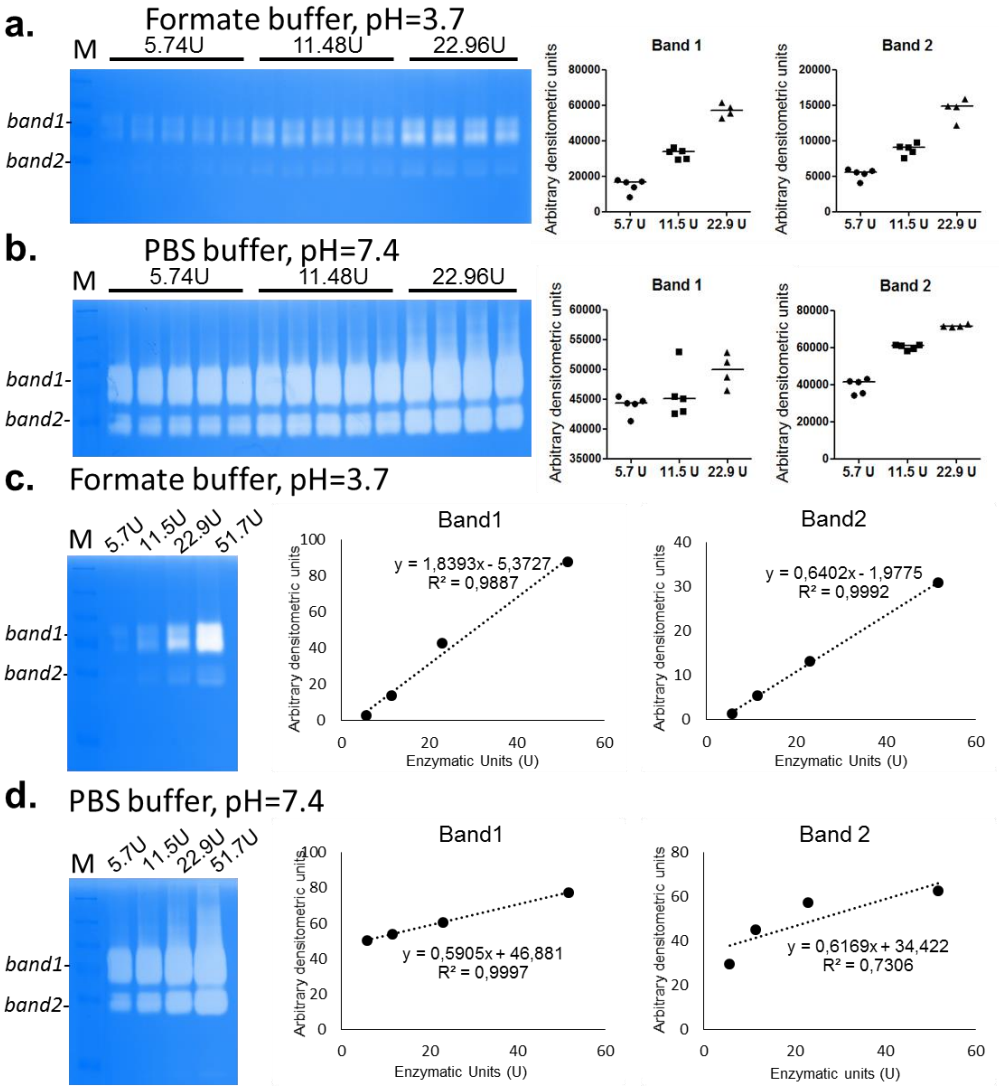

Fig. 1. Evaluation of enzymatic activity of BTH by zymography.
a. Different samples of enzyme were loaded in three different concentrations (5.7, 11.5 or 22.9 Units) and developed in formate buffer, pH=3.7. The quantification of the bands is shown in charts on the right; **b.** The same samples as above were developed in PBS at pH=7.4, and the densitometric analysis is shown on the right; **c** and **d**. To verify the linearity of the assay, different amounts of enzymatic units were loaded and developed in formate buffer (**c**) and PBS (**d**). Curves based on the densitometric analysis of the zymograms are shown on the right side. The slopes were obtained in excel. M represents the protein molecular weight marker.

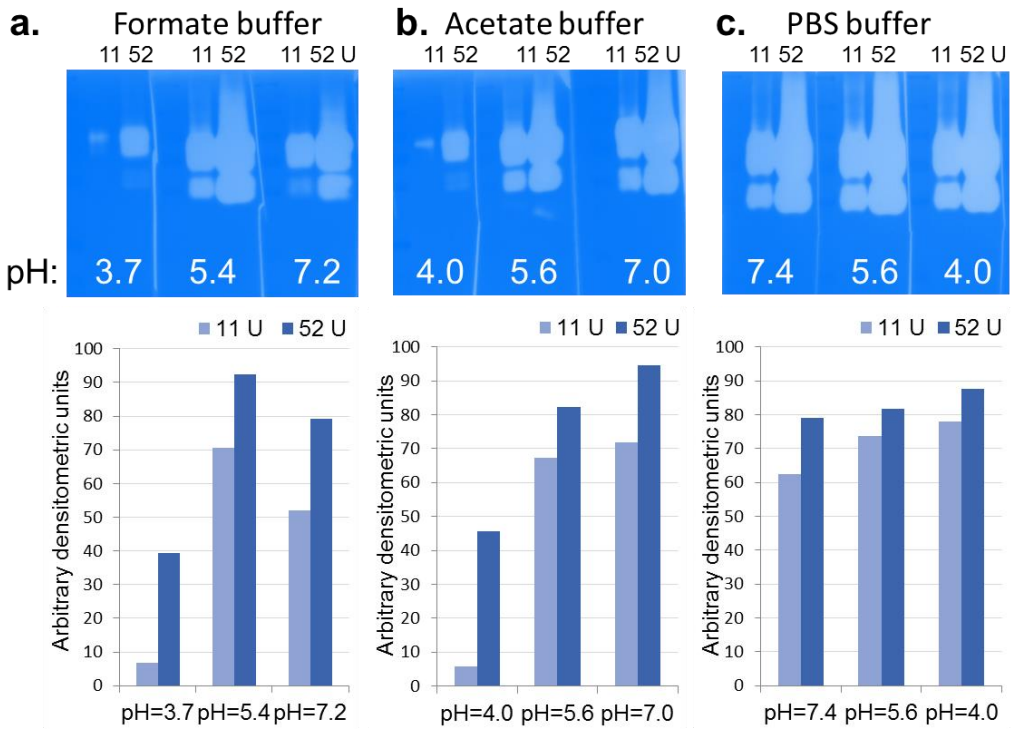

Fig. 2. Enzymatic activity of BTH is influenced by pH.
Two enzyme concentrations (lane1- 11U, lane2- 52U) were loaded in HA gel and incubated for 3h, at 37°C in formate buffer (**a**), acetate buffer (**b**) and PBS (**c**) at different pH values, as is marked on the figure. The intensities of the bands were quantified and graphed below.

In the acetate buffer (Fig.2b), the zymogram and the graph based on its quantification show an increase of enzymatic activity at higher pH values.

In Fig. 2c, the enzymatic activity developed in PBS buffer apparently does not vary with the pH.

Based on all these data, BTH seems to function better at a higher pH in these tested conditions, regardless on the nature of the buffer.

As we also have access to another method that implies a different mechanism, we tested BTH in the same conditions by hyaluronidase colorimetric assay.

By colorimetry, BTH shows better activity at lower pH

The colorimetric method for hyaluronidase activity implies the determination of NAG obtained by digesting the substrate with BTH in given conditions of pH and ionic strengths. In Fig. 3, we characterized the behavior of NAG in the same buffer, but varying the pH, in order to determine the potential

interference of the pH with the read-out system. Each NAG curve had a good linearity, regardless of the buffers or the pH used. Moreover, the read values for the absorbance for each condition do overlap, suggesting that the color reaction is not sensitive to these changes (Fig. 3a, b, c).

The enzymatic curve in acetate buffer (Fig. 3a) shows a decrease in activity with increasing the pH value with the maximum rate of reaction at pH=4. The reaction rates decrease with the increasing of the pH values.

A similar behavior can be observed for the BTH in other two buffers tested, PBS (Fig. 3b) and formate (Fig. 3c), where higher reaction rate is obtained at lowest pH tested, 4.0 and 3.7, respectively. At the highest pH tested (7.0, 7.2, or 7.4 for acetate, formate and PBS, respectively), the reaction rates remains nearly unchanged irrespective of increasing the enzymatic units added in the reaction.

These data indicate that for the colorimetric assay, BTH has an analogous comportment regardless of the different buffers used, at low pH values the enzyme reaction rate showing the best results, while at higher pH values loosing almost completely the activity.

BTH is a well described enzyme that has already wide applications [9, 10, 15]. However, there is a lot of variability in the results from previous studies that state several pH optima for this enzyme [11, 12, 13]. To clarify this issue, we tested the enzyme activity in similar conditions in parallel by zymography and colorimetric assay.

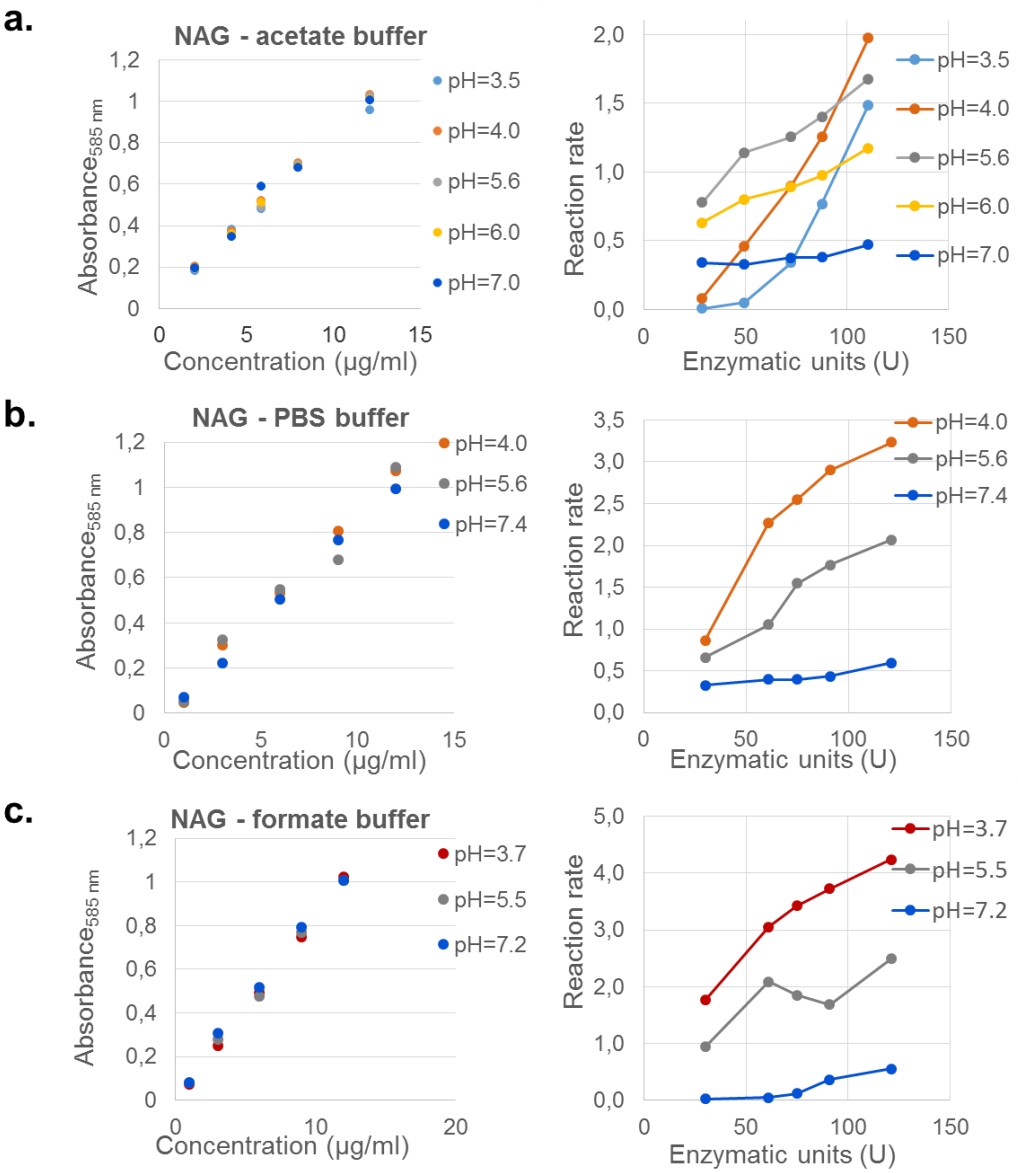

Fig. 3. Evaluation of BTH enzymatic activity by colorimetry. NAG concentrations were plotted against the absorbance of the NAG-pDMAB complex at 585nm obtained during reactions in different buffers with various pH values (**a.** acetate buffer at pH=3.5, 4.0, 5.6, 6.0, 7.0, **b.** PBS buffer at pH=4.0, 5.6, 7.4 and **c.** formate buffer at pH=3.7, 5.5, 7.2). Enzymatic curves where reaction rate was plotted against the number of enzymatic units are shown on the right for the corresponding buffers and pH (**a.** acetate buffer at pH=3.5, 4.0, 5.6, 6.0, 7.0, **b.** PBS buffer at pH=4.0, 5.6, 7.4 and **c.** formate buffer at pH=3.7, 5.5, 7.2).

Our data suggest that the zymography method can be used in applications such as hyaluronidase inhibitors screening, as it seems like a robust and sensitive method for determining small differences of enzymatic activity (in the range of single units). Moreover, we have the possibility of performing these tests in a wide range of pH, depending on application. For example, when one would like to address the behavior of the enzyme in the presence of inhibitors, like for example bioactive complexes rich in glycosaminoglycans [16] at a physiological pH, we can employ the zymography method. Moreover, these kinds of approaches can be applied also to test other enzymes, from other sources (biological samples, like blood serum or synovial fluids).

Conclusions and discussion

A surprising finding of our study was the discrepancy of pH optima that BTH has in the two different assays by which the enzymatic activity was tested, zymography and colorimetric method. The results show BTH having pH optima at around 4.0 by colorimetric method, but between 5.6 and 7.0 by zymography, summarized in Table 1. In these assays, the other conditions were kept unchanged (the substrate, the enzyme and the buffers were the same). So how can we explain this difference in the behavior of BTH?

Table 3) Summary of enzymatic activity (EA) variation based on different pH values. n.d.= not determined.

BUFFER	pH	EA ZYMOGRAPHY	EA COLORIMETRIC
Formate	3.7	↑	↑↑↑
	5.5	↑↑↑	↑↑
	7.2	↑↑↑	↑
Acetate	3.5	n.d.	↑↑
	4.0	↑	↑↑↑
	5.6	↑↑↑	↑↑
	6.0	n.d.	↑↑
	7.0	↑↑↑	↑
PBS	4.0	↑↑↑	↑↑↑
	5.6	↑↑↑	↑↑
	7.4	↑↑↑	↑

One explanation might come from the fact that the two assays look from different angle at the enzyme activity.

Zymography uses HA substrate immobilized in polyacrylamide gel, the enzyme is applied to this gel, is run based on electric current in non-reducing

conditions and gets trapped at the corresponding molecular weight in gel. To recover conformation and implicitly activity, the enzyme is renatured with TritonX-100 (that replaces the denaturing action of SDS) and then incubated in the corresponding buffer. In these conditions, the enzyme directly degrades the substrate from the immediate vicinity, and once this action takes place, the degraded substrate is released from the gel mesh, getting diluted out in the developing buffer.

Perhaps, all these actions that maintain the enzyme immobilized in the polyacrylamide gel may confer a specific conformation that allows a better accessibility of the substrate in the catalytic site. This conformation may be different than the conformation of BTH in solution, like it has in the colorimetric method. This may confer different accessibility to the substrate and implicitly different enzymatic activity.

This hypothesis is supported also by the finding of Meyer and colleagues [1]. In that study they show that BTH is a soluble fragment from the membrane-bound PH-20, an enzyme essential for fertilization. In a complementary study, they show that BTH has the best activity at pH=4.0, while PH-20 has a pH optima in the neutral range. Thus, BTH immobilized in gel may behave as the membrane-bound PH-20, having a similar conformation, and this way requiring a higher pH as an optimum condition.

Alternatively, previous studies show that BTH functions as a transglycosylase at higher pH [5]. In the colorimetric reaction, the enzyme is continuously in contact with intact as well as with degraded substrate, then at higher pH, as this transglycosylase activity may be prevalent, at the end of the incubation time there would be less NAG to react with p-DMAB, which would translate in less activity. This is very different from the zymography situation, where the degraded HA leaves immediately the site and is diluted out in the developing buffer, getting this way inaccessible for the transglycosylase.

Our data here gives important alternatives for characterizing pH optimum conditions for BTH enzyme activity, and also raises new interesting questions. Further studies that look at the potential conformation change of BTH between these two assays conditions will elucidate this incongruence.

Moreover, based on these results, we open the possibility for new applications of the assays for screening hyaluronidase inhibitors represented by bioactive complexes rich in glycosaminoglycans as those recently described by our group [16].

Conflict of interests

The authors declare no conflict of interest.

Notations and/or **Abbreviations**

BTH – bovine testes hyaluronidase; HA – hyaluronic acid/hyaluronan; SDS – sodium dodecyl sulphate; NAG - N-acetyl-D-glucosamine; NaCl – sodium chloride; PBS – phosphate buffer saline

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