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# Isolation and enzymatic characterization of the first reported hyaluronidase from Yak (*Bos grunniens*) testis

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**Abstract**−A novel hyaluronidase (BgHya1) from Yak *Bos grunniens* testis was isolated and shown to have comparatively high activity on sodium hyaluronate. However, surveys on BgHya1 are still limited. The enzyme was purified through gel filtration on Sephacryl S-100 and cation-exchange on SP Sepharose fast flow; the purity was confirmed by a reverse phase FPLC Shodex $C_4$ column. The specific activity of the purified BgHya1 was 20.4 U/mg assayed by the colorimetric method against 0.85 U/mg for the crude enzyme, representing a 24-fold purification. It was a monomeric protein of 55 kDa estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Sephacryl S-200. It exhibited maximum activity in the presence of 0.15 M NaCl at 37 °C, pH 3.8, and a specificity to sodium hyaluronate higher than that of chondroitin-4-sulfate, chondroitin-6-sulfate, and dermatan. The $K_m$ value for BgHya1, using sodium hyaluronate as substrate, was 0.106 mg/mL. Activity of BgHya1 was inhibited mildly by $Ca^{2+}$ and $Fe^{2+}$, and significantly by $Fe^{3+}$, $Mg^{2+}$, EDTA, urea, heparin, and 0.5 M NaCl. It was not affected by $Cu^{2+}$, $Zn^{2+}$, $Co^{2+}$, ascorbic acid, PMSF, DTT, glutathione (reduced), or L-cysteine. BgHya1 was shown to be heat unstable in the range of 4-45 °C. In terms of storage stability, 92% of the activity was retained after four weeks at 4 °C, and 58% at room temperature. In addition, adding BSA (1.0 mg/mL) to the enzyme sample prior to freezing resulted in complete retention of enzyme activity. This work yielded a high purity hyaluronidase, the first one isolated from *Bos grunniens* by-product.

Keywords: *Bos grunniens*, Hyaluronidase, Purification, Biochemical Characterization

## INTRODUCTION

Hyaluronidase has been used therapeutically to increase speed of absorption, reduce discomfort due to subcutaneous/intramuscular injection of fluid, promote resorption of excess fluids and congestion in the tissues, and increase the effectiveness of localized anesthesia. It has also been used to catalyze the hyaluronan found in the extracellular matrix, especially in soft connective tissues [1]. The catalyst reaction is completed by cleavage at either $\beta$-1,4 or $\beta$-1,3 glycosidic bonds [2]. The major final reaction products interact with various receptors of the extracellular matrix, which plays an important role in cell invasion, anti-angiogenesis, and wound repair [3,4]. A series of hyaluronidases (e.g., Hylase® Dessau, Neopermease®, Wydase®) have been widely used in various fields, such as plastic surgery, ophthalmology, internal medicine, oncology, and dermatology [5-8]. However, these hyaluronidases purified by various methods are a complex mixture of proteins [9,10]; therefore, it is necessary to find better resources for producing hyaluronidase.

Yak (*Bos grunniens*) is a unique livestock resource in western China. It is distributed along the Qinhai-Tibetan Plateau at altitudes above 3,000 m. Chinese yak, which now accounts for approximately 95% of the world's total yak production (14.7 million) [11,12], has a strong ability to adapt to the extreme environment of the Qinhai-Tibetan Plateau. This cold-loving animal is well known as a source of pollution-free products characterized by low fat, high protein, and a favorable fat composition. All of these attributes of yak are criteria demanded by today's discerning consumer. It is commonly used for human consumption and for the production of Halal food, and the abundant resource of by-products makes its utilization scope and opportunities enormous. However, while large quantities of by-products are produced during the processing of yak, the testes are typically discarded. It is essential to take measures to ensure its effective utilization with high added value, not only for environmental conservation, but also for its huge economic benefits.

Hyaluronidase has been isolated and characterized from the following resources: liver, kidney, heart, spleen [2,13], bovine testes, boar reproductive tract, and dog testes [9,14,15]. As stated in the literature, hyaluronidases from different mammal species differ in molecular composition and functional properties, even among those belonging to the same genus. In addition, hyaluronidases applied in clinical medicine need more specific activity and higher purity, but not complex proteins. To the best of our knowledge, BgHya1 has never been discussed in any previously reported hyaluronidase purification processes.

Our aim was to develop an effective isolation process based on chromatography to obtain a novel hyaluronidase with higher activity and purity from *Bos grunniens* testes. The enzymatic characterization of BgHya1 investigated here may contribute to the optimization of therapies for clinical application and help in understanding the numerous pathophysiological processes in which the enzyme is involved. It can also help achieve additional high-value utilization of yak by-products, protect the environment, and reduce resource waste.

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## MATERIALS AND METHODS

### 1. Materials

The *Bos grunniens* testes were bought in Qinhai Province, PR China. All of the samples, which were obtained from healthy, disease-free yak, were washed with chilled distilled water to remove extraneous materials and then immediately frozen and kept at −80 ºC until further analysis. Sephacryl S-100, Sephacryl S-200, and SP Sepharose Fast Flow columns were obtained from GE (Uppsala, Sweden). Reverse phase Shodex $C_4$ column was obtained from Showa Denko KK, (Kawasaki, Japan). Bovine testicular hyaluronidase, sodium hyaluronate, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan, ascorbic acid, phenyl methyl sulfonyl fluoride (PMSF), dithiothreitol (DTT), reduced glutathione, L-cysteine, ethylenediaminetetraacetic acid (EDTA), heparin, urea, bovine serum albumin (BSA), and all other chemicals used, unless otherwise stated, were obtained from Sigma Chemical Company (St. Louis, MO). All aqueous solutions were prepared using water filtered by a Milli-Q water system (Millipore, Bedford, MA).

### 2. Preparation of Crude Enzyme

The testis samples (100 g) were thawed and homogenized at 18,000 *g* with 250 mL of 100 mM sodium acetate buffer (pH 3.8) containing 150 mM NaCl for 2 min. The resulting homogenates were centrifuged at 16,000 *g* for 30 min at 4 ºC. After centrifugation, the supernatant was mixed with ammonium sulfate. The fraction between 30 and 70% saturation was collected. The precipitate was dissolved in a minimal volume of chilled distilled water and dialyzed with a membrane (8-14 kDa MWCO) overnight. It was then centrifuged at 16,000 *g* for 20 min. The supernatant was lyophilized to obtain the crude enzyme for the chromatography step.

### 3. Chromatography

The purification of the BgHya1 was performed in two successive steps. All purification steps were carried out at 4 ºC. The absorbance was monitored at 280 nm using a FPLC ÄKTA Purifier UPC-10 system (GE).

The crude enzyme (100 mg) was evenly dispersed in 1 mL 50 mM sodium acetate buffer, pH 5.4, containing 150 mM NaCl (buffer A), and centrifuged at 16,000 *g* for 15 min. The supernatant was loaded into a Sephacryl S-100 gel filtration column (1.6 cm×70 cm), equilibrated, and eluted with buffer A at 0.5 mL/min. Then, the fractions with hyaluronidase activity were pooled, dialyzed against 50 mM sodium acetate buffer, pH 5.4 (buffer B), and then injected into a SP Sepharose Fast Flow cation-exchange column (1.6 cm× 20 cm) equilibrated with buffer B. Step elution was performed by the sequential addition of buffer B at increasing ionic strengths, from 0 to 0.5 M of NaCl, at 2.0 mL/min. Fractions with hyaluronidase activity were pooled and dialyzed. Purity of the isolated hyaluronidase BgHya1 was confirmed by reverse-phase FPLC using a Shodex $C_4$ column (0.46 cm×25 cm) equilibrated with 0.1% trifluoroacetic acid (TFA). The increasing gradient was performed with 0-100% buffer C (60% acetonitrile, 0.1% TFA) at 0.5 mL/min.

### 4. Hyaluronidase Assay

BgHya1 activity was evaluated by a modification of the colorimetric method of Reissig et al. [16]. p-Dimethylaminobenzaldehyde (DMAB) reagent (10 g DMAB) was dissolved in 100 mL of an acid mixture containing 87.5 mL glacial acetic acid and 12.5 mL 10 M HCl. Tetraborate reagent was prepared by dissolving $K_2B_4O_7 \cdot 4H_2O$ at 0.8 M, without any adjustment of pH to 9.10, and thus the pH was 9.87. Sodium hyaluronate solution (1.5 mg/mL in 100 mM sodium acetate buffer, pH 3.8, containing 150 mM NaCl and BSA of 1.0 mg/mL) was equilibrated at 37 ºC for 30 min. All of the other reagents used were prepared by the method of Reissig et al. [16]. The reaction mixture consisted of 400 μL of sodium hyaluronate and 100 μL of the enzyme solution. After incubation at 37 ºC for 15 min, the reaction mixture was heated in a boiling water bath for 5 min to stop the enzyme reaction. After cooling it in tap water, a color reaction was started by adding 110 μL tetraborate reagent. It was heated in a boiling water bath for 4.5 min and then cooled in tap water; 3 mL of DMAB reagent was added and incubated at 37 ºC for 20 min to allow color development to occur. After centrifugation at 16,000 *g* at 4 ºC for 10 min to remove the precipitate, the absorbance of the clear supernatant at 585 nm was measured against that of a blank test carried out in the same manner, except that the enzyme reaction mixture was not incubated.

One unit of hyaluronidase activity was defined as the amount of enzyme required to liberate 1 μmoL of reducing N-acetyl-D-glucosamine per minute by using N-acetyl-D- glucosamine as a standard.

### 5. Protein Concentration Determination

The amount of protein in the investigation enzyme preparation and the chromatography fractions was determined by the method described by Bradford [17], with BSA as a standard protein.

### 6. SDS-PAGE

SDS-PAGE was carried out according to the method described by Laemmli [18]. The samples obtained from the purification process were subjected to 12.5% polyacrylamide gel electrophoresis using tris-glycine buffer at pH 8.8 as a running buffer. After electrophoresis, the gel was stained with Coomassie Blue R-250.

### 7. Molecular Weight Determinations

The molecular weight of the purified BgHya1 was determined by gel filtration technique using Sephacryl S-200 according to the method of Öberg and Philipson [19], using 50 mM sodium acetate buffer, pH 5.4 (containing 150 mM NaCl), at 0.5 mL/min. A chromatographic glass column (1.6 cm×70 cm) was calibrated with ribonuclease (13.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), and bovine serum albumin (67 kDa). Blue dextran was used to determine the void volume (Vo). A calibration curve was obtained by plotting Ve/Vo against their respective logarithmic molecular weights.

### 8. Optimum pH and Temperature

All samples used for the biochemical characterization of the BgHya1 were purified from the final step of chromatography.

BgHya1 activity was assayed with a pH range of 2.0-9.0 by preparing the substrate of sodium hyaluronate in various buffer systems (i.e., 100 mM sodium acetate for pH 2-6, 100 mM sodium phosphate for pH 7.0, and 100 mM Tris-HCl for pH 8-9) prior to adding the BgHya1 at 37 ºC. To investigate the optimum temperature of the BgHya1. Enzyme activity was assayed at different temperatures ranging from 4 ºC to 70 ºC using sodium hyaluronate (1.5 mg/mL) as the substrate. The assay was conducted with 100 mM sodium acetate buffer (containing 150 mM NaCl) under optimal pH. BgHya1 activity was assayed as described in the hyaluronidase assay section. Relative activity was estimated considering 100% the highest activity measured in the above assay.

### 9. Thermal Stability

To determine thermal stability, the BgHya1 was incubated at vari-

ous temperatures (4, 15, 24, 37, 45, 50, 55, 60, and 70 °C) for 60 min in a temperature-controlled water bath. The heat-treated samples were rapidly cooled in an ice bath and residual activity was assayed as described in the hyaluronidase assay section. Relative activity was estimated considering 100% the highest activity detected in this assay.

**10. Effect of NaCl on Enzyme Activity**

The BgHya1 was incubated in the presence of NaCl at varying final concentrations, from 0 to 0.5 M, at 37 °C for 60 min. The residual activity was assayed as described in the hyaluronidase assay section. Relative activity was estimated considering 100% the highest activity detected.

**11. Storage Stability**

Stability of the BgHya1 under different storage conditions was assayed. Activity was determined after storage of the enzyme solution (containing 1.0 mg/mL BSA) at 4 °C and room temperature during days 0-28, respectively. Activity of the BgHya1 after repeated freezing and thawing of the enzyme solution was also measured. Immediately after the determination of enzyme activity, the solution was stored at −20 °C for at least one day before activity was re-determined after thawing of the solution at room temperature.

**12. Effect of Metal Ions and Inhibitors**

Samples of the purified BgHya1 were pre-incubated with various metal ions ($Fe^{3+}$, $Mg^{2+}$, $Ca^{2+}$, $Fe^{2+}$, $Cu^{2+}$, $Zn^{2+}$, and $Co^{2+}$ at 50 mM) and a series of potential inhibitors (ascorbic acid, PMSF, DTT, reduced glutathione, L-cysteine, EDTA, heparin, and urea) at different concentrations (Table 3), for 60 min at 37 °C. Afterwards, the enzyme reaction was started by the addition of sodium hyaluronate solution (1.5 mg/mL in 100 mM sodium acetate buffer, pH 3.8, containing 150 mM NaCl and BSA of 1.0 mg/mL). The mixture was equilibrated at 37 °C for 15 min. The enzymatic activity was determined as described in the hyaluronidase assay section. Blank tests were performed simultaneously under similar conditions, but without metal ions or potential inhibitors.

**13. Kinetic Parameters**

The Michaelis-Menten constant ($K_m$) of the BgHya1 was determined from the Lineweaver-Burk plot. The enzymatic reaction was carried out according to the method described above by varying the sodium hyaluronate concentration between 0.1 and 1.5 mg/mL of 100 mM sodium acetate buffer containing 150 mM NaCl. The assays were performed in triplicate.

**14. Substrate Specificity**

To determine substrate specificity, 100 μg of different substrates (sodium hyaluronate, chondroitin-4-sulfate, chondroitin-6-sulfate, and dermatan) were dissolved in 67 μL of 100 mM sodium acetate buffer (pH 3.8, containing 150 mM NaCl, 1.0 mg/mL BSA) and incubated with 17 μL of BgHya1 at 37 °C for 15 min. The colorimetric method of Reissig et al. [16] was used to determine activity, as described in the hyaluronidase assay section. Similar experiments were performed for bovine testicular hyaluronidase as a control.

## RESULTS AND DISCUSSION

**1. Purification and Molecular Weight of BgHya1**

The BgHya1 was purified in two steps. In the first step, the crude enzyme was purified by gel filtration (Fig. 1(a)). A total of five peaks were eluted from the Sephacryl S-100 chromatographic separation.

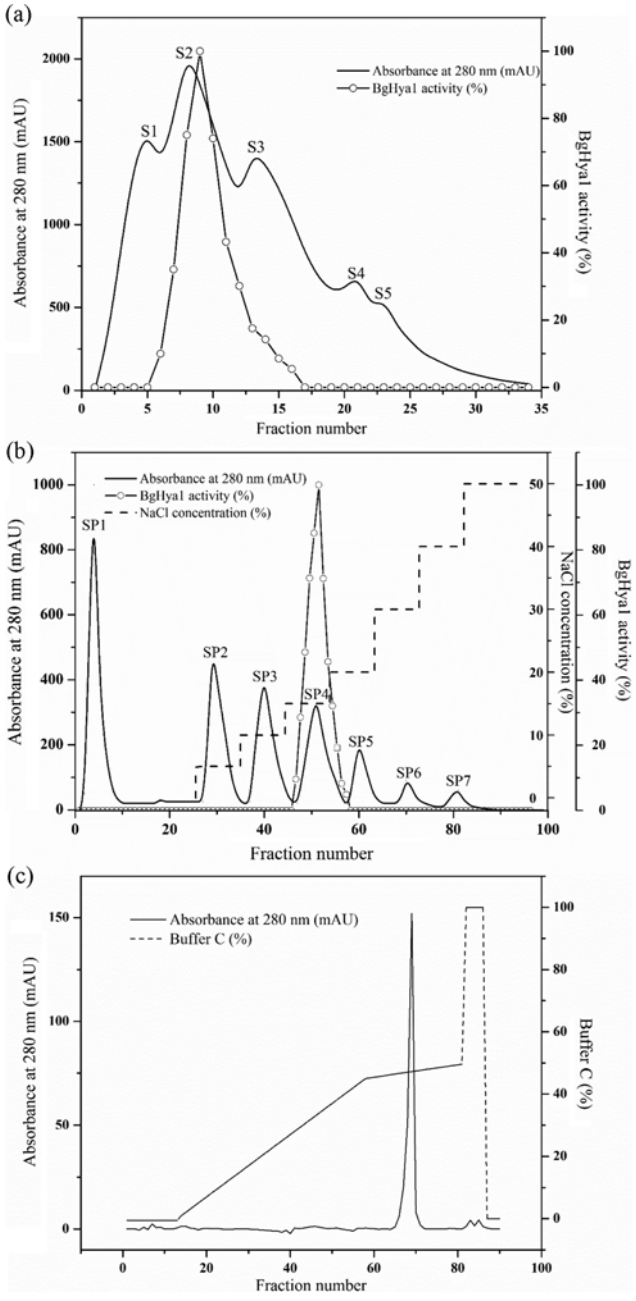


**Fig. 1. Isolation of hyaluronidase from *Bos grunniens* testis. (a) Gel filtration (Sephacryl S-100) profile. The elution buffer was 50 mM sodium acetate, containing 150 mM NaCl, pH 5.4, at a flow rate of 0.5 mL/min. (b) Cation exchange chromatogram profile. The fraction in S2 containing hyaluronidase activity was pooled and further separated on cation exchange column (SP Sepharose Fast Flow). The column was pre-equilibrated with 50 mM sodium acetate buffer, pH 5.4. Step elution was performed by sequential addition of the same buffer at increasing ionic strengths from 0 to 0.5 M NaCl. Fractions of 3 mL/tube were collected at a flow rate of 3.0 mL/min. (c) The fraction from SP4 was collected and confirmed on a reverse phase FPLC Shodex $C_4$ column equilibrated with 0.1% TFA. Elution was done by applying a linear gradient of buffer B from 0 to 100% (60% acetonitrile, 0.1% TFA), at a flow rate of 0.5 mL/min. The target protein was eluted with a single peak for the identification of BgHya1 activity.**

**Table 1. Summary of purification process of BgHya1**

| Step | Protein (mg) | Total activity (U) | Specific activity (U/mg) | Purification | Yield (%) |
|---|---|---|---|---|---|
| Crude enzyme | 100.00 | 85.00 | 0.85 | 1.00 | 100 |
| Sephacryl S-100 | 8.61 | 56.31 | 6.54 | 7.69 | 66.25 |
| SP sepharose fast flow | 0.55 | 11.22 | 20.40 | 24.00 | 13.20 |

Proteins with high molecular weight were eluted mainly in two fractions, named S1 and S2, with the highest BgHya1 activity found in S2, while the later peaks consisted of low molecular weight peptides (from S3 to S5). The S2 fractions with hyaluronidase activity were detected and pooled with a 66.25% yield and specific activity of 6.54 U/mg, which was a 7.69-fold increase over the crude enzyme. Subsequently, fractions from the first step were submitted to a SP Sepharose fast flow column step elution; seven fractions were obtained. The fraction named SP4 was found to be positive for BgHya1 activity (indicated by a smooth line in Fig. 1(b)). The pooled fractions from SP4 had a specific activity of 20.4 U/mg. A summary of the purification process is shown in Table 1. A quantity of 0.55 mg of purified BgHya1 was obtained from 100 mg of crude sample, with a yield of 13.2%. Specific activity increased 24-fold after purification. Comparing the specific activity of the other hyaluronidases using the same colorimetric assay method, it becomes obvious that the enzyme activity per mg of protein of BgHya1 is about 2.9 times higher than that of bovine testicular hyaluronidase H3884 Type IV-S from Sigma (shown in Table 4), and 8.16 times higher than that of Neopermease® (bovine testicular hyaluronidase produced in Austria; 2.5 U/mg) [10], and 5.62 times higher than that of purified bull seminal plasma (3.63 U/mg) [20].

The SP4 fraction was estimated by SDS-PAGE to be 55 kDa (Fig. 2), which was consistent with the result as determined by Sephacryl S-200 (Fig. 3). The SP4 fraction was also applied into a reverse phase FPLC Shodex $C_4$ column to check the degree of purity of the BgHya1.

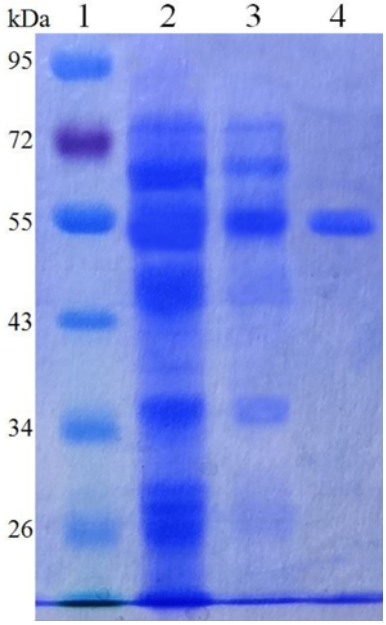


**Fig. 2. SDS-PAGE of BgHya1. Lanes 1: MW standards; 2: Crude enzyme; 3: BgHya1 from S2; 4: BgHya1 from SP4.**

A single peak with strong hyaluronidase activity was eluted (Fig. 1(c)). Compared with other hyaluronidases, it revealed two close bands with approximate molecular weights of 61 kDa and 67.1 kDa from bovine testicular hyaluronidase [9] and 58 kDa and 33 kDa from Neopermease [10], indicating that BgHya1 is generally similar in molecular mass, but with a relatively higher purity.

**2. Optimum pH**

As shown in Fig. 4(a), the purified BgHya1 had a typical bell-shaped profile covering a broad pH range, from 2 to 9. BgHya1 showed maximum activity at pH 3.8, which decreased by half at pH 6.0 and was virtually abolished at pH 9.0. Hyaluronidases from snake, bee, scorpion, and lizard venoms are optimally active in *vitro* at a pH range of 4.0 to 6.0. Hyaluronidases are generally classified as acid active (active between pH 3 and 4) or neutral active (active between pH 5 and 6) [7,21]. As it is optimally active at pH 3.8, BgHya1 belongs to the former class. The optimal pH for BgHya1 was similar to that found for hyaluronidases from various sources. It is usually in the acidic range of 3.8-5 [22-25], whereas the maximum bovine testicular hyaluronidase activity was reported as weak acidic and neutral pH [26]; most bacterial sources had optimum activity in the near neutral range of pH 6-7. These discrepancies may result from differences in the enzyme assay, especially with respect to the composition of the buffer, which is known to affect the activity of hyaluronidase from bovine testes diversely [27].

**3. Optimum Temperature and Thermal Stability**

The temperature-activity profile is shown in Fig. 4(b). The activity of purified BgHya1 was higher between 24 and 50 °C. The optimal temperature (37 °C) turned out to be more similar to that of the

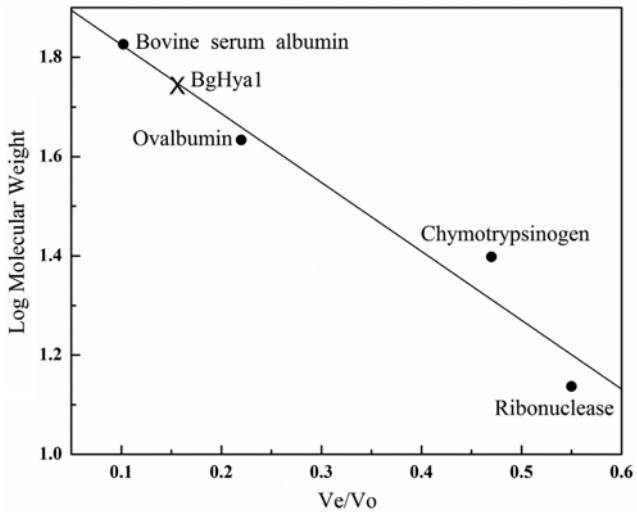


**Fig. 3. The molecular weight of BgHya1, as calculated from the calibration curve of a Sephacryl S-200 column. Standard proteins were previously described in the Materials and Methods Section.**

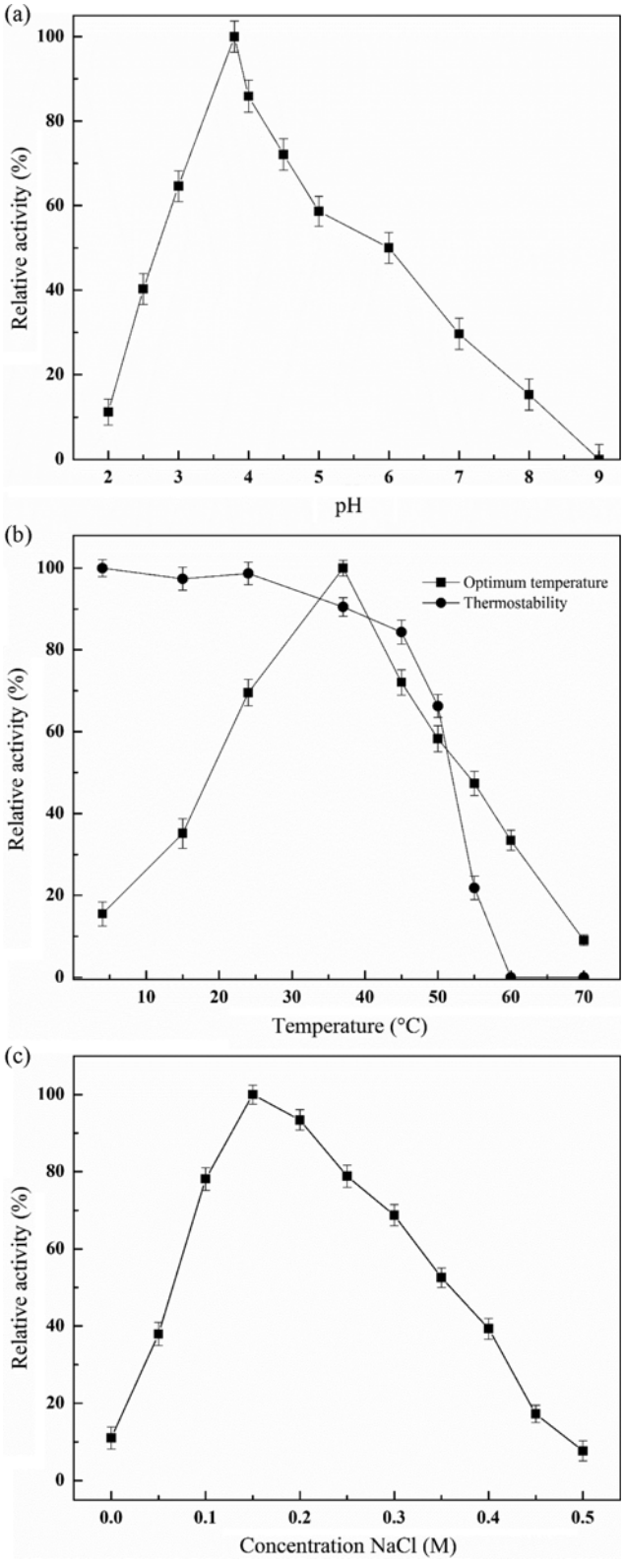


**Fig. 4. Biochemical characterization of BgHya1. (a) Profile of the pH optimum. (b) Profile of the optimum temperature and thermostability. (c) Profile of the concentration of NaCl.**

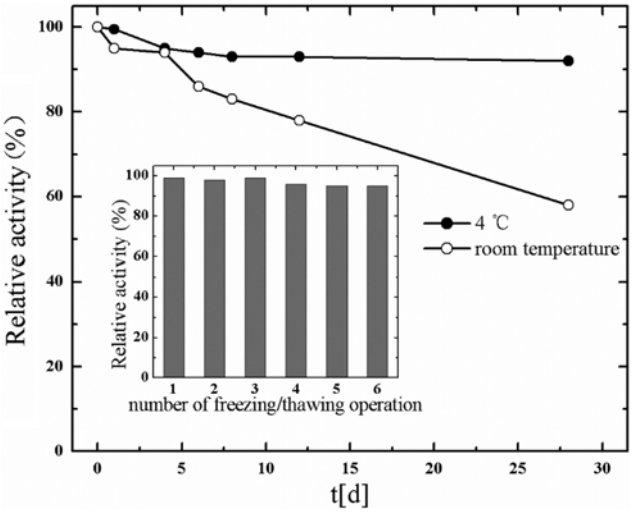


**Fig. 5. Storage stability of BgHya1. The activity was determined after storage of the enzyme solution (containing BSA 1.0 mg/ml) at 4 °C (—●—) and room temperature (—○—), respectively. Inset: Activity of the BgHya1 after repeated freezing and thawing of the enzyme solution. Immediately after the determination of the enzyme activity, the solution was stored at –20 °C for at least 1 day, before activity was redetermined after thawing of the solution at room temperature.**

bovine testicular hyaluronidase [28]; it was also in agreement with hyaluronidase from *Naja naja* [29], *Bos taurus*, and *Apis mellifera* [25]. Hyaluronidases from the scorpion venoms *T. serrulatus* and *P. gravimanus* were found in the relatively higher range of 37-40 °C [1,21,23,30]. In addition, it was noted that BgHya1 activity decreased significantly above 60 °C. This phenomenon was similar to those reported for bacterial hyaluronidase, which was almost completely destroyed at 60 °C. As shown in Fig. 4(b), BgHya1 activity was stable in the range of 4-45 °C, but it had a sharp drop from 45 °C to 55 °C. Above 60 °C, the enzyme was totally inactivated. Other hyaluronidases reported from different sources lose native conformation when heated to 60 °C [28,29,31,32].

**4. Effect of NaCl on Enzyme Activity**

BgHya1 activity was greatly influenced by the concentration of NaCl, and maximum activity was observed in the presence of 0.15 M NaCl, while concentrations over 0.5 M caused 92% inhibition (Fig. 4(c)). However, the NaCl concentrations in the reaction mixture with optimum activity reported for bovine testicular hyaluronidase and sheep testicular hyaluronidase were 0.65 M and 0.9 M, respectively [33]. Yang and Srivastava [28] reported that bull-sperm hyaluronidase had an absolute NaCl requirement for its activity, but the optimum values were not given. Therefore, NaCl is a vital requirement for both enzyme activity and BgHya1stability.

**5. Storage Stability**

Hyaluronidases exhibit considerable variation in storage stability. Some preparations rapidly lose activity when stored above 4 °C [34], while the hyaluronidase from *N. naja* is stable and maintains enzyme activity for over 15 days at 37 °C [29]. In the present study, as shown in Fig. 5, 92% of the activity was retained after four weeks when the enzyme solution was kept at 4 °C, whereas storage at room temperature over the same period resulted in a continuous decrease to 58% (day 28) compared to the activity measured at the beginning of the experiment (day 1). In addition, multiple freezing and thawing caused a serious loss of BgHya1 activity. However, the addi-

**Table 2. Effect of different metal ions on BgHya1 activity**

| Metals (50 mM) | Relative activity (%) |
|---|---|
| Control | 100±2.21 |
| $Fe^{3+}$ | 20±4.12 |
| $Mg^{2+}$ | 46±3.21 |
| $Ca^{2+}$ | 65±2.61 |
| $Fe^{2+}$ | 77±3.34 |
| $Cu^{2+}$ | N.D. |
| $Zn^{2+}$ | N.D. |
| $Co^{2+}$ | N.D. |

* N.D.: Not detectable. The results are expressed as mean±S.E.

tion of BSA to the enzyme sample prior to freezing resulted in complete retention of enzyme activity (Fig. 5). Similar findings have also been reported in Norway lobster (*Nephrops norvegicus*) hyaluronidase [33].

**6. Effect of Metal Ions and Inhibitors**

Among the metal ions investigated (Table 2), the activity of BgHya1 was inhibited by $Fe^{3+}$>$Mg^{2+}$>$Ca^{2+}$>$Fe^{2+}$, with the result for $Fe^{3+}$ and $Fe^{2+}$, which reduced about 80% and 23% of the activity, respectively. Ferric chloride has been used as a flocculant for hyaluronidase extraction from shrimp processing waste waters [35], and ferric ions have been reported to be potent inhibitors of human kidney hyaluronidase, but to a much lesser extent [36]. $Ca^{2+}$ and $Mg^{2+}$ have been described as inhibitors of hyaluronidase from the venoms of the *Potamotrygon motoro* stingray [37], *Cerastes cerastes* viper [38], *Crotalus durissus terrificus* rattlesnake [40] and *Hippasa partita* spider [40]. BgHya1 was not affected by $Cu^{2+}$, $Zn^{2+}$, or $Co^{2+}$, similar to early reports of bull sperm hyaluronidase [28]. BgHya1 was unaffected by ascorbic acid, PMSF, DTT, glutathione (reduced), and L-cysteine (Table 3), which might be related to the enzyme structure stabilized by two-disulfide bridges [41]. In addition, BgHya1 was completely inhibited by chelation reagents at a concentration of 10 mM EDTA and urea, suggesting that the activity was metal dependent (Table 3). In contrast, the hyaluronidase from *Palamneus gravimanous* scorpion venom was unaltered by EDTA and urea [23]. Heparin, as an inhibitor, had a wide inhibitory spectrum to hyaluronidases from the venoms of the *Agkistrodon acutu*s snake [42], *Heterometrus fulvipus* and *Palamneus gravimanus* scorpions [23,24], and *Synanceia horrida* stonefish [43], and BgHya1 as well. This finding might suggest that the activity of hyaluronidase was inhibited by means of a noncompetitive mechanism by interacting with surface amino groups of the enzyme [44,45] or by prohibiting the binding of the hyaluronan substrate [46].

**Table 3. Effect of different chemicals on activity of BgHya1**

| Chemicals | Inhibition (%) |
|---|---|
| None | 0 |
| EDTA | |
| 1 mM | 41±2.28 |
| 5 mM | 75±2.49 |
| 10 mM | 102±3.01 |
| Urea | |
| 1 mM | 29±3.21 |
| 5 mM | 71±2.64 |
| 10 mM | 100±3.35 |
| Heparin | |
| 0.015 mg/ml | 25±3.11 |
| 0.15 mg/ml | 43±2.87 |
| 1.5 mg/ml | 90±3.06 |
| Others | |
| Ascorbic acid | N.D. |
| PMSF | N.D. |
| DTT | N.D. |
| Glutathione (reduced) | N.D. |
| L-cysteine | N.D. |

Others are at a concentration of 10 mM. N.D.: Not detectable. The results are expressed as mean±S.E.

**7. Kinetic Parameters**

The $K_m$ value of BgHya1 at pH 3.8 and 37 °C was calculated from the Lineweaver-Burk plot to be 0.106 mg/mL, with sodium hyaluronate as a substrate (Fig. 6). This value is similar to those of human serum hyaluronidase (0.087 mg/mL) and rat liver hyaluronidase (0.08 mg/mL) [47,48], as determined by the colorimetric Morgan-Elson method, and lower than those of *Synanceia horrida* stonefish hyaluronidase (709 μg/mL) [42], sheep hyaluronidase (4.25 mg/mL), scampi hyaluronidase (0.42 mg/mL), and bovine testis hyaluronidase (3.2 mg/mL) [33]. Compared to the $K_m$ of those hyaluronidases, BgHya1 has a comparatively high affinity for sodium hyaluronate.

**8. Substrate Specificity**

Table 4 shows that compared with bovine testicular hyaluronidase, BgHya1 exhibited higher hydrolysis rates (66%, 81%, 89%, 95%) when chondroitin-4-sulfate, chondroitin-6-sulfate, and dermatan were used as substrates, respectively. BgHya1 also had the highest hydrolysis rate on sodium hyaluronate, which is similar to those reported for snake venom hyaluronidases, which mainly produce tetrasaccharides [49]. It is speculated that this result is due to

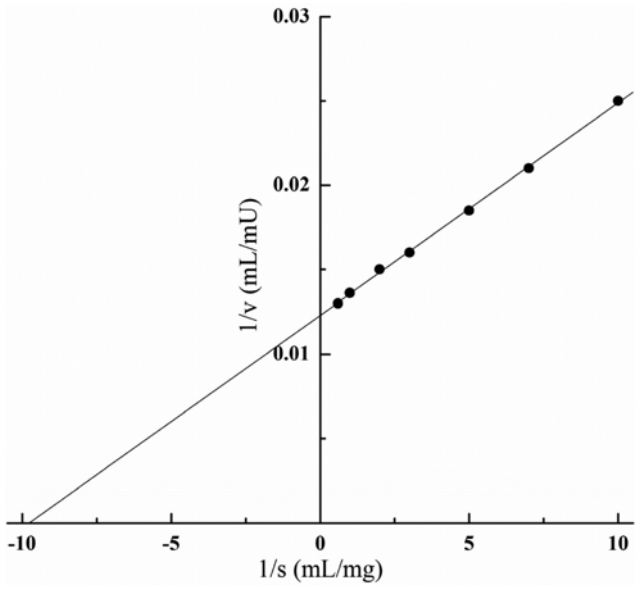


**Fig. 6. Lineweaver-Burk plot of the BgHya1 activity with the substrate sodium hyaluronate.**

**Table 4. Substrate specificity of BgHya1 and bovine testicular hyaluronidase**

| Substrate (100 μg) | Relative activity of bovine testicular hyaluronidase (%) | Relative activity of BgHya1 (%) |
|---|---|---|
| Sodium hyaluronate | 34 | 100 |
| Chondroitin-4-sulfate | 19 | 28 |
| Chondroitin-6-sulfate | 11 | 19 |
| Dermatan | 5 | 7 |

Bovine testicular hyaluronidase from Sigma (H3884 Type IV-S, 750-3,000 units/mg solid)

differences in polymer sizes and isomeric patterns. Sodium hyaluronate has a greater molar mass than other substrates, and it presents a higher number of repeating units of N-acetyl-D-glucosamine and D-glucuronic acid to be cleaved, facilitating the access of hyaluronidase to the hydrolysis sites. Another difference is that sodium hyaluronate is formed by N-acetyl-D-glucosamine, while the other tested substrates are formed by N-acetyl-D-galactosamine-sulfate [38].

## CONCLUSIONS

Information regarding the use of yak wastes is limited. This work was the first to develop an effective purification process based on chromatography to obtain a hyaluronidase from a new source—yak (*Bos grunniens*) by-product. As a novel source of hyaluronidase, BgHya1 exhibited higher activity than commercially available samples. Based on partial characterization in a set of biochemical assays, the BgHya1 purified from yak testes was classified as a true hyaluronidase. During yak processing, a large amount of the testis is discarded. Thus, this material can be the cheapest source for the production of BgHya1. Therefore, yak testis may be a suitable candidate for exploring commercial hyaluronidase for further use. In addition, its use would reduce environmental pollution.

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