

A hyaluronidase from the Snake Venom of *Agkistrodon Blomhoffii* Ussurensis of Changbai Mountain: Isolation and Characterization

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Abstract

Snake venom is a complex mixture of protein and peptide toxins. Hyaluronidase a 'spreading factor' has not been studied extensively in snake venom. In this paper, we describe the purification and characterization of a hyaluronidase from the snake venom of *Agkistrodon blomhoffii ussurensis* of Changbaimountain. Hyaluronidase has been purified by the successive chromatography on a DEAE-Sephadex A-25, on CM- Sephadex C-25 and on Sephadex G-75 columns on the basis of the purification method of thrombin-like enzyme (TLE). The molecular mass was found to be 35.0 kDa by SDS-polyacrylamide gel electrophoresis. Hyaluronidase was 5.66 percent of the snake venom of *Agkistrodon blomhoffii ussurensis* of Changbai mountain, the activity of mass (u/g) was 437218.5. The pH optimum for the enzyme was in the rang of 3.0-4.0 and 6.0-6.5, maximum activity was obtained at 37°C, and the optimum concentration of Na⁺(mol/L) was in the rang of 0.3-3.0, the Km for hyaluronidase, using hyaluronic acid as substrate, was 4.633×10^{-3} mg/ml. The hyaluronidase had not the inhibition effect to growth of the Hela cell and HepG-2 cell in vitro through the experiment.

Keywords: *Agkistrodon blomhoffii ussurensis* of Changbai mountain, Hyaluronidase, Hyaluronic acid

1. Introduction

Hyaluronidases are a ubiquitously expressed group of very dissimilar enzymes that naturally cleave hyaluronic acid (HA), a high molecular weight glycosaminoglycan, which is a major component of extracellular matrix (ECM) of vertebrates that connects protein filaments, collagen fibers and the connective tissue cells (Laurent T.C., 1989; Laurent T.C., 1992, pp.2397-2404). Some of these enzymes have the ability to degrade additional glycosaminoglycans, but at a slower rate (Frost G.I., 1996, pp.419-434).

Hyaluronidase has been considered as an invariant factor in the venoms of snakes, bees, scorpions, stonefish and lizards (Meyer K., 1971, pp.307-320; Kreil G., 1995, pp.1666-1669) and is popularly referred to as a 'spreading factor' as it facilitates easy diffusion of systemic toxins in to the circulation of the prey. Rapid hydrolysis of long linear megastructure of hyaluronan into fragments of varied molecular size drastically reduces the viscosity of the envenomed milieu favoring rapid diffusion of toxins into circulation which were otherwise diffused much slowly (Girish K.S., 2002, pp.105-110; Girish K.S., 2004, pp.193-202; Girish K.S., 2006, pp.1433-1440).

Kenzo Kudo and Anthony (Kudo K., 2001, pp.154-162) was comprehensive to report hyaluronidase activity from the venom of *Agkistrodon contortrix contortrix* (Southern Copperhead), on a Sephacryl S-200 HR column and CM-Sephadex C-25 column. The hyaluronidase's molecular weight is about 60 kD, the activity of mass (u/g) is 243000, the optimal temperature is 37 °C, the optimum pH is 6.0, the optimum concentration of Na⁺(mol/L) is in the rang of 0.3 - 3.0. But the concentration of hyaluronidase in the venom is very small, from 500 mg crude venom only 0.3 mg of purified hyaluronidase could be obtained.

The 70 percent of thrombin-like enzyme (TLE) was purified from the snake venom of *Agkistrodon blomhoffii ussurensis* of Changbai mountain, the first purification was DEAE-Sephadex A-25 chromatography, the penetrated liquid through DEAE-Sephadex A-25 column as waste, but the waste has hyaluronidase. In this paper, hyaluronidase has been purified by the successive chromatography on a CM-Sephadex C-25 and on Sephadex G-75 columns on the basis of the purification method of thrombin-like enzyme (TLE). We have not found the report of purification of hyaluronidase from the snake venom of *Agkistrodon blomhoffii ussurensis* of Changbai mountain.

2. Materials and methods

2.1 Materials

Lyophilized crude venom from *Agkistrodon blomhoffii ussurensis* of Changbai mountain was provided by pharmaceutical limited company of Kaitai, Hubei province of china; DEAE-Sephadex A-25, CM-Sephadex C-25, Sephadex G-75 was from Pharmacia; bovine testicular hyaluronidase was from Sigma; HA was from biological limited company of Jiangyin, Jiangsu province of China; heal cell and hepG-2 cell was provided by Neural Molecular Biology Laboratory, Faculty of Life Science and Technology, Kunming university of science and technology; All other chemicals and reagents were of the first grade commercially available.

2.2 Methods

2.2.1 Venom

The Lyophilized crude venom from *Agkistrodon blomhoffii ussurensis* of Changbai mountain was macerated and dissolved in phosphate buffer 20mM, pH 7.6, and immediately centrifuged at 3000rpm for 10 min. Venom was stored at -20°C until use.

2.2.2 DEAE- Sephadex A-25 column chromatography

The crude venom was loaded on a DEAE- Sephadex A-25 column (2.5×30 cm) previously equilibrated with 20mM tris-Hcl buffer, pH 7.6, and flowed with the same buffer at a flow rate of 48 ml/h, collected the penetrated liquid through DEAE-Sephadex A-25 column within 6 hours. The collected liquid was lyophilized to powder, the mass of powder is 0.3g.

2.2.3 CM-Sephadex C-25 column chromatography

The powder of 2.2.2 was loaded on a CM-Sephadex C-25 column (1.6×20 cm) previously equilibrated with 20mM phosphate buffer, pH 6.4, and flowed with the same buffer. Column was eluted stepwise using same buffer containing NaCl of 0–1000mM. Fractionation was carried out at room temperature at a flow rate of 30 ml/h and fractions were collected, the collected fractions was Lyophilized to powder.

2.2.4 Sephadex G-75 column chromatography

The Lyophilized fractions of 2.2.3 was loaded on a Sephadex G-75 column (1×40cm) previously equilibrated with 20mM phosphate buffer, pH 6.4, and flowed with the same buffer. Fractionation was carried out at room temperature at a flow rate of 30 ml/h and fractions were collected, the collected fractions was Lyophilized to powder.

2.2.5 Hyaluronidase activity

The hyaluronidase activity was determined turbidimetrically by the method of Ferrante (Kudo K., 2001, pp.154-162; Ferrante ND, 1956, pp.303-306). Aliquots of 50 ml of the enzyme solution were added to 70 mg of hyaluronan in 0.45 ml of 0.2 M sodium acetate buffer, pH 6.0, containing 0.15 M NaCl. The mixture was incubated at 37°C for 15 min, and then the reactions were terminated by the addition of 1 ml of 2.5% hexadecyltrimethylammonium bromide in 2% NaOH solution. After 5 min, the optical density of each sample was read by spectrophotometer at 400 nm and then blank-corrected. All assays were performed in duplicate. Bovine testes hyaluronidase was also used for comparison. One unit is defined as the amount of enzyme that will cause the same turbidity reduction as 1.0 unit of international standard preparation.

2.2.6 SDS-polyacrylamide gel electrophoresis

SDS-PAGE (15%) was carried out according to the method of Laemmli (Laemmli U.K., 1970, pp.680-685). Molecular weight standards from 116 to 14.4 kDa were used. Electrophoresis was carried out using Tris (25 mM), glycine (192mM) and SDS (0.1%) for 3 h at 90V at room temperature. After electrophoresis, gel was stained with 0.1% Coomassie brilliant blue R-250 for detection of the protein bands and destained with 25% ethanol and 10% acetic acid.

2.2.7 pH and temperature kinetics and effect of NaCl on enzyme activity

For determination of the optimum pH for enzyme activity, 0.2 M sodium acetate buffer containing 0.15 M NaCl was used. The sodium acetate solution has buffer capacity from 3 to 11. For the optimum temperature study, the enzyme activity at various temperatures was measured under the standard assay conditions. For the optimum NaCl concentration study, sodium acetate buffer, pH 6.0, containing various NaCl concentrations was used.

2.2.8 Protein estimation

Protein concentration was determined according to the method of Lowry et al. using bovine serum albumin (BSA) as standard.

2.2.9 The activity of anti-tumour in vitro

The activity of anti-tumour in vitro was determined turbidimetrically by the method of MTT, and the inhibition ratio of hyaluronidase to growth of heal cell and hepG-2 cell in vitro was calculated by Graphad Prism 5.0 software and Excel software(Pauwels R, 1988, pp.309-321; Park JG, 1987, pp.5875-5879).

3. Results

3.1 Purification of hyaluronidase

A three-step protocol was standardized for hyaluronidase purification. The first step involved the DEAE-Sephadex A-25 chromatography on the basis of the purification method of thrombin-like enzyme (TLE) (Fig. 1A), the penetrated liquid was collected and lyophilized to powder. The second step involved the CM-Sephadex C-25 chromatography and resolved into four peak of protein. The second peak containing hyaluronidase activity was eluted with a linear gradient of NaCl (Fig. 1B). The third step involved the Sephadex G-75gel filtration chromatography and resolved into two peak of protein, the second peak containing hyaluronidase activity (Fig.1C).

SDS-PAGE showed that the enzyme migrated as a single band, and the molecular weight of the hyaluronidase was about 35 kDa (Fig. 2). Fig. 3 are the standard turbidimetrical curve of HA and hyaluronidas. Hyaluronidase was 5.66 percent of the snake venom of Agkistrodon blomhoffii ussurensis of Changbai mountain. A summary of the purification procedure is given in (Table 1). The enzyme was purified to 76.6-fold with a yield of 4.33-fold, having a specific activity of 4.37×10^5 u/g of protein. We can found there is the inhibitor of hyaluronidase from Table 1. The inhibitor was acidic substance.

The pH activity profile of purified hyaluronidase was determined in a pH range from 3 to 11 using sodium acetate buffer. The enzyme had two optimal pH range: 3.0-4.0; 6.0-6.5 (Fig. 4A). Degradation effect of HA is maximal in the pH range from 3 to 4 (Hokputsa S, 2003, pp.111-117; Severian Dumitriu, 1998, 313-335), so mangabe hyaluronidase have the maximal activity in the pH range from 3.0 to 4.0. The influence of temperature on hyaluronidase activity was determined between 4 and 60 °C at pH 6.0. The optimal temperature for hyaluronidase activity was 37 °C and the activity decreased significantly apart from 37 °C (Fig. 4B). The enzyme activity was greatly influenced by sodium ions, and it reached its maximal activity in the range from 0.2 to 3.2M sodium ions (Fig. 4C). The K_m (1.09×10^{-3} mg/ml) values were calculated from the fig of Hanes of the hyaluronidase with the substrate hyaluronic acid(Xu, X, 1982, pp.973-981) (Fig. 5).

3.2 The activity of anti-tumour in vitro

The hyaluronidase of the snake venom of Agkistrodon blomhoffii ussurensis of Changbai mountain had not activity of anti-tumour to growth of heal cell and hepG-2 cell in in vitro(Fig. 6).

4. Discussion

Pathophysiological mechanism of sanke venom in duced tissue damage and spreading facilitated diffusion of systemic toxins is currently gaining importance. Venom contains mixture of toxins and envenomation-induced effects are due to synergistic effects of these toxins. Metalloprotease and hyaluronidases are the main components involved in local tissue damage and gravitational spreading of the venom. The pathology involves the degradation of proteins and glycosaminoglycans in the extracellular matrix (ECM) surrounding the blood vessels and capillaries (Xu, X, 1982, pp.973-981; Baramova, E.N., 1989, pp. 63-71; Maruyama, M., 1992, pp.1387-1397; Girish K.S., 2002, pp.105-110).

In this study with the snake venom of Agkistrodon blomhoffii ussurensis of Changbai mountain, we found that three-step fractionation on DEAE- Sephadex A-25, columnCM-Sephadex C-25 colum and Sephadex G-75 column resulted in the purification of a protein with hyaluronidase activity on the basis of the purification method of thrombin-like enzyme (TLE). The final yield of 5.66% (protein by weight) and of 4.34-fold of

76.6-fold purified hyaluronidase will signify the difficulty associated in working with the enzyme. There is the inhibitor of hyaluronidase, and the inhibitor was acidic substance.

SDS-polyacrylamide gel electrophoresis showed that hyaluronidase purified from the snake venom of *Agkistrodon blomhoffii ussuriensis* of Changbai Mountain as a single band with an estimated molecular mass of 35 kDa.

The optimal pH for the enzyme activity was different from that found for hyaluronidase from a variety of snake venom. There were two ranges: 3.0-4.0; 6.0-6.5. Degradation effect of HA is maximal in the pH range from 3 to 4 (Sanya Hokputsa, 2003, pp.111-117), so mangabe hyaluronidase have the maximal activity in the pH range from 3.0 to 4.0. The optimum temperature was found to be 37°C at pH 6.0, and it is in agreement with hyaluronidase from *Agkistrodon contortrix contortrix* (Southern Copperhead) (Xu xun, 1983, p.680-681). The enzyme activity was greatly influenced by sodium ions, and it reached its maximal activity in the range from 0.2 to 3.2M sodium ions.

The purified hyaluronidase from the snake venom of *Agkistrodon blomhoffii ussuriensis* of Changbai mountain showed Michaelis kinetics with hyaluronic acid as substrate. The K_m of 1.09×10^{-3} mg/ml indicates that the enzyme has comparatively high affinity for hyaluronic acid compared with other hyaluronidases. This value was substantially lower than the reported for *Agkistrodon acutus* (6.2×10^{-3} mg/ml).

There are two diametrically opposite views about the role of hyaluronidase on the activity of anti-tumour. Some studies think that the expression increases of HA in the serum and ECM have a relationship with the progress and transfer of malignant tumor (Lee J Y, 2000, p.581; Zhang L, 1995, p.428). Excessive synthesis of HA promotes the growth of cancer cells (Kosaki R, 1999, p.1141), and hyaluronidase inhibits malignant tumor by decomposing HA, so hyaluronidase is taken as the products of tumor inhibiting genes (Csoka AB, 2001, p.499; De Maeyer E, 1992, p.657). However, other researches discovered that hyaluronidase has a stimulation to growth, invade and transfer of tumor cells (Lin G, 2001, p.95). We found the hyaluronidase had not the inhibition effect to growth of the Hela cell and HepG-2 cell in vitro through the experiment. So we can principally determine that the hyaluronidase has not effect to growth of the tumour cells, it will have effect to the tumour cells only in the human body and with the HA. But whether it has the promotion effects or the inhibition effects should be researched later.

In conclusion, this study presents the first purification of a hyaluronidase from the snake venom of *Agkistrodon blomhoffii ussuriensis* of Changbai Mountain. This enzyme exhibited high affinity for hyaluronic acid. Further structural and functional analyses might provide an insight for the better understanding of the role.

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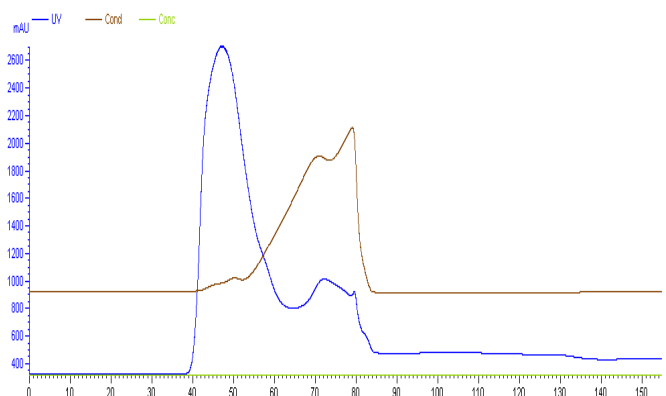
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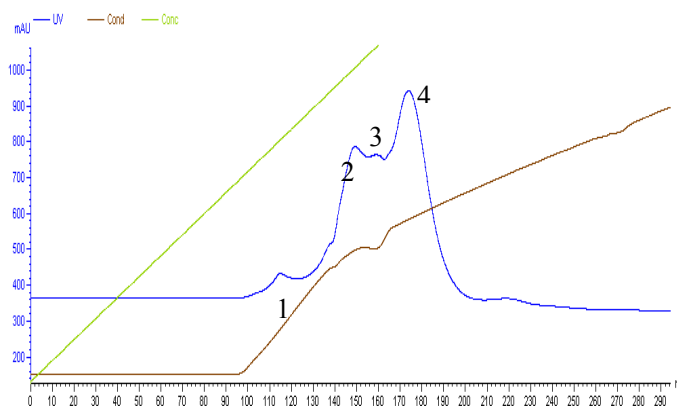
Table 1. Summary of purification of hyaluronidase from the snake venom of *Agkistrodon blomhoffii ussurensis* of Changbai mountain

Purification step	Total protein (mg)	Total activity (u)	Specific activity (u/g)	Purification (fold)	Yield (fold)
Crude venom	800	4.57×10^3	5.71×10^3	1	1
DEAE-Sephadex A-25	300	3.64×10^4	1.21×10^5	21.2	7.96
Sephadex G-75	45.3	1.98×10^4	4.37×10^5	76.6	4.34

A



B



C

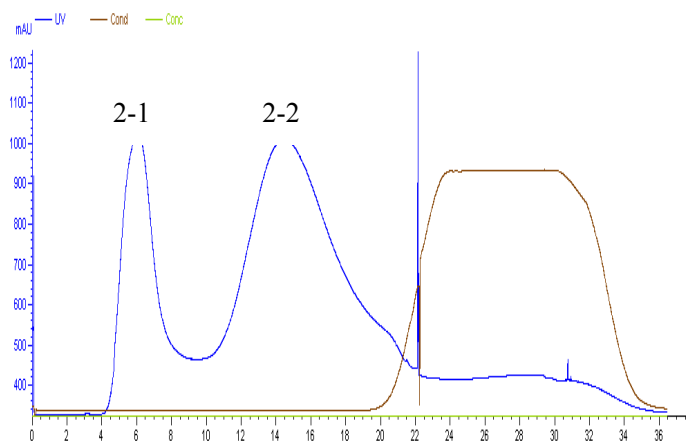


Figure 1. Isolation of hyaluronidase from the snake venom of *Agkistrodon blomhoffii ussurensis* of Changbai mountain. (A) Elution profile from DEAE-Sephadex A-25 chromatography. The column (2.5×30 cm) was eluted with 20mM tris-HCl buffer, pH 7.6 at a flow rate of 48 ml/h, and 45ml fractions were collected. Protein elution was monitored at 280nm. The collected fractions were concentrated and applied onto CM-Sephadex C-25 column for further fractionation. (B) Elution profile from CM-Sephadex C-25 column chromatography. The column (1.6×20 cm) was equilibrated with 20mM phosphate buffer, pH 6.0, at a flow rate of 30 ml/h. The column was washed with the same buffer and eluted with a linear gradient of 0–1.0M NaCl, resolved into four peaks of protein. The second peak containing hyaluronidase activity, and was concentrated and applied onto Sephadex G-75 column for further fractionation. (C) Elution profile from Sephadex G-75 chromatography. The column (1×40cm) was eluted with 20mM phosphate buffer, pH 6.0, containing at a flow rate of 30 ml/h, resolved into two peaks of protein, the first peak containing hyaluronidase activity. Protein elution was monitored at 280nm

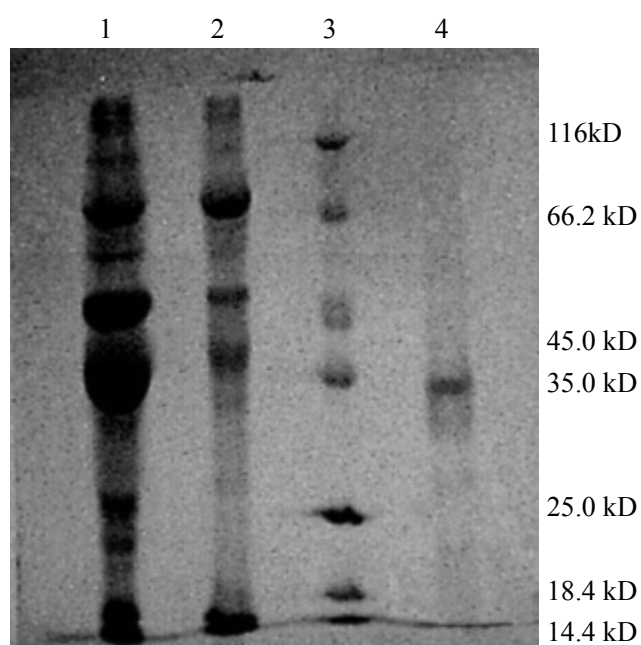
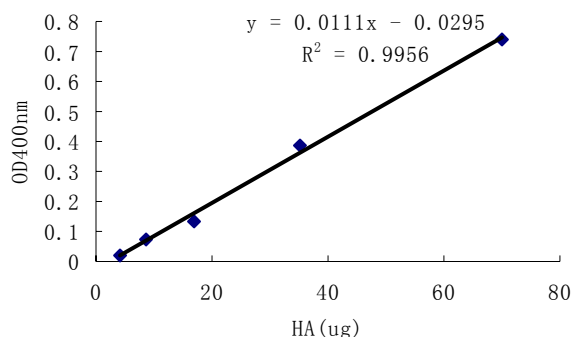


Figure 2. SDS-PAGE of the purified hyaluronidase. (Lane 1) Crude the snake venom of *Agkistrodon blomhoffii ussurensis* of Changbai mountain. (Lane 2) Purified enzyme after DEAE- Sephadex A-25 chromatography. (Lane 3) Molecular weight markers. (Lane 4) Purified hyaluronidase

A



B

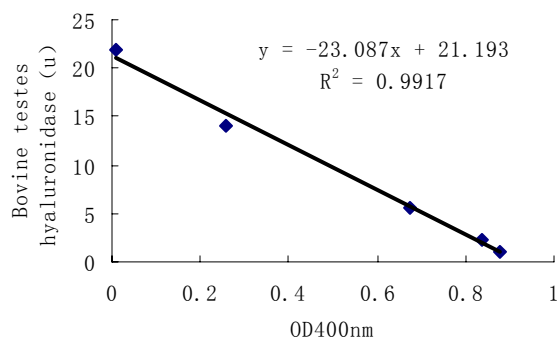
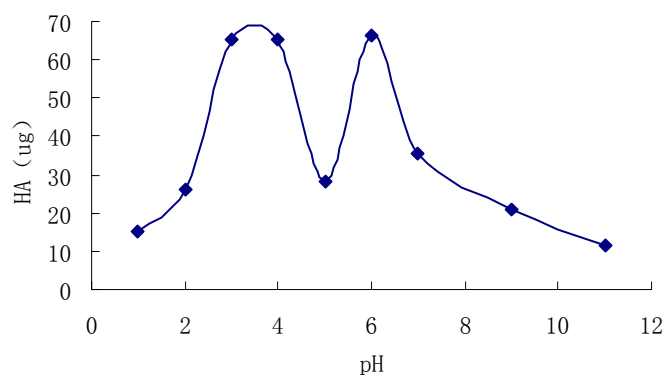
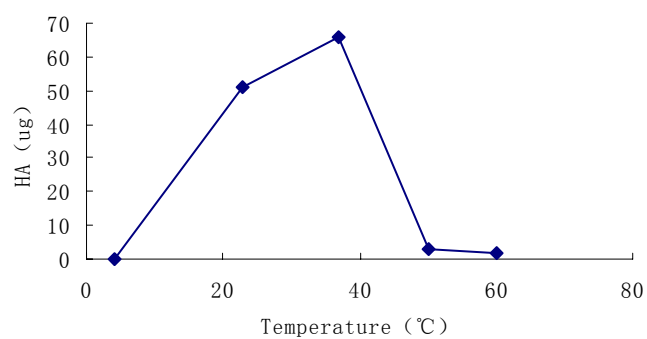


Figure 3. Determination of enzyme activity of the purified hyaluronidase from the snake venom of *Agkistrodon blomhoffii ussurensis* of Changbai mountain. (A) Different mass of hyaluronan in 0.45 ml of 0.2 M sodium acetate buffer, pH 6.0, containing 0.15 M NaCl was incubated at 37°C for 15 min, and then the reactions were terminated by the addition of 1 ml of 2.5% hexadecyltrimethylammonium bromide in 2% NaOH solution. After 5 min, the optical density of each sample was read by spectrophotometer at 400 nm and then blank-corrected. All assays were performed in duplicate. (B) Aliquots of 50 ml of the different concentration of bovine testes hyaluronidase solution were added to 70 mg of hyaluronan in 0.45 ml of 0.2 M sodium acetate buffer, pH 6.0, containing 0.15 M NaCl. The mixture was incubated at 37°C for 15 min, and then the reactions were terminated by the addition of 1 ml of 2.5% hexadecyltrimethylammonium bromide in 2% NaOH solution. After 5 min, the optical density of each sample was read by spectrophotometer at 400 nm and then blank-corrected. All assays were performed in duplicate.

A



B



C

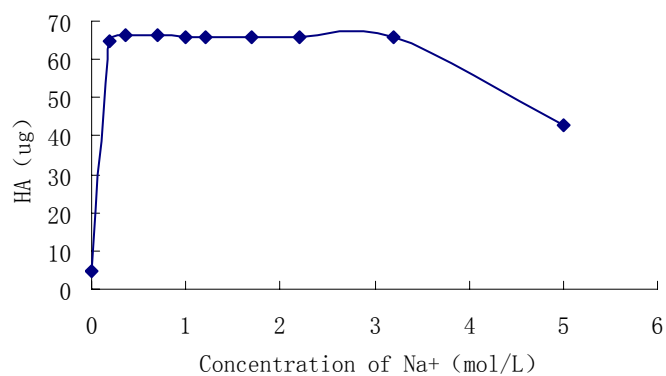


Figure 4. The effect of pH (A), temperature (B), and NaCl concentration

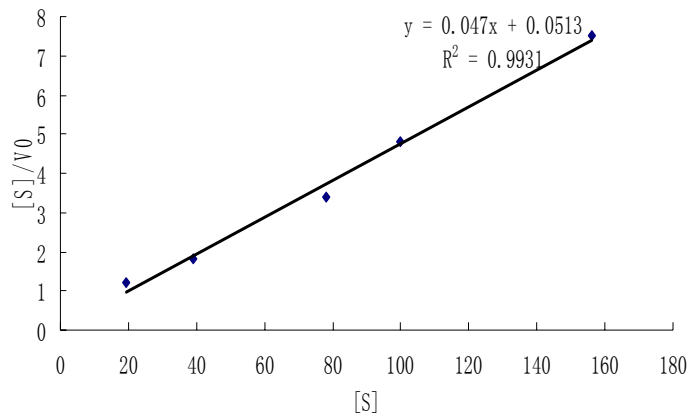
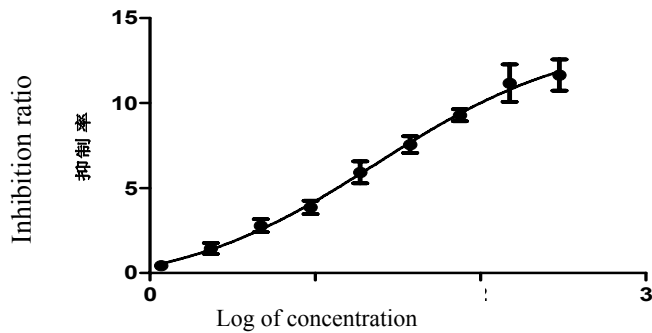


Figure 5. Fig of Hanes of the hyaluronidase with the substrate hyaluronic acid.Experiments were performed at 37 °C and pH 6.0

A



B

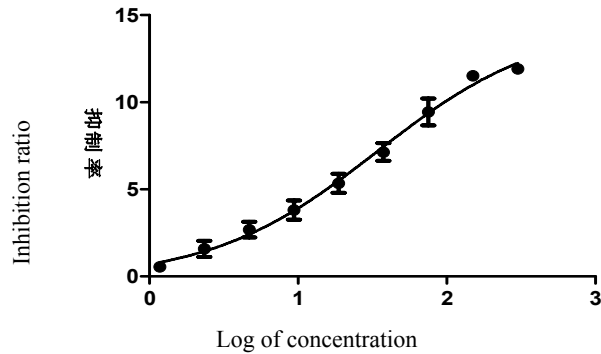


Figure 6. The activity of hyaluronidase of anti-tumour in vitro.(A)The effect of different concentration of hyaluronidase to growth of heal cell in vitro.(B) The effect of different concentration of hyaluronidase to growth of hepG-2 cell in vitro