

Preparation, Characterization and Anticancer Applications of HAase from *Flavobacterium heparinum*

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Abstract

Hyaluronidase (HAase) is attracting considerable attention in the field of medical cosmetics and exhibits considerable potential. In this study, a novel HAase (FH-HAase) was obtained from ultrasonication cells of *Flavobacterium heparinum* cultivated in fermentation broth containing hyaluronic acid. FH-HAase was separated and purified through affinity chromatography and ion exchange chromatography. The final purification fold of enzyme obtained was 48.76, the yield was 43.08%, and the specific activity was 33.06 IU/mL. The purified enzyme was displayed as a single band on electrophoresis, and its molecular weight was determined to be 79.6 KDa by LC-MS. The enzyme was stable at pH of 6.5-7.5 and temperature below 30°C. Hyaluronic acid is the optimal substrate for enzyme degradation, while chondroitin sulfate and dermatan sulfate can also be degraded. The purified HAase showed potent anticancer activities against melanoma B16F10 cells with low toxicity against HaCaT cell. The cell viability of HAase-treated B16F10 cells was found to be in a dose dependent manner. This is the first report, to our knowledge, on preparing HAase from microorganism *Flavobacterium heparinum* and the application of it in inhibiting tumor cell growth.

Introduction

Hyaluronan (HA) is the most common glycosaminoglycan in the dermis, composed of alternating units of N-acetyl-D-glucosamine and D-glucuronic acid[1]. Hyaluronidase (HAase) is a kind of glycosidase widely distributed in nature, which degrades hyaluronic acid by acting on β -1,3 or β -1,4 glycosidic bonds[2].

HAase has been detected in many biological materials, such as animal testicles and venom, as well as in various bacteria and fungi, since its first discovery in 1929 by Duran Reynals [3] in extracts from mammalian testes. HAases from different sources are different protein molecules, and their degradation characteristics towards substrates are also different[2–4].

Over the past few decades, hyaluronidase has been widely used in many medical fields, such as plastic surgery, surgery, ophthalmology, internal medicine, tumor treatment, dermatology, and gynecology, some of which have been approved by the US FDA as clinical drugs[5–7].

In this report, we discovered the HAase in *F. heparinum*, increased the yield of the enzyme by fermentation optimisation, purified the HAase through columns chromatography, and conducted enzymatic properties studies. Thereafter, we found FH-HAase could selectively inhibit the activity of Melanoma B16F10 cells.

Materials and methods

Materials

Experiments were performed by using hyaluronic acid (Shaanxi Bolin Biotechnology, China); tryptone, beef meal, and nutrient agar (Oxoid,UK); Cellufine Sulfate (CHISSO, Japan), UniGel-30CM (Cytiva, USA); Melanoma B16F10 cells (B16F10), Human immortalised keratinocytes (HaCaT), human ovarian cancer cells (SK-OV-3), human hepatocellular carcinoma cells (HepG2), human bladder migratory cell carcinoma cells (T-24), and human lung cancer cells (A549) (Shanghai Cell Bank, China), coomassie brilliant blue, PMSF, DTT (Sigma,USA), electrophoresis molecular weight marker kits(MBI, New England). These chemicals and all others were analytical grade.

Optical measurements were made with a spectrophotometer (UV-2100, Unico, China). For experimental work, incubator shaker (Xinrui automatic apparatus, China) were used.

Assays

Protein assays

Protein was measured by means of Lowry[8]. Assays were performed using a UNIC 2100 spectrophotometer.

Enzyme assays

HAase activity was measured by the increase in ultraviolet absorption at 232 nm, according to the procedure of et al[9].

Optimization of hyaluronidase production by *Flavobacterium heparinum*

The strain used in this study was *Flavobacterium heparinum* (ATCC 13125). The microorganism was maintained on slants contained (w/v) (%): tryptone 1.0, beef extract 0.3, NaCl 0.5, agar 1.5 at 4°C.

The bacterial cells on slant culture were transferred into a 0.5-L shake flasks contained 100 mL seed medium which is the same as slant except no agar added. The culture was incubated on a 2.5-cm stroke shaker at 150 rpm for 20–24 h. Shake flasks contained fermentation medium contained (w/v) (%): HA (0.1–0.3%), peptone (1.0%); Na₂HPO₄(0.5%); CaCl₂ (0.05%) were inoculated from seed culture in exponential growth at a dose of 5%. Cultures were grown in shake flasks for 24h, then harvested.

Optimal dosage of HA

The microorganism was cultured in fermentation medium contained HA at different concentration, 0.1, 0.15, 0.2, 0.25 and 0.3% at 25°C, 150 rpm for 24 h. The cell free centrifugate was obtained at 4000 rpm centrifugation for 30 min and used for enzyme assay [10, 11]

Optimum incubation temperature

To examine the effect of fermentation temperatures on the production of hyaluronidase enzyme, different temperatures were used, 15°C, 20°C ,25°C and 30°C in flasks. All flasks were shaking incubated

at 150 rpm for 24 h[10, 11].

Optimum incubation pH

To examine the effect of starting pH on the production of hyaluronidase enzyme, different pH values were used, 4, 5, 6, 6.5, 7, 8 and 9 in flasks. All flasks were incubated at 25°C at 150 rpm for 24 h [10, 11].

Fermentation period

The suitable fermentation period supporting the maximum production of enzyme was investigated at different periods under the optimum conditions, 6–72 h at 150 rpm [10, 11].

Crude enzyme recovery

From one liter of fermentation broth that had been incubated under the optimum conditions (pH 7 and 21°C for 48 h), about 4 g wet cell pellet was obtained by centrifugation for 15 min at 10000×g at 4°C. This pellet was suspended in 100ml of 10mM Tris-HCl buffer at pH 7.0 and 4°C. Cell suspension (20ml at a time) was placed into a 30-ml glass cup and sonicated with cooling for 10 min at 150 watts using a 50% pulsed mode. The disrupted cells were centrifuged at 15000×g for 30 min at 4°C and the pellet discarded. The 100ml of supernant, obtained by sonification and centrifugation, contained 1.5 mg/ml protein.

Purification of FH-HAase

The crude enzyme was loaded onto a Cellufine Sulfate column (1.6×8 cm), which was pre-equilibrated with 5 column volumes of 25 mM Tris-HCl buffer (pH 7.0, containing CaCl_2 at 10 mM). The column was washed stepwisely by three column volumes of 25 mM Tris-HCl buffer (pH7.0, containing NaCl at 200 mM, 300 mM, 400 mM respectively). Fractions with HAase activity were collected and dialysed against 25 mM Tris-HCl buffer (pH7.0, containing CaCl_2 at 10 mM) overnight with two changes[12].

Lyase activity purified by Cellufine Sulfate column was loaded to a UniGel-30CM column (1.6×4 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH7.0, containing CaCl_2 at 10 mM). The column was washed stepwisely by three column volumes of 10mM Tris-HCl buffer (pH7.0, containing NaCl at 120mM, 140mM, 160mM, respectively). Fractions with HAase activity were collected and dialysed against 10 mM Tris-HCl buffer (pH 7.0, containing CaCl_2 at 10 mM) overnight with two changes[13].

Lyase activity purified by UniGel-30CM column was reloaded to UniGel-30CM column (1.6×4 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH7.0, containing CaCl_2 at 10 mM). The column was washed by a Tris-HCl buffer linear gradient of 0-500mM (pH7.0, containing CaCl_2 at 10 mM) at 1.0ml/min flow rate. Fraction of every 3ml elution were collected. The pooled fractions with HAase activity were dialysed overnight against 10 mM Tris-HCl buffer (pH7.0, containing CaCl_2 at 10 mM), then lyophilized or used for properties investigation straightly[13].

Absorbance at 280 nm, protein content and HAase activity were monitored for each fraction and each purification step.

Characterization of the purified HF-HAase

To assess the purity by electrophoresis, discontinuous SDS-PAGE was performed on the HF-HAase using a modification of a procedure previously described by Laemmli[14]. The gels were fixed with 12% (w/v) trichloroacetic acid, rinsed with distilled water and stained with a Rapid Coomassie Stain solution, and destained. The following proteins were used as SDS-PAGE electrophoresis molecular weight standards: rabbit phosphorylase b (97,400), bovine serum albumin (66,200), rabbit actin (43,000), bovine carbonic anhydrase (31,000), trypsin inhibitor (20,100), and hen egg white lysozyme (14,400).

The molecular weight of FH-HAase was determined by liquid chromatography-mass spectrometry on a U3000 UPLC-Q liquid-mass spectrometry system. Purified HF-HAase (10 μ L, 1 μ g/ μ L protein) was applied on a UPLC column (ACQUITY UPLC Protein BEH C4, 300 Å, 1.7 μ m, 2.1 $\times$ 50 mm, column temperature 70°C), and eluted by mobile phase A (aqueous solution containing 0.1% formic acid) and mobile phase B (acetonitrile solution containing 0.1% formic acid) at flow rate of 0.3 mL/min. The liquid phase gradient was set as follows: 0–3 min, 2% B; 3–10 min, 2%~95% B; 10–13 min, 95% B; 13-13.1 min, 95%~2% B; 13.1–15 min, 2% B. Mass spectrometry acquisition: Sheath gas flow rate (45), Aux gas flow rate (10), Spray voltage (3.5kV), Capillary temp. (320°C), S-Lens RF Level (55), Aux gas heater temp. Scan Type (Full MS), Microscans (10), Resolution (17500), AGC target (3e6), Maximum IT (200 ms), Number of scan ranges (1), Scan range Spectrum (400–4000 m/z), Data type (Profile)[15].

To analysis N-terminal, the purified enzyme (100 μ g) was loaded on seven tracks of SDS-PAGE gel (15% polyacrylamide). After electrophoresis, the gel was electrophoretically transferred onto PVDF membrane cartridge. N-terminal amino acid sequence analysis was performed using an applied Biosystems sequencer (ABI491A, PE Co.USA)[16].

The activity pH optimum for HF-HAase was obtained by using Tris-HCl (4.5–9.5). HAase assay solutions were made by diluting a 10 μ L sample of the purified lyase (2–3 mg/ml protein concentration) with 90 μ L of Tris-HCl buffer at 50 mM, pH 7.0, and placed on ice until required for assay. The activities of HAase at different pH values were then determined[17].

Temperature for optimum activity was determined for HF-HAase at pH7.0 in Tris-HCl buffer in 5°C increments at temperatures between 25°C and 85°C. The temperature was adjusted in a temperature-regulated spectrophotometer and equilibrated for 10 min before the assay was started.

To study the effect of temperature and pH on HF-HAase stability, enzyme assay stock solutions were prepared in buffer and placed in water baths at 25°C or in a fridge at 4°C, -20°C, -25°C, samples were taken out 30min later to measure remaining enzyme activity[17].

To study the effect of different buffer solutions on enzyme activity, 1 mg/mL of HA solution was resolved by 5, 25, 50, and 100 mM sodium acetate-acetic acid, PBS, and Tris-HCl buffer respectively. Activity of a

purified HAase sample was determined by using substract solutions as above[18].

To study the effect of metal ions on enzyme activity, CaCl_2 , MnCl_2 , MgCl_2 , KCl , NaCl , BaCl_2 , CuCl_2 , ZnCl_2 and FeCl_3 solutions were added at concentrations of 10, 25, 50, and 100 mM respectively. Activity of the mixed enzyme solutions were measured with the enzyme activity of the blank group defined as 100% [18].

To study the substrate specificity of HF-HAase, 1 mg/mL of HA, CS, DS, heparin, serine, inulin, gum arabic, galactose, CMC-Na, dextran, D-alginate, and K-carrageenan solution in 25 mM Tris-HCl (pH7.0, addition with 10 mM CaCl_2). Activity of a purified HAase sample was determined by using substract solutions as above[18].

Cytotoxicity of FH-HAase on normal and cancer cells

B16F10, HaCaT, SK-OV-3, HepG2, T-24, and A549 were evaluated for the cytotoxicity of FH-HAase. Cell viability in these cells was assessed using the MTT colorimetric assay[19]. The half-maximal inhibitory concentrations (IC_{50}) were analysed using Graph Pad Prism10.0 software.

Cellular mitochondrial membrane potential, reactive oxygen species ROS

B16F10 cells (2×10^5 cells/well) in 6-well plates were treated with 20, 30, and 40 $\mu\text{g/mL}$ FH-HAase for 12 h at 37°C , 5% CO_2 to detect membrane potential and ROS. JC-1, DCFH-DA, and Hoechst 33342 staining solution were added for 20 to 30 min, washed by PBS 2 times, photographed using a fluorescence microscope. Trypsin digested and blown cells were harvested by centrifugation (1500 rpm), resuspended in 1 mL of JC-1 for determination of membrane potential level, 1 mL of DCFH-DA for ROS assay[20–22].

Result and discussion

Optimization of hyaluronidase production

Effect of various HA concentration

The *Flavobacterium heparinum* was cultured in fermentation medium contained HA at different concentration, 0.1, 0.15, 0.2, 0.25 and 0.3% at 25°C , 150 rpm for 24 h. And the maximum production of HAase enzyme (1.75 U/mL) was obtained at 0.15% HA (Fig. 1A).

Effect of initial pH

Based on data present in (Fig. 1B), the optimum pH for HAase was at 8 with enzyme activity of 1.6 U/mL. The enzyme activity of HAase at pH 7 or 7.5 was similar to pH 8. However, at pH 6.5 and 8.5 there was much lower production of hyaluronidase enzyme. Medium pH is essential factor in the production of any metabolic substrate as it influences the properties of the medium, solubility of materials and ionic

state of hyaluronic acid as a bacterial substrate [23]. Neutral pH was recorded for hyaluronidase optimum production by Patil et al. and Kadhum [24], which is inconsistent with our results. In contrast, Sahoo et al.[10]recorded the optimum production of hyaluronidase at pH 5.5.

Effect of incubation temperatures

The optimum temperature was investigated by incubating *Flavobacterium heparinum* at different temperatures and the maximum production of HAase enzyme (3 U/mL) was obtained at 15°C (Fig. 1D). Further elevation in incubation temperature resulted in low production of enzyme due to its denaturation [25]. Patil et al. [24] recorded that the maximum production of hyase enzyme (284 U/mL) was at 37°C. Mahesh et al. [23] also reported that *Streptococcus mitis* recorded the highest production of enzyme at mesophilic range of temperature.

Effect of various incubation periods

The time course for HAase production showed the maximum activity of enzyme (3.2 U/mL) was within 48 h (Fig. 1C). The same results were obtained by Sahoo et al. [10] who found that the highest production of hyase by *Streptococcus mitis* was after 48 h incubation.

Purification of FH-HAase

The initial specific activity of HAase in the crude enzyme was estimated to be 0.68 U/mg protein (Table 1). The specific activity rose to 6.62 U/mg protein after Cellufine Sulfate column chromatography and estimated to be 16.37 and 32.32 U/mg protein with yields of 50.6% and 43.1% and 24.1 and 47.5-fold increased over the initial crude enzyme after UniGel-30CM column chromatography eluted by NaCl gradient and UniGel-30CM rechromatography eluted by Tris-HCl gradient.

Purification of hyase enzyme using DEAE cellulose and Sephacryl columns was recommended by Abdel-Monsef et al. [26] as a simpler and faster method. An enzyme hyaluronidase (hyase) producing halotolerant bacterium was isolated from dental caries and identified as *Brevibacterium halotolerans* DC1. Hyase was purified using salt precipitation, DEAE cellulose ion exchange, and Sephadex G-100 gel filtration chromatography. The enzyme was purified to 13-fold with 67.19% recovery of activity and 26.37 U/mg of specific activity[27]. Another report on the purification of hyase from the *Bacillus sp. A50* strain found that the enzyme has 102.14 U/mg protein, 25.38%, and 21-fold for specific activity, yield and fold, respectively [28].

Table 1
Summary of purification of HAase enzyme by *F. heparinum*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	2433	1650	0.68	1	100
Cellufine Sulfate	180	1192	6.62	9.7	72.2
UniGel-30CM (NaCl)	51	835	16.37	24.1	50.6
UniGel-30CM (Tris-HCl)	22	711	32.32	47.5	43.1

Characterization of HF-HAase

Molecular weight by LC-MS

The results of liquid chromatography (ACQUITY UPLC Protein BEH C4, 300Å, 1.7 µm, 2.1×50 m) showed that the enzyme sample contained three protein peaks (Fig. 3A), among which peak3 with the highest abundance was the target enzyme. Molecular mass of the enzyme (peak3) based on mass spectrometry analysis showed molecular weight of 79616.96 Da (Fig. 3B).

N-terminal amino acid sequencing

No N-terminal amino acid was detected in the purified FH-HAase. Zimmerman J J F[29] also failed to obtain the N-terminal amino acid of heparinase from *F. heparinum*. Our attempts on FH-HAase from the same strain also failed presumably due to blocked N termini.

activity and stability

Activity profiles of the purified enzyme was investigated at range of pH5-9 buffer systems at 30°C, or at temperature range of 25–75°C at pH 7.0. Results shown the optimal pH and temperature of the enzyme was 6.5 (Fig. 4A) and 45°C (Fig. 4C) respectively.

The thermo-stability variations were investigated by preincubating the purified enzyme in 10mM Tris-HCl buffer, pH7.0, at range of 30–45°C for 10h. The remaining activity values were determined under the standard assay conditions. Results indicated the enzyme was stable in 10h under 30°C, but was inactivated rapidly at 35°C or above (Fig. 4D). No significant retained activity was found after incubation at temperature above 45°C for 1 h. The purified enzyme remained comparatively stable in the region of pH 6.5–7.5 at 30°C for 24 h, but was inactivated out of this range (Fig. 4B).

The purified FH-Hase enzyme activity remains stable in sodium acetate buffer and Tris HCl buffer at concentrations of 5–50 mM (Fig. 4E). PBS buffer cannot be used for this enzyme, as it leads to a 50% decrease in enzyme activity at 5mM and a 100% decrease at concentrations above 25mM (Fig. 4E).

As shown in (Fig. 4F), K⁺, Na⁺, Ca²⁺, Mg²⁺, Ba²⁺, Mn²⁺ + showed little effect on enzyme activity in the concentration range of 10-100mM. However, Cu²⁺ + and Zn²⁺ + showed a significant inhibitory on enzyme activity, with no enzyme activity detected (CuCl₂) at 10mM or only 10% enzyme activity remaining (ZnCl₂).

Substrate specificity

As shown in Table 2, FH-HAase showed the strongest activity towards HA. The enzyme also showed obvious activity (78% vs HA) on chondroitin sulphate (CS) and (30% vs HA) on dermatan sulphate (DS). It did not show hydrolytic activity towards other substrates, such as heparin, inulin, gum arabica, CMC-Na, Dextran, trehalose and K-Carrageena (Table 2).

Table 2
Substrate specificity of FH-HAase

Substrate	Relative activity (%)
Hyaluronic acid (HA)	100
Chondroitin Sulfate (CS)	78
Dermatostatin Sulfate (DS)	30
heparin	2
inulin	1
gum arabica	1
CMC-Na	0
Dextran	0
trehalose	0
K-Carrageenan	0

Applications of FH-HAase

In vitro anticancer activity

The cytotoxicity of FH-HAase at 10-90ug/ml against tumor cells of SK-OV-3, HepG2, T-24 and A549 lines and normal HaCaT cells was evaluated. FH-HAase showed no cytotoxic effect on SK-OV-3, HepG2, T-24 and A549 cells by MTT assay. However, Melanoma B16F10 cells were significantly inhibited by HAase (Fig. 5A) with a IC₅₀ of 28.63 ug/mL. Indition, HaCaT cells were inhibited at 60-90ug/ml HAase (Fig. 5A) with a IC₅₀ of 86.12 ug/mL (Fig. 5B). This enzyme exhibits specific and strong cytotoxic activity against B16F10, but low toxicity to HaCaT cells, suggesting the potential of the enzyme in the treatment of melanoma.

Following treatment with different doses of the purified HF-HAase, the unchanged morphology of B16F10 cells were demonstrated in (Fig. 6A). The observation of inverted microscope showed that B16F10 cells untreated with HAase exhibit as spindle shaped, well-defined, and cytoplasmic transparent intact cells. After 24 h of HAase treatment, microscopic examination revealed that many cells appear as elongated, oddly shaped cells mixed with cell fragments. The microscopic changes in cell morphology reveal the intuitive process of enzyme treatment leading to cell death.

Furthermore, a fluorescence phase contrast microscope was used to demonstrate the vitality of B16F10 cells, as shown in (Fig. 6B, C and D). Compared to untreated cells, blue fluorescence indicated nuclei in cells under Hoechst 33342 fluorescence microscopy. The images showed that the intracellular nucleic acid substances of many cells appear as swollen and fragmented masses (Fig. 6B). The morphological changes of intracellular nucleic acid substances caused by the addition of FH-HAase revealed that enzyme treatment lead to cell death due to apoptosis.

Effect on mitochondrial ROS and membrane potential changes in B16F10 cells

DCFH-DA fluorescence microscope (Fig. 6C) showed intracellular reactive oxygen species (ROS) in B16F10 cells increased in 24h when treatment with HAase. The accumulation of intracellular ROS increased significantly with the increase of HAase concentration at 20–40 µg/mL.

JC-1 fluorescence microscope (Fig. 6D) showed mitochondrial membrane potential of B16F10 cells was severely impaired after HAase(20, 30, and 40 µg/mL) treatment. The collapse of the mitochondrial membrane potential of the cells increased significantly with the increase of HAase concentration at 20–40 µg/mL.

Previous studies have shown that mitochondrial dysfunction led to structural changes in mitochondria of B16F10 cells, impairment of membrane potential, and instability of electron transport reactions, which resulted in the accumulation of ROS and the activation of apoptotic pathways[30]. Excessive ROS overwhelmed the mitochondrial antioxidant defence mechanisms, leading to oxidative stress which induced disruption of mitochondrial membrane potential[31].

In this study, the mitochondrial membrane potential of melanoma cells collapsed under the intervention of HAase, and the intracellular content of ROS increased significantly, which may be due to the initiation of intrinsic apoptotic pathway. This was confirmed by the dense staining observed with Hoechst 33342 fluorescent staining. These results provide a general mechanism for the use of enzymes in the treatment of melanoma.

Conclusion

Various HAases have been reported from microbial origin, such as SE-StAB[32], *Streptococcus pneumoniae*, *Clostridium perfringens*, *Staphylococcus aureus*, *Propionibacterium acnes*, and *Streptococcus anisopliae*[33,34]. This is the first report of HAase from *Flavobacterium*

heparinum. The highest production of FH-HAase was supported by pH 8 and temperature 15°C, and 48 h incubation period. The enzyme was purified as a single protein band through three steps of chromatography. Its molecular weight was determined by GC-MS to be 79.6 KDa. Temperature, pH, and thermal stability were found to have an impact on the activity of FH-HAase. It showed stability throughout a broad pH and temperature range, making it useful for several applications. FH-HAase showed a potential anticancer activity on B16F10 cell with IC50 values of 28 µg/ml with low toxicity on normal HaCaT cells. It resulted in cell shrinkage, nuclear condensation, mitochondrial membrane potential collapse and excessive ROS damage to mitochondrial function, resulting in apoptosis in melanoma cancer treated cells. Therefore, FH-HAase has the potential to become a novel anti-cancer agent for the treatment of melanoma.

Declarations

Credit authorship contribution statement

Bo Dou: Data curation, Visualization, Formal analysis, Writing–original draft. Ruiqi Wu: Writing–review & editing. Xiaolai Ma: Writing–review & editing, Supervision, Conceptualization. Xiaoqun Duan: Supervision, Funding acquisition.

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Data availability

Data will be made available on request.

Funding Declaration

The authors did not receive funding from any organization for the submitted work.

Declaration of competing interest

The authors have no competing financial interest.

Consent for publication

All authors read and agreed to the submission of this research work to this journal.

Ethic approval and consent to participate

Not Applicable.

Clinical trial number

Not applicable.

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Figures

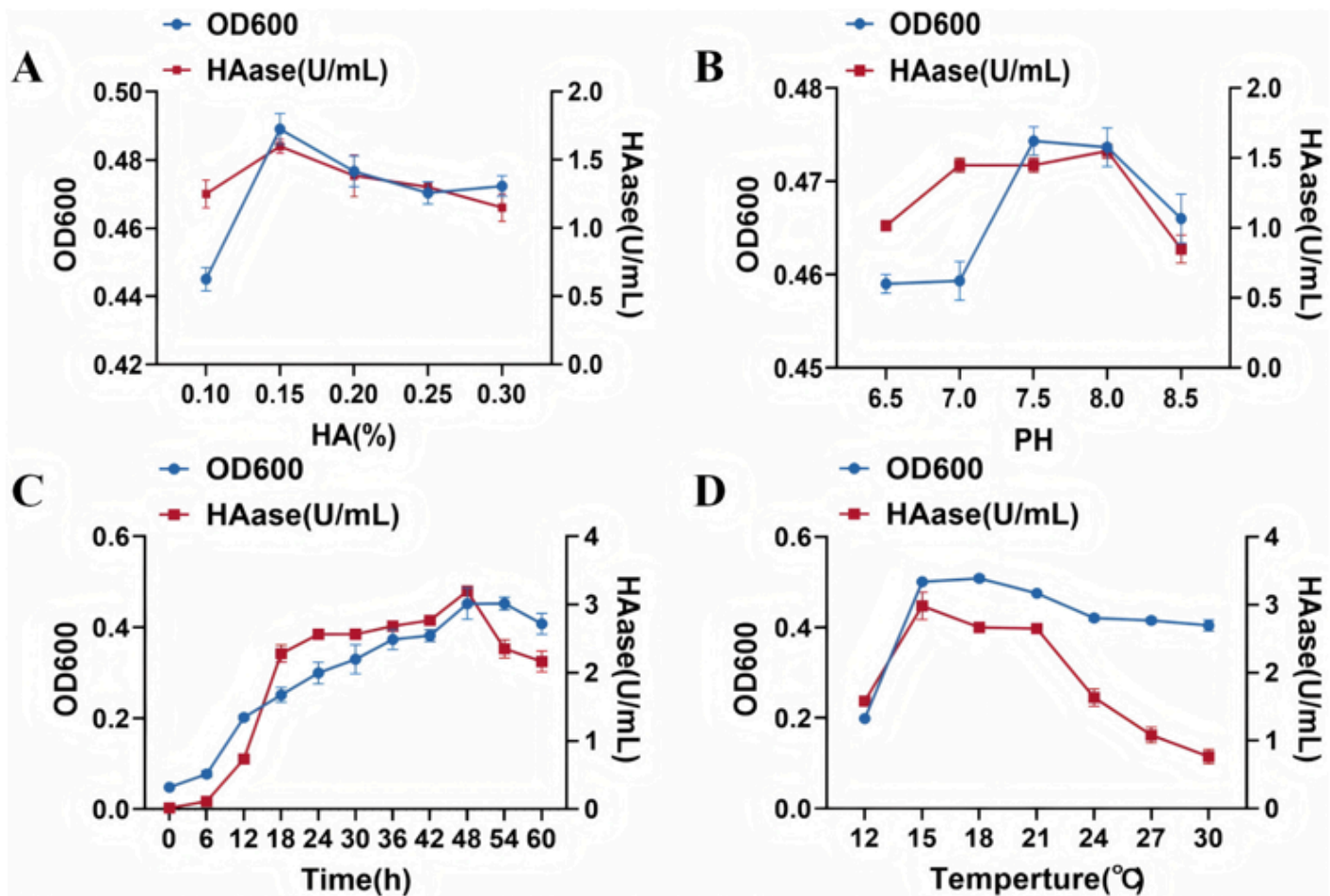


Figure 1

Optimization of HAase enzyme production by *Flavobacterium heparinum*. (A) Temperature, (B) pH, (C) HA concentration and (D) incubation period. Significant differences are indicated by different letters between the bars at $p < 0.05$ using Duncan's multiple range test

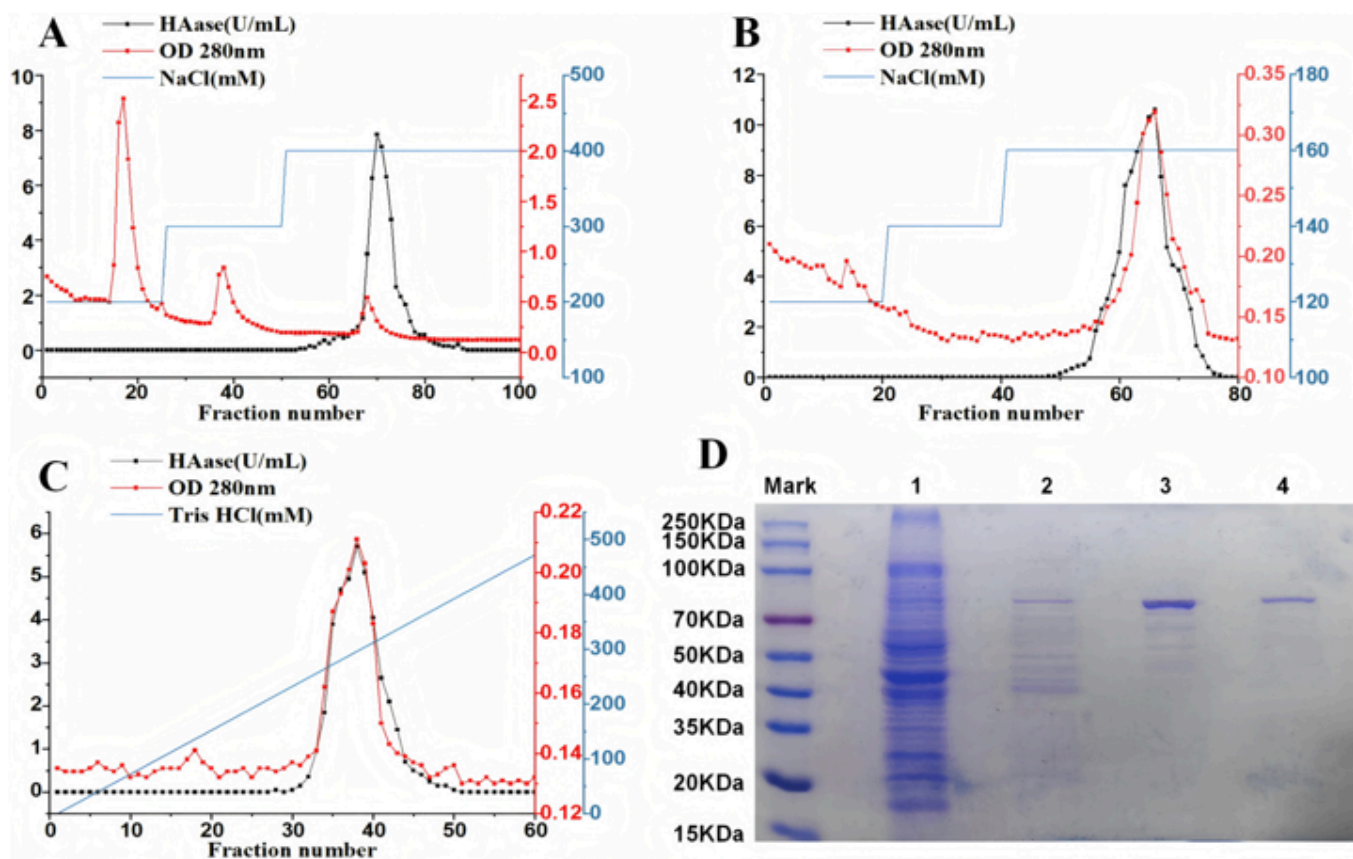


Figure 2

HAase elution profile on a Cellufine Sulfate column(A), UniGel-30CM column (by NaCl gradient) (B), UniGel-30CM column (by Tris-HCl gradient) (C). (D) 12% SDS-PAGE electrophoresis during hyaluronidase purification steps: A protein marker was shown in lane Marker, crude enzymes were shown in lane 1, bands eluted from a Cellufine Sulfate column, UniGel-30CM column(NaCl), and UniGel-30CM column (Tris-HCl) were shown in lane 2,3 and 4, the purified hyase has 80 KDa as a relative molecular weight

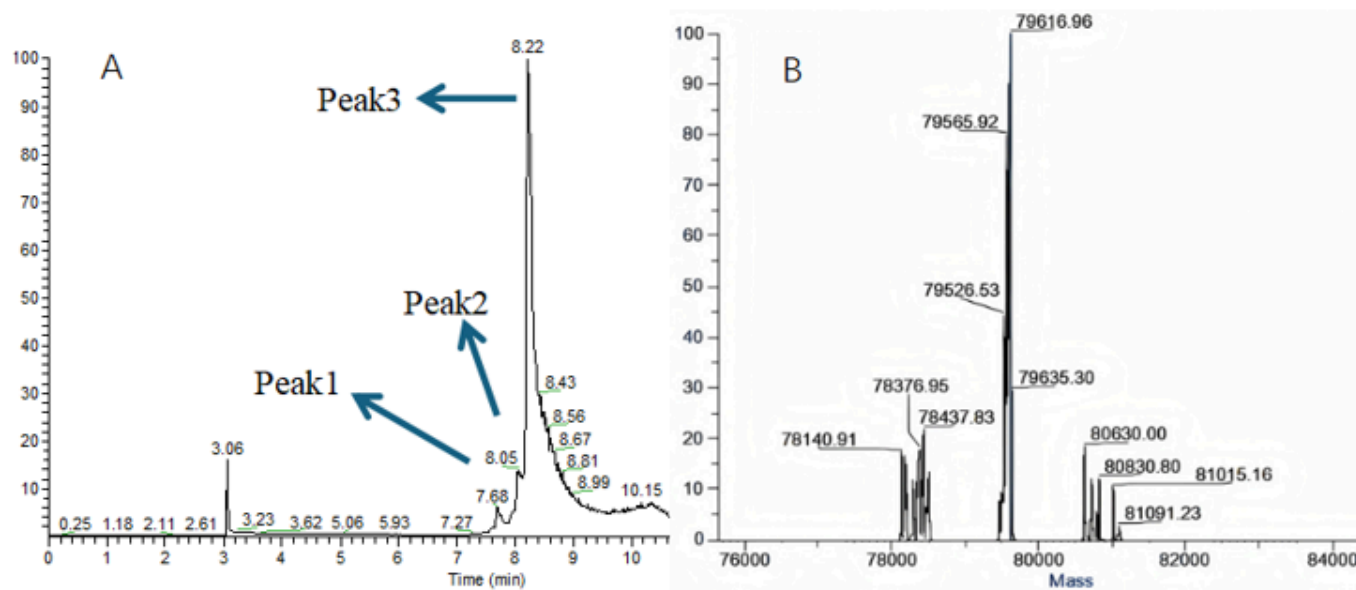


Figure 3

LC-MS of FH-HAase molecular weight: (A) TIC profile of the enzyme sample, (B)Molecular mass of the enzyme based on mass spectrometry analysis

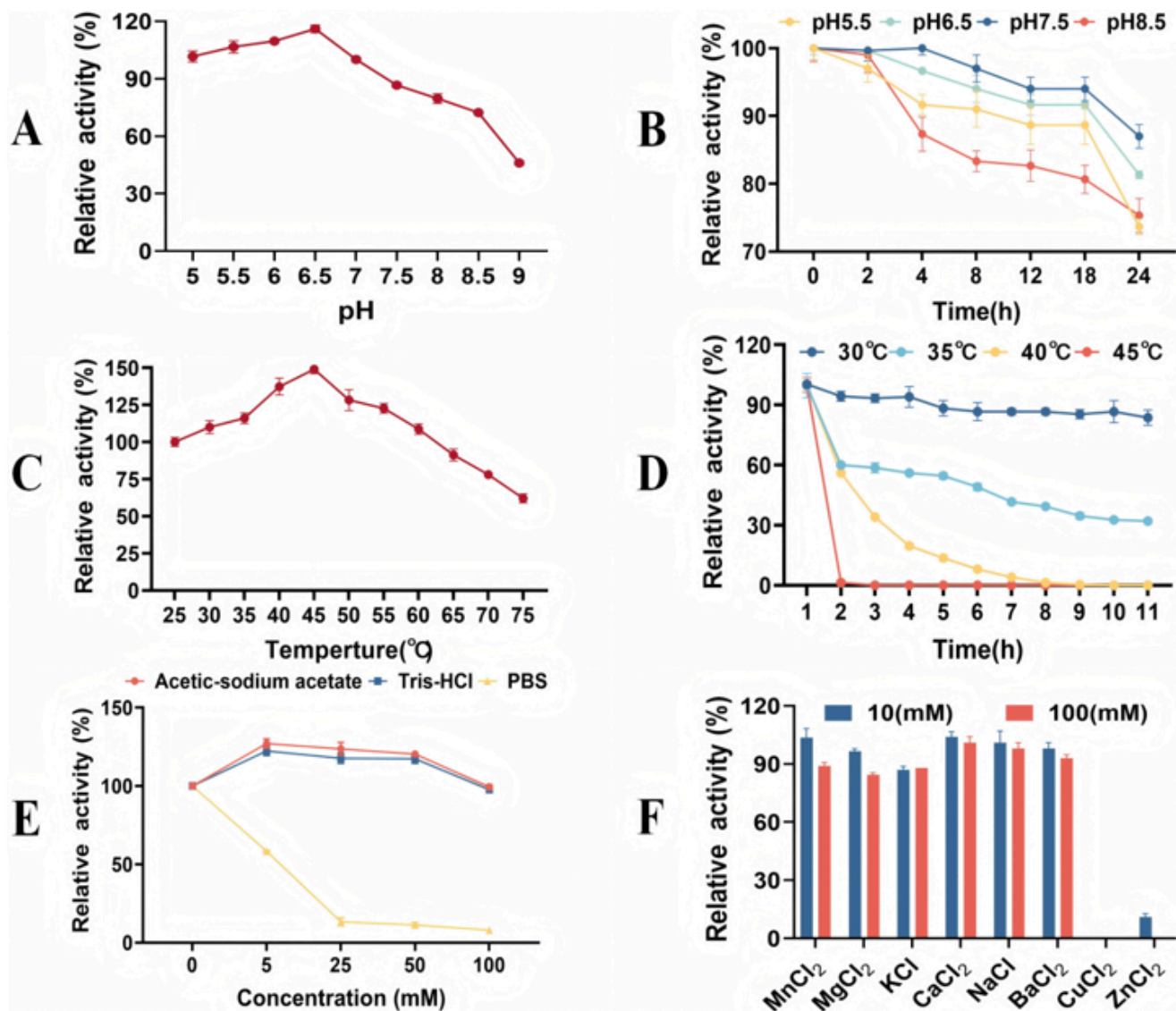


Figure 4

Characterization of FH-HAase activity and stability. Effect of pH (A) and temperature (C) on the enzyme activity, Stability of the enzyme at different pH(B) and temperature (D); Effect of different buffers (E) and metal ions (F) on the enzyme activity

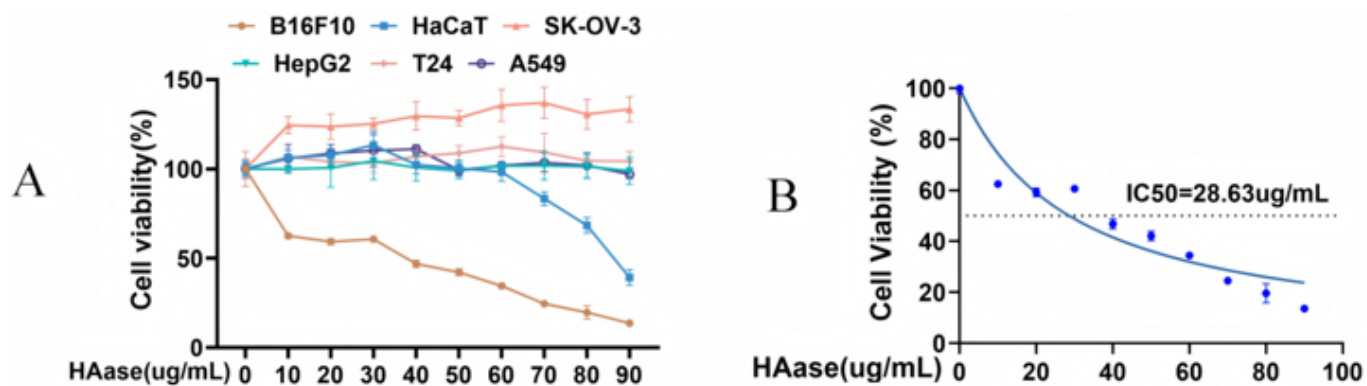


Figure 5

Cytotoxicity of FH-HAase on various cancer cells and normal cell using MTT assay. (A) Cell viability after 24h; (B) IC₅₀ of HAase on normal and cancer cells. The mean values from three experiments expressed as mean $\pm$ SE. Significant differences are indicated by different letters between the bars at $p < 0.05$ using Duncan's multiple range test

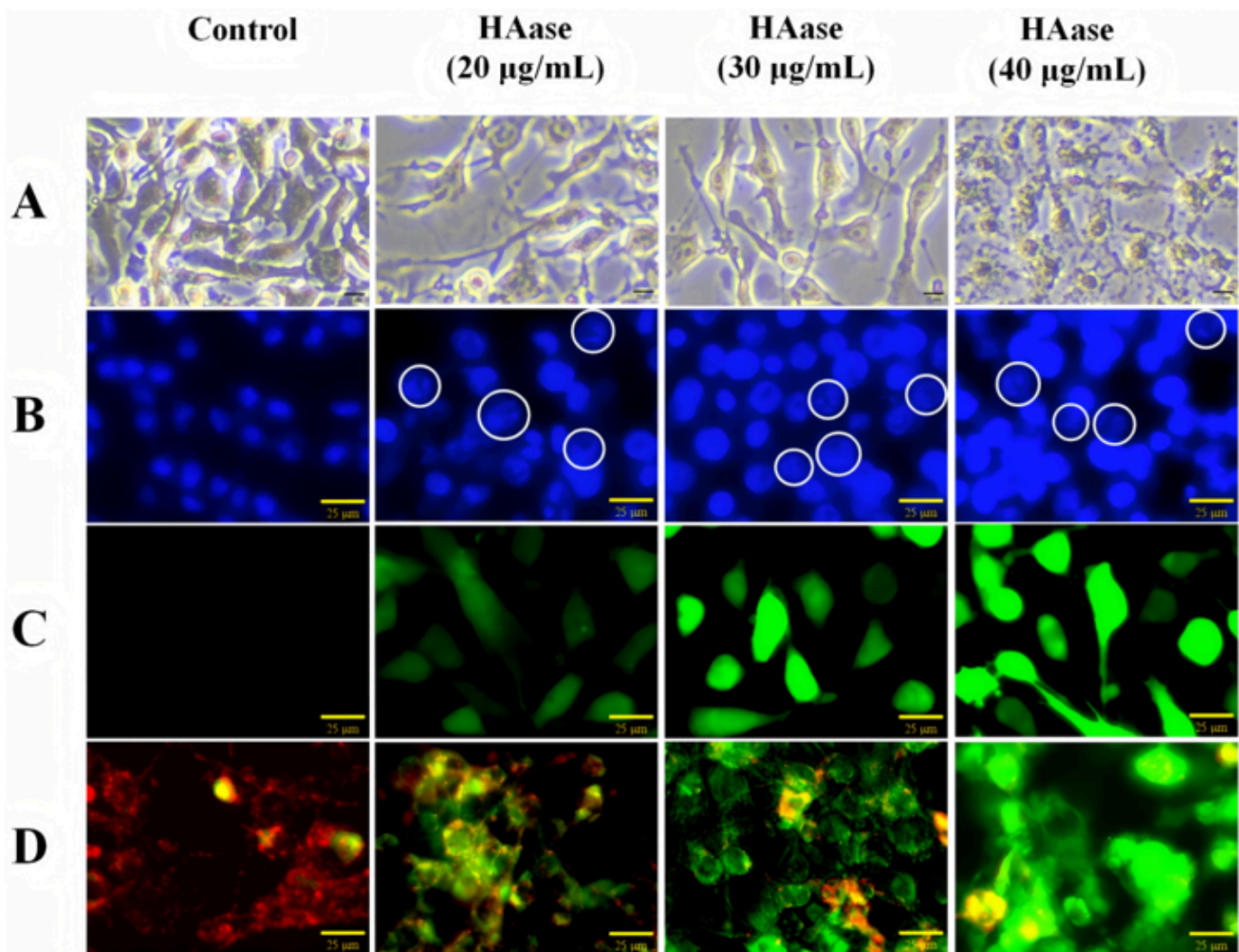


Figure 6

Morphological changes of FH-HAase-treated B16F10 cells for 24h at concentrations of 20,30 and 40µg/mL as compared to untreated cells. (A) Changes in the morphology of the cells under phase contrast microscope. (B) Blue fluorescence indicated nuclei in cells under Hoechst 33342 fluorescence microscopy. (C) Green fluorescence indicated mitochondrial ROS in the cells under DCFH-DA fluorescence microscope. (D) Red to green fluorescence transition indicated membrane potential changes in the cells under JC-1 fluorescence microscope. Magnification was 100 µm and 200 µm scale bar

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