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Article

Study of the fibrinolytic activity of serrapeptase and its *in vitro* thrombolytic effects

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Serrapeptase, a proteolytic enzyme, has been used for the adjuvant treatment of many diseases. However, its fibrinolytic activity is still uncertain. Herein, the fibrinolytic activity of serrapeptase and its *in vitro* thrombolytic effects were investigated. The results showed that the fibrinolytic activity of serrapeptase was 1295 U/mg, and the specific activity was 3867 U/mg of protein when its proteolytic activity toward casein was 2800 U/mg. The optimum temperature and pH for serrapeptase activity were 37–40°C and 9.0, respectively. At 1 mmol/L, Zn^{2+}, Mn^{2+} and Fe^{2+} could activate the fibrinolytic activity of serrapeptase, while K^{+}, Cu^{2+}, sodium dodecyl sulfate (SDS) and ethylene diamine tetraacetic acid (EDTA) inhibited it. *In vitro* tests showed that serrapeptase could completely prevent blood coagulation at 150 U/mL, and the percentage of blood clot lysis reached 96.6% at 37°C after 4 h at 300 U/mL. These results indicate that serrapeptase has excellent fibrinolytic activity, and can be used as a health food or candidate drug for the prevention or treatment of thrombotic diseases.

Keywords: Serrapeptase. Protease. Fibrinolytic activity. *In vitro* thrombolysis.

INTRODUCTION

Thrombotic diseases pose a serious threat to human health, and are characterized by fibrin clots that block blood flow, resulting in hypoxia, ischemia and necrosis of affected tissues or organs. Common thrombotic diseases include ischemic heart disease, high blood pressure, and acute myocardial infarction (Martin, Ma, Key, 2020). Thrombotic diseases not only have a high mortality and long-term disability rate, but their incidence is much higher than that of cancer (Zhou *et al*., 2019). With changes in dietary habits and increased life span, the incidence of thrombotic diseases has increased rapidly in recent years. Cardiovascular diseases (CVDs) have been identified as the leading cause of death worldwide. The majority of CVDs, including acute coronary syndrome and ischemic stroke, are thrombotic diseases (Goto, Tomita, 2013). According to World Health Organization statistics, approximately 17.9 million people died from CVDs in 2016, which represented 31% of all global deaths (World Health Organization, 2017).

The fibrinolytic enzymes are thrombolytic agents that can dissolve fibrin clots, and are expected to be the best drugs to treat thrombolytic diseases. The first generation of fibrinolytic enzymes for clinical therapy began with the discovery of streptokinase in the 1930s (Tillett, Garner, 1933). The plasminogen activators (e.g. tissue-type plasminogen activator and urokinase) and plasmin-like proteins (e.g. nattokinase and lumbrokinase) are examples of fibrinolytic enzymes (Chung *et al*., 2010). Many microbial proteases have expected advantages in treating thrombotic disease because they are suitable for oral administration and have high fibrinolytic activity. Therefore, it is of great significance to develop new fibrinolytic enzymes from microorganisms for the prevention of thrombotic diseases.

Serrapeptase (EC 3.4.24.40), also known as serrapeptidase, serratiopeptidase, serralysin and serratia-protease, is an extracellular alkaline metalloprotease first isolated from *Serratia marcescens* E15 (Miyata *et al*., 1970). It was called a "miracle enzyme" because of its powerful proteolytic activity (Rawat, Daharwal, Singh, 2008). Serrapeptase has excellent anti-inflammatory and

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anti-swelling effects, promotes the excretion of sputum and pus, and has analgesic effects. It has been used to treat postoperative inflammation, paranasal sinusitis, milk retention mastitis and periodontitis, and also bronchitis, tuberculosis and bronchial asthma (Bhagat, Agarwal, Roy, 2013; Ethiraj, Gopinath, 2017; Sivaramakrishnan, Sridharan, 2018).

In recent years, recognition of the efficacy of serrapeptase has led to growing interest in its clinical use. The internet market has also fueled its popularity in the worldwide dietary supplement industry, where it is used for its anti-inflammatory effects, respiratory support, and as an adjunct to antibiotic therapy (Devi, Naine, Vaithilingam, 2019). However, even though serrapeptase has been used for the treatment of atherosclerosis, the fibrinolytic activity of serrapeptase has not been reported in detail, and it has not been widely used in the treatment and prevention of thrombotic diseases.

In our previous study (Mei *et al*., 2020), a *S. marcescens* strain isolated from the intestine of the Chinese silkworm (*Bombyx mori*) produced serrapeptase with a molecular weight of 50 kDa. The proteolytic activity of this serrapeptase was 1505 U/mL when fermented in a 10-L fermenter. The activity of the dry enzyme separated through ammonium sulfate precipitation was approximately 2800 U/mg and the specific activity was approximately 3739 U/mg of protein. However, the fibrinolytic activity of this serrapeptase is not clear. Therefore, its fibrinolytic activity, anticoagulant and thrombolytic activities *in vitro* were investigated in the current study.

MATERIAL AND METHODS

Materials and chemicals

Serrapeptase was prepared through fermentation of *S. marcescens* CCTCC No: M2015780 (stored at Guangdong Microbial Culture Center, China). The enzyme powder was obtained through ammonium sulfate precipitation, dialysis desalination and freeze-drying. The proteolytic activity toward casein was 2800 U/mg and the specific activity was 3739 U/mg of protein (SDS-PAGE of the serrapeptase is shown in Figure 1). Thrombin and bovine fibrinogen were purchased from Sigma-Aldrich LLC. Urokinase (fibrinolytic activity ≥50000 IU/mg, specific activity ≥120000 IU/mg) was purchased from Aladdin Reagent (Shanghai) Co., Ltd. Fresh blood was collected from a healthy adult rabbit for the experiments. All other chemicals used in the study were of analytical or biological grade and commercially available.

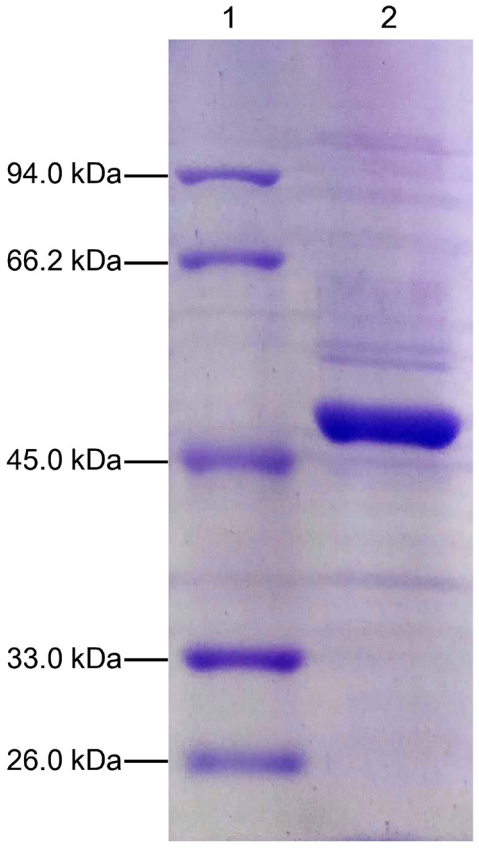

FIGURE 1 - SDS-PAGE of the serrapeptase purified by ammonium sulfate precipitation Lane 1: standard molecular mass markers; Lane 2: serrapeptase.

Determination of fibrinolytic activity

Fibrinolytic activity was determined through the fibrin plate method with some modifications (Astrup, Müllertz, 1952). A total of 10 mL of 0.25% (w/v) bovine fibrinogen dissolved in Tris–HCl buffer (50 mM, pH 8.0) was mixed into 10 mL of 1.5% (w/v) agarose solution preheated to 55°C, and then 40 U thrombin dissolved in the same Tris–HCl buffer was added and mixed well. A total of 5 mL of the mixture was added to a Petri dish

with a diameter of 60 mm, after it was coagulated at room temperature, and stored at 4°C until use.

Six holes with a diameter of 2 mm were punched on the fibrin plate, and then 5 μL of the serrapeptase solution (in 50 mM Tris-HCl buffer, pH 8.0) to be tested was dropped into each hole. The plate was incubated at 37°C for 18 h. The fibrinolytic activity of the serrapeptase was estimated by measuring the dimension of the clear zone on the fibrin plate and comparing it with a standard curve made by varying the activity units of urokinase.

Effects of temperature on the fibrinolytic activity and stability of serrapeptase

To determine the optimal temperature for fibrinolytic activity of serrapeptase, 0.5 μL of serrapeptase solution (600 U/mL) was added to a fibrin plate, and maintained at different temperatures (25–60°C) for 18 h, and the residual fibrinolytic activities were measured. To determine the thermal stability of serrapeptase, the residual fibrinolytic activities of serrapeptase solution (600 U/mL) were measured on the fibrin plate after storage at −20°C, 4°C or 37°C for 1–7 d.

Effects of pH on the fibrinolytic activity and stability of serrapeptase

To determine the optimal pH for fibrinolytic activity of serrapeptase, fibrin plates were prepared using buffers at pH values 4.0–11.0. The residual fibrinolytic activities of serrapeptase were measured on these fibrin plates. To determine the pH stability of serrapeptase, it was dissolved in buffers at different pHs, stored at 4°C for 6 h, and then the residual fibrinolytic activities were measured.

Effects of metal ions and chemical reagents on the activity of serrapeptase

The effects of metal ions and chemical reagents on serrapeptase activity were determined by adding metal ions or chemical reagents to a serrapeptase solution (in 20 mM Tris-HCl buffer, pH 8.0, 600 U/mL) to final concentrations of 1 or 5 mM. The residual fibrinolytic activity was measured on a fibrin plate.

Anticoagulant activity test of serrapeptase *in vitro*

A total of 1 mL of serrapeptase solution with fibrinolytic activity of 0, 60, 120, 180, 240, or 300 U/mL was loaded into a sterile test tube, and 1 mL of fresh whole blood, which was collected from healthy rabbits, was added into the test tube. The mixture was gently shaken to mix it and incubated at 37°C. When no liquid flowed out if the test tube was completely inverted, coagulation was considered to have occurred and the coagulation time was recorded.

Thrombolytic activity test of serrapeptase *in vitro*

Fresh rabbit blood was loaded into a sterile Petri dish. After coagulation, the upper serum was discarded and the clot was washed twice using physiological saline. The clot was cut into small pieces (approximately 0.5 g per piece). The small clots were weighed (W1) after the liquid on their surface was absorbed using absorbent paper. Then they were placed into sterile test tubes, and 1 mL of serrapeptase solution with fibrinolytic activity of 0, 60, 120, 180, 240, or 300 U/mL was added. The test tubes were incubated at 37°C in a water bath. After 12 h, the clots in the test tubes were carefully removed, and were weighed again (W2). The percentage clot lysis was calculated in accordance with the following formula:

Statistical analysis

All experiments were performed in triplicate, and results are reported as the mean ± standard deviation.

RESULTS AND DISCUSSION

Fibrinolytic activity of serrapeptase determined on a fibrin plate.

The fibrinolytic activity of serrapeptase at a concentration of 0.5 mg/mL was measured on a fibrin plate, and the clear zone formed at 37°C after 18 h is shown in Figure 2.

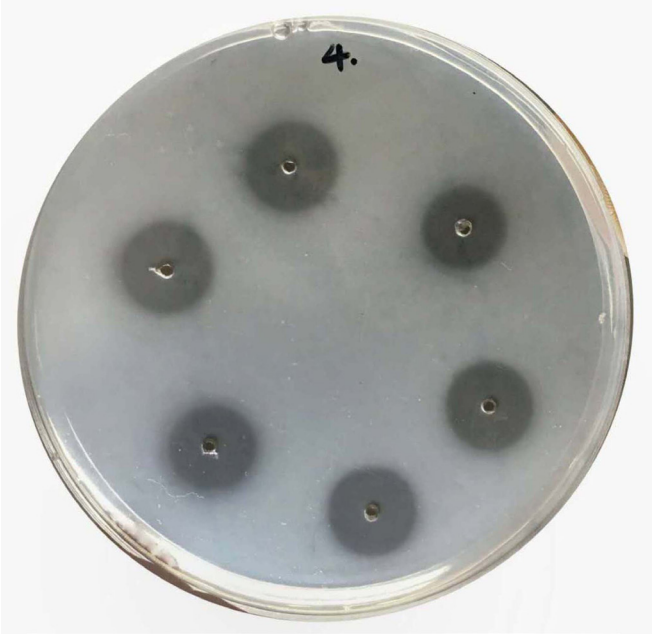

FIGURE 2 - Fibrinolytic activity of serrapeptase shown on a fibrin plate.

Based on a standard curve of urokinase fibrinolytic activity, the fibrinolytic activity of the serrapeptase was 1295 U/mg. The protein content of the enzyme powder was 33.5%, and so its specific activity was 3867 U/mg of protein. Among the thrombolytic enzymes currently used, the specific activity of urokinase and streptokinase for injection is 120000 IU/mg and 90000 IU/mg, respectively (Chinese Pharmacopoeia Commission, 2015), and the specific activity of nattokinase, which is used as a health food, has been reported as less than 2000 U/mg when it is purified through ammonium sulfate precipitation (Chang *et al*., 2000; Peng *et al*., 2003; Wang *et al*., 2009; Wang, Wu, Liang, 2011). It can be seen that the fibrinolytic activity of serrapeptase is much lower than that of urokinase and streptokinase, which is because of their high purity. If serrapeptase is further purified through gel chromatography or ultrafiltration, its specific fibrinolytic activity will be higher than that determined at present. As with nattokinase, if serrapeptase is only used as a health food, this fibrinolytic activity is sufficient for making capsules. Fewer purification steps would reduce the production cost of serrapeptase.

Effects of temperature on the fibrinolytic activity and stability of serrapeptase

The effects of temperature on the fibrinolytic activity of serrapeptase are shown in Figure 3A. With an increase in temperature, the fibrinolytic activity of serrapeptase gradually increased. When the temperature was 37–40°C, the fibrinolytic activity reached the maximum. However, when the temperature continued to increase, the fibrinolytic activity decreased rapidly. Therefore, the optimal temperature for serrapeptase was 37–40°C, which is approximately the temperature of the human body.

The effect of temperature on the stability of serrapeptase is shown in Figure 3B. The fibrinolytic activity of serrapeptase was more than 90% after being stored at −20°C for 7 d, while only 52.0% of the activity remained after 7 d at 4°C. The inactivation rate was faster at 37°C, and almost all of the activity was lost after 5 d. This indicates that serrapeptase is sensitive to high temperature in water solution and should be preserved in dried form.

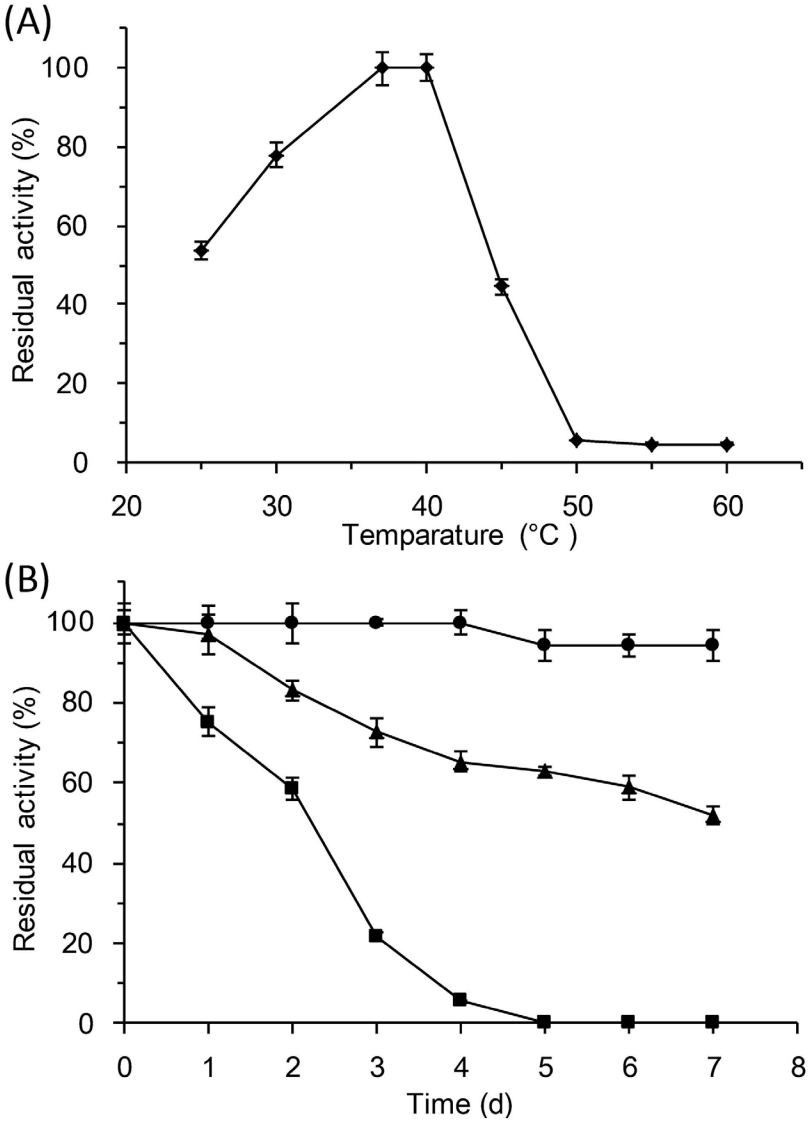

FIGURE 3 - Effects of temperature on the fibrinolytic activity and stability of serrapeptase. (A) Fibrinolytic activity of serrapeptase; (B) Stability of serrapeptase.

Effects of pH on the fibrinolytic activity and stability of serrapeptase

The effect of pH on the fibrinolytic activity of serrapeptase is shown in Figure 4. With an increase in pH, the residual activity of serrapeptase gradually increased. When the pH was 9.0, the enzyme activity was the highest, and when the pH was increased more, the activity decreased rapidly. The results suggest that the optimal pH for the fibrinolytic activity of serrapeptase is 9.0, and under acidic conditions, it was inhibited. However, when the pH was higher than 9.0, the activity diminished rapidly.

The stability of serrapeptase, as shown in Figure 4, demonstrated the same phenomenon as its activity under different pH conditions. In a pH range between 4.0 and 9.0, the fibrinolytic activity of serrapeptase was stable, while when pH was higher than 9.0, it decreased quickly.

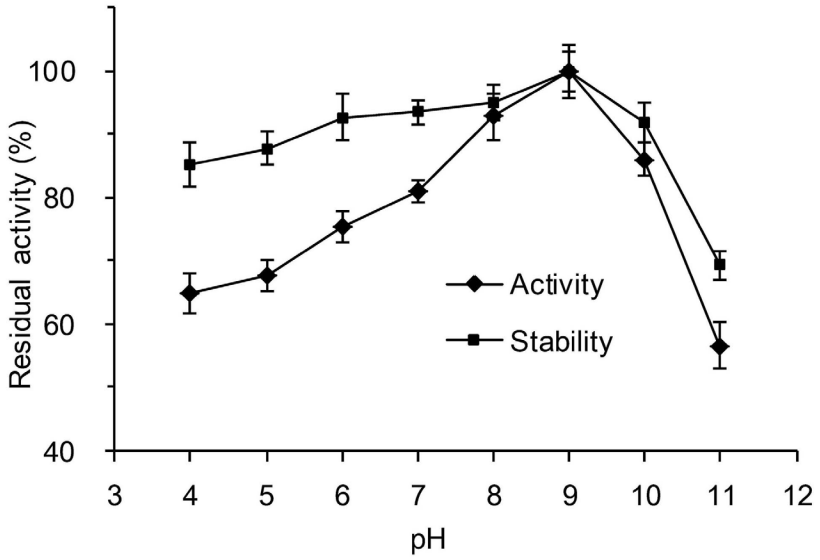

FIGURE 4 - Effect of pH on the fibrinolytic activity and stability of serrapeptase.

Effects of metal ions and chemical reagents on the activity of serrapeptase

As shown in Table I, Mg^{2+}, Mn^{2+} and Fe^{2+} at a concentration of 1 mmol/L had activation effects on the fibrinolytic activity of serrapeptase, and Zn^{2+} produced an activation effect at a concentration of 5 mmol/L. Al^{3+}, Sr^{3+}, Na^{+}, and Ca^{2+} had almost no effect on enzyme activity, while K^{+} and Cu^{2+} could inhibit enzyme activity, and Cu^{2+} had a significant inhibitory effect on serrapeptase, and the enzyme activity was only 25.8% at a concentration of 5 mmol/L. Moreover, sodium dodecyl sulfate (SDS) and ethylene diamine tetraacetic acid (EDTA) significantly inhibited the fibrinolytic activity of serrapeptase at a concentration of 1 mmol/L. Therefore, during the storage and use of serrapeptase, contact with K^{+}, Cu^{2+}, SDS and EDTA should be avoided.

TABLE I - Effects of metal ions and chemical reagents on the fibrinolytic activity of serrepetidase

Chemicals	Residual activity of serrepetidase	
	Concentration at 1 mmol/L	Concentration at 5 mmol/L
None	100.0±1.72	100.0±1.72
Mg^{2+}	108.5±5.41	94.4±6.64
Zn^{2+}	103.6±3.34	111.8±6.25
Ca^{2+}	97.2±4.97	97.2±7.88
Fe^{2+}	111.0±6.35	94.4±5.70
K^{+}	86.4±1.79	86.4±3.55
Mn^{2+}	116.8±5.82	106.4±2.17
Sr^{3+}	93.4±4.73	96.6±4.82
Na^{+}	94.0±4.74	81.9±4.13
Cu^{2+}	69.7±3.55	25.8±0.86
Al^{3+}	93.4±2.82	99.8±4.07
SDS	75.3±3.88	
DTT	100.0±4.02	
EDTA	79.64±1.64	

SDS: Sodium dodecyl sulfate, DTT: Dithiothreitol, EDTA: Ethylene diamine tetraacetic acid.

Anticoagulant activity of serrapeptase *in vitro*

The anticoagulant activity of serrapeptase at different enzyme activity levels in fresh blood is shown in Figure 5.

The fresh blood coagulated completely in the control group after 4 min, but coagulation time was significantly prolonged by adding serrapeptase. When the activity was 30 U/mL, the blood coagulated after 9 min, and when the activity was 60, 90 and 120 U/mL, it did not completely coagulate after 60 min, and the amount of clotting decreased with the increase in enzyme activity. When the activity was 150 U/mL, the blood did not coagulate at all. Therefore, it can be concluded that serrapeptase has a good anticoagulant effect *in vitro*.

FIGURE 5 - Anticoagulant effects of serrapeptase on fresh blood after 10 min *in vitro*.

Thrombolytic activity of serrapeptase *in vitro*

A piece of clot (approximately 0.5 g) was added to 1 mL of serrapeptase solution with different fibrinolytic activities. The thrombolytic effect *in vitro* is shown in Figure 6. It can be seen that the blood clot demonstrated minimal dissolution in the control group. However, the addition of serrapeptase at 60, 120, 180, 240 or 300 U/mL resulted in significant dissolution of the blood clots. The higher the activity, the higher the percentage of clot lysis. The percentage of clot lysis is shown in Figure 7. When the fibrinolytic activity of serrapeptase reached 300 U/mL and it was maintained at 37°C for 4 h, a blood clot of 0.522 g could be dissolved almost completely, which showed that serrapeptase had strong thrombolytic activity *in vitro*.

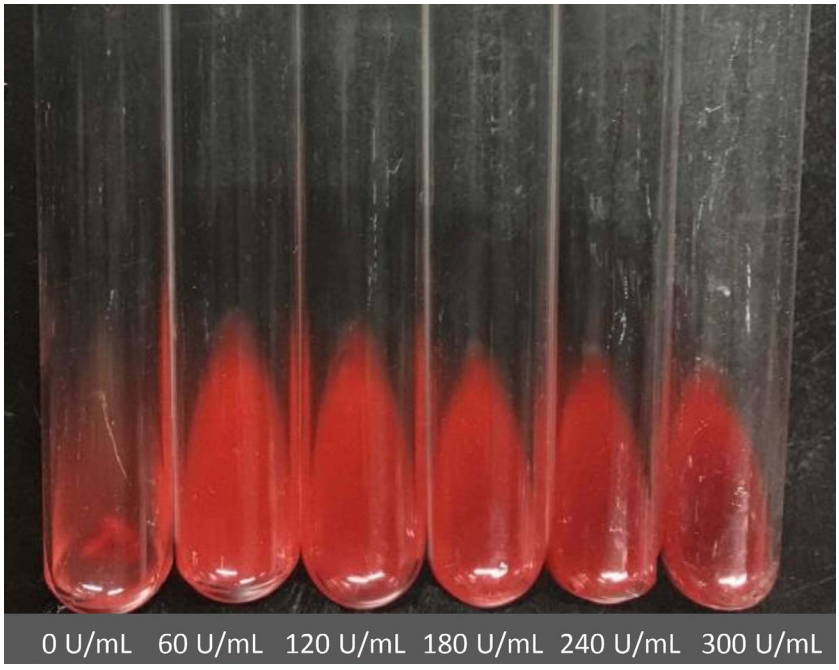

FIGURE 6 - Thrombolytic effects of serrapeptase on blood clots *in vitro.*

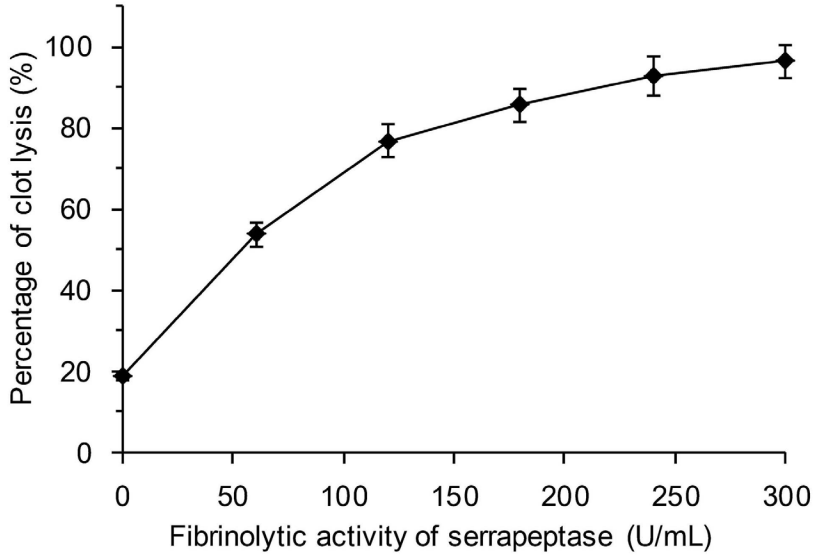

FIGURE 7 - Percentage of blood clot lysis produced by adding serrapeptase.

CONCLUSION

Although serrapeptase has been developed and used clinically for many years, it is currently used as a prescription drug in most countries, and the proportion of its use as a health food is not high. For example, there is no domestic serrapeptase health food available in the Chinese market. Several serrapeptase health foods produced in the United States are available on the online market, such as "Serrétia" produced by Arthur Andrew Medical Inc., "Serrapeptase" produced by Doctor's Best, Inc. and "Serrateric" produced by Deerland Enzymes, Inc. They are mainly used for the treatment of sinusitis and bronchitis. The results of the current study showed that serrapeptase had good fibrinolytic activity, and also showed excellent anticoagulant and thrombolytic activity

in vitro, indicating that it could be used as a health food or candidate drug for the prevention and adjuvant treatment of thrombotic diseases.

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CONFLICT OF INTEREST STATEMENT

None of the authors of this study has any financial interest or conflict with industries or parties.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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