Fibrinolytic Enzyme Production by *Bacillus subtilis* KH-4 Isolated from *Deonjang*

JM Kim, BJ Suh, SW Ahn*, MS Kim** and SH Oh***

*Department of Food and Nutrition, College of Health Sciences, Korea University, Seoul 136-703, Korea*
*Neurotide LTD, Chunchon 200-161, Korea*
**Biotech. Division, Pacific Corporation, Gyonggi 425-120, Korea*
***Department of Food Engineering, Ansan College of Technology, Gyonggi 425-792, Korea*

Abstract

A strong fibrin-specific fibrinolytic enzyme was produced from *Bacillus subtilis* KH-4 isolated from *Deonjang*, a Korean fermented soybean paste similar to Japanese miso. The addition of glucose as a carbon source resulted in the highest levels of caseinolytic and fibrinolytic activities. Likewise, the addition of yeast extract as the nitrogen source resulted in the highest caseinolytic and fibrinolytic activities (3473.2 unit and 47.4 munit, respectively). It was observed that out of all metal ion sources only calcium (chloride) enhanced caseinolytic and fibrinolytic activities, with increases of 4949.3 unit and 58.2 unit/mg, respectively. The optimal temperature for the production of the enzyme was found to be 40°C in the optimal medium (glucose 20 g, yeast extract 5 g, CaCl₂ 1 g, and NaCl 2 g). The maximum fibrinolytic activity was observed at the late stationary phase. *B. subtilis* KH-4 produced a fibrinolytic enzyme at 40°C, after 30 h growth, which increased up to 54 h and then remained constant. These results suggest that *Deonjang* has potential as a source of physiologically active anti-thrombotic enzymes.

Key words: fibrinolytic enzyme, caseinolytic activity, *Bacillus subtilis*

INTRODUCTION

Fibrin is a major structural component of blood clots. Accordingly, fibrinolytic enzymes have therapeutic potential for the treatment of thrombosis in man. Lately, there has been great interest in the search for new thrombolytic agents from various origins with particular reference to microbial sources.

Typical thrombolytic agents used therapeutically include urokinase (1) and a tissue-type plasminogen activator (tPA) (2). They are plasmin activators that convert plasminogen to plasmin which degrades fibrin. These activators are of human origin and generally safe, but are expensive. There have also been reports of fibrinolytic enzymes derived from microorganisms (3-7). Among them, streptokinase produced from *Streptococcus haemolyticus* (4,6) and staphylokinase produced from *Staphylococcus aureus* (5,7) have been studied most extensively. They are also plasminogen activators and are generally safe for intravenous administration.

If a fibrinolytic enzyme is produced by food-grade microorganisms in a fermented food, regular consumption of that food might help prevent thrombosis and other related diseases. Oral administration of the fibrinolytic enzyme nattokinase (8), revealed to be the same as subtilisin NAT (9) and which is produced from *Bacillus* NAT in the traditional Japanese fermented food (Natto), has been reported to enhance fibrinolytic activity in plasma and the production of tPA (10). A fibrinolytic enzyme produced from *Bacillus subtilis* has also been reported (11), but was a less effective plasminogen activator activity than nattokinase. Other novel fibrinolytic enzymes have been isolated and characterized from *Bacillus* sp. CK 11-4 from *Chungkookjang* (a traditional Korean fermented soybean paste) and from *Bacillus* sp. KA 38 from *Jeotgal* (a traditional Korean fermented fish sauce) (12,13).

*Bacillus* sp. KH-4, which produces a strongly fibrinolytic enzyme, was isolated in our laboratory from *Deonjang*, a traditional Korean fermented-soybean, for study as a therapeutic thrombolytic agent. This study investigated the production of a new fibrinolytic enzyme by *B. subtilis* KH-4 and examined the effect of growth conditions, including carbon and nitrogen sources, for the rate of production of the fibrinolytic enzyme.
MATERIALS AND METHODS

Microorganism isolation and cultivation

*B. subtilis* KH-4 cultures with fibrinolytic enzyme activity were isolated from *Doenjang* samples collected from various regions of Korea. The isolated strains were cultivated in the basal medium containing 5% soybean powder, 0.5% yeast extract, 2% NaCl, 0.1% KH₂PO₄, and 0.01% MgSO₄·7H₂O. The pH was adjusted to 7.0 with 1 M HCl or 1 M NaOH. For seed culture, one colony per plate was inoculated into 5 mL of basal media and incubated at 37 °C in a shaking water bath for 24 h. The seed culture broth (1 mL) was transferred to 50 mL of basal medium in a 500 mL shake flask and incubated at 37°C for 24 h.

Enzyme assay

Fibrinolytic activity was determined by the fibrin plate assay (14) after some modifications. The fibrinogen (plasminogen-free) solution (2.5 mL of 1.2% (w/v) was prepared with human fibrinogen (Sigma; St. Louis, USA) in 0.1 M sodium phosphate buffer, pH 7.4) was mixed with 0.1 mL of thrombin solution (100 NIH U/mL; Sigma) and 7.4 mL of 1% (w/v) agarose solution in a petri dish (100 by 15 mm). After the dish was allowed to stand for 30 min at room temperature to form fibrin clots, five holes were made on the fibrin plate by suction using a capillary glass tube (50 mm-diameter). Twenty microliters of sample solution was dropped into each hole and incubated at 37°C for 12 h. After measuring the dimension of the clear zone, the number of units was determined according to standard curve by using plasmin.

Caseinolytic activity was assayed by the following procedure. A mixture (1 mL) containing 0.7 mL of 0.1 M sodium phosphate buffer (pH 7.5), 0.1 mL of 2% casein, and 0.1 mL of enzyme solution was incubated for 10 min at 37°C, mixed with 0.1 mL of 1.5 M trichloroacetic acid, allowed to stand for 30 min, and then centrifuged at room temperature. The A₂₇₅ for the supernatant was measured and converted to tyrosine equivalents. One unit of caseinolytic activity was defined as the amount of enzyme releasing 1 mol of tyrosine equivalent per min.

Effect of carbon, nitrogen, and metal ion sources

To determine the optimal culture conditions, variations in carbon sources, nitrogen sources, pH and temperature were studied in basal medium. In order to select the optimal carbon source, the fibrinolytic activity was determined after 2 days of incubation in the basal medium containing 2% of various carbon sources. The effect of nitrogen source on the fibrinolytic activity was investigated by incubating in the basal medium containing 2% glucose and 0.5% nitrogen source. The effect of temperature and pH on the fibrinolytic activity was also investigated by incubating in the optimal medium at different temperatures and pHs.

RESULTS AND DISCUSSION

Isolation of *B. subtilis* KH-4

From the Korean fermented soybean paste, *Doenjang*, 15 microorganisms were isolated. One strain, KH-4, showed strong fibrinolytic activity and weak caseinolytic activity in the culture supernatant. This strain was an aerobic, gram-positive and endospore forming rod that grew well in NaCl at concentrations up to 15%. Since the preliminary morphological and biochemical characteristics of this bacterium coincide with *B. subtilis*, we designated it as *B. subtilis* KH-4.

Recently, Sumi et al. (15) have reported that orally administered urokinase, result in some level of fibrinolytic activity in the blood of dogs and humans for more than 6 h. A similar result has been reported for another fibrinolytic enzyme lumbrokinase, which was isolated from earthworms (16). These results suggest the possibility of using orally administered drugs for treating or preventing thrombosis. In this respect, recent studies by Sumi and coworkers (15) and Kim et al. (13) deserve attention. Both groups have isolated proteolytic enzymes with strong fibrinolytic activity from traditional fermented foods, such as *Natto* and *Shiokara* (8) and *Chungkooksang* and *Jeotgal* (12,13).

Effect of carbon, nitrogen, and metal ion sources

To study the effect of carbon source, 20 g/L of each carbon source was added to the basal medium. Monosaccharides were better carbon sources than di- and polysaccharides for the production of caseinolytic activity (Fig. 1). The addition of glucose and fructose resulted in the highest levels of caseinolytic activity, whereas the addition of starch resulted in the lowest activity among the carbon sources. The nitrogen sources were also important for the production of fibrinolytic activity (Fig. 1).

![Fig. 1. Effect of different carbon source on the production of fibrinolytic enzyme by B. subtilis KH-4.](image-url)
sources. Among the carbon sources promoting caseinolytic activity, the addition of glucose and sucrose increased fibrinolytic activity above the basal medium, with glucose addition inducing the highest activities of both caseinolytic and fibrinolytic enzymes.

Although starch appears to stimulate the production of proteolytic enzymes by other bacteria and actinomycetales (16,17), starch did not exhibit any enhancing effect on the production of the enzyme in B. subtilis KH-4, but rather was inhibitory.

The effect of nitrogen source on caseinolytic and fibrinolytic activity was studied by incubating in the medium containing glucose (20 g/L) and various nitrogen sources (5 g/L). As shown in Fig. 2, the addition of yeast extract yielded the highest levels of both caseinolytic activity and fibrinolytic activity (3473.2 units and 47.4 munits, respectively). The addition of either ammonium sulfate or sodium nitrate resulted in very low enzymatic activity. Bascaran et al. (18) also reported that yeast extract was a good nitrogen source for the enhancement of fibrinolytic activity. It was reported by Chitte and Dey (19) that the addition of haemoglobin and fibrin, though costly, increased the enzymatic activity 4–10% above the control. However, it was observed that haemoglobin and fibrin are not proper for commercial production of fibrinolytic enzymes.

The effect of metal ions on caseinolytic and fibrinolytic activity was studied by incubating in the medium containing glucose (20 g/L), nitrogen source (5 g/L) and metal ion source (1 g/L). It was observed that out of all the metal ion sources only calcium chloride enhanced caseinolytic and fibrinolytic activities, with activities of 4949.3 and 58.2 units/mg, respectively (Fig. 3). These results corroborated the previous observation by Chitte and Dey (19). It was reported by Longinova et al. (16) that solutions with trace metals and phosphate enhanced the production of different proteases, and it is possible that they would also increase the production of the fibrinolytic enzyme by B. subtilis KH-4.

The optimal medium composition obtained in this study was as follows (g/L): glucose 20 g, yeast extract 5 g, CaCl₂ 1 g, and NaCl 2 g.

**Effect of temperature and initial pH**

Temperature effects on fibrinolytic activity in B. subtilis KH-4 were evaluated by cultivating in the optimal medium at different temperatures (Fig. 4). The optimal temperature for enzyme production was 40°C. The enzyme activity at 30°C was 85% of that at the optimal temperature, and 48.9% and 26.9% at 20 and 50°C, respectively. Heo et al. (20) and Jang et al. (21) reported that the optimum temperature for fibrinolytic activity was 37°C and the activity was sharply decreased above 50°C.

Although the maximum production of the enzyme by B. subtilis KH-4 was at pH 7–8, with an optimum of pH 8.0, at pH 5.0 and 9.0 more than 70% of the optimum was produced (Fig. 4), which corroborated the previous observation by Heo et al. (20). The fibrinolytic activity of Bacillus sp. from Chungkoojang was highest at a pH 8.0.
range of 6–8 with pH 8.0 maximizing activity; the fibrinolytic activity of Bacillus sp. from Jeot-Gal, was sharply decreased above pH 8.0 (21).

**Cultivation under optimal culture conditions**

Fig. 5 shows various time courses of fibrinolytic activity in a flask culture under optimal culture conditions. The cell growth reached a maximum after 24 h of cultivation. Caseinolytic activity showed a similar time course as fibrinolytic activity. The maximum fibrinolytic activity was observed at the late stationary phase. There were no significant changes in fibrinolytic activity after 54 h of cultivation, suggesting that fibrinolytic activity may remain stable in fermented foods. Bacillus sp. isolated from Jeot-Gal produced a fibrinolytic enzyme at 37°C, after 24 h growth, which increased up to 72 h and then remained constant (21). But Streptomyces megasporus SD5 produced a high level of fibrinolytic activity at the late logarithmic phase (19).

These results suggest that Deonjang has potential as a source of physiologically active fibrinolytic enzymes.

**ACKNOWLEDGEMENTS**

This work was supported by Ansan College of Technology, Korea, during the program year of 2002.

**REFERENCES**


(Received October 14, 2002; Accepted December 10, 2002)