Nutraceuticals & Food
Vol. 7, p.417~420 (2002)

Nutraceuticals and Food

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

JM Kim, HJ 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, $CaCl_2$ 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-thromotic 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 *Staphylococcusaureus* (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 sublitis* 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 *Doenjang*, 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.

†Corresponding author. E-mail: sungoh@ansantc.ac.kr
Phone: +82-31-490-6152, Fax: +82-31-490-6152

MATERIALS AND METHODS

Mircoorganism 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% KH_2PO_4, and 0.01% $MgSO_4$-$7H_2O$. 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 A_{275} 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 desingnated 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 lumbrokianse, 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 *Chungkookjang* 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

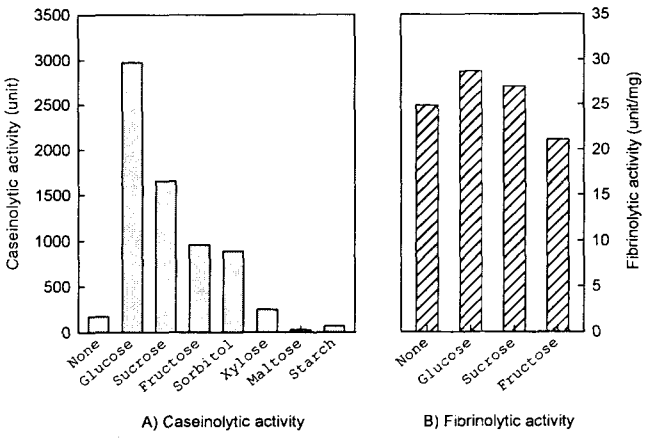

Fig. 1. Effect of different carbon source on the production of fibrinolytic enzyme by *B. subtilis* KH-4.

ources. Among the carbon sources promoting caseinolytic ctivity, the addition of glucose and sucrose increased ibrinolytic activity above the basal medium, with glucose ıddition inducing the highest activities of both caseinolytic ınd fibrinolytic enzymes.

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

The effect of nitrogen source on caseinolytic and fib-inolytic activity was studied by incubating in the medium ontaining glucose (20 g/L) and various nitrogen sources 5 g/L). As shown in Fig. 2, the addition of yeast extract ielded the highest levels of both caseinolytic activity ınd fibrinolytic activity (3473.2 units and 47.4 munits, espectively). The addition of either ammonium sulfate ır sodium nitrate resulted in very low enzymatic activity. Bascaran et al. (18) also reported that yeast extract was ı good nitrogen source for the enhancement of fibrinolytic ıctivity. It was reported by Chitte and Dey (19) that the ıddition of haemoglobin and fibrin, though costly, increased he enzymatic activity 4~10% above the control. However, he use of haemoglobin and fibrin are not proper for com-ınercial production of fibrinolytic enzymes.

The effect of metal ions on caseinolytic and fibrinolytic ıctivity was studied by incubating in the media containing glucose (20 g/L), nitrogen source (5 g/L) and metal ion ource (1 g/L). It was observed that out of all metal ion ources only calcium chloride enhanced caseinolytic and ibrinolytic activities, with activities of 4949.3 and 58.2 ınits/mg, respectively (Fig. 3). These results corroborated he previous observation by Chitte and Dey (19). It was eported by Longinova et al. (16) that solutions with trace netals and phosphate enhanced the production of dif-

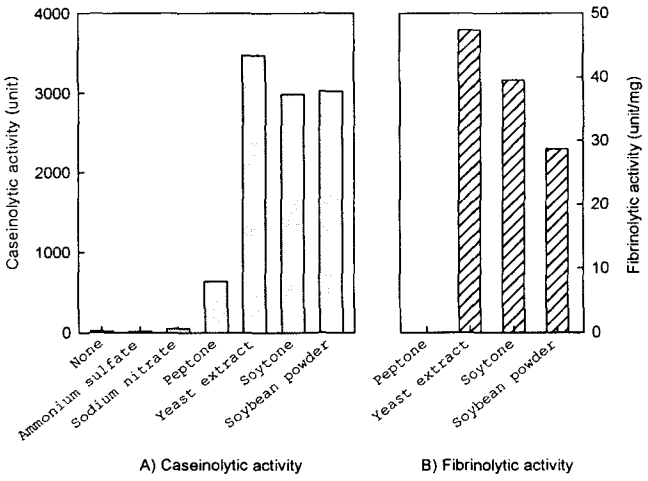

Fig. 2. Effect of different nitrogen source on the production of fibrinolytic enzyme by *B. subtilis* KH-4.

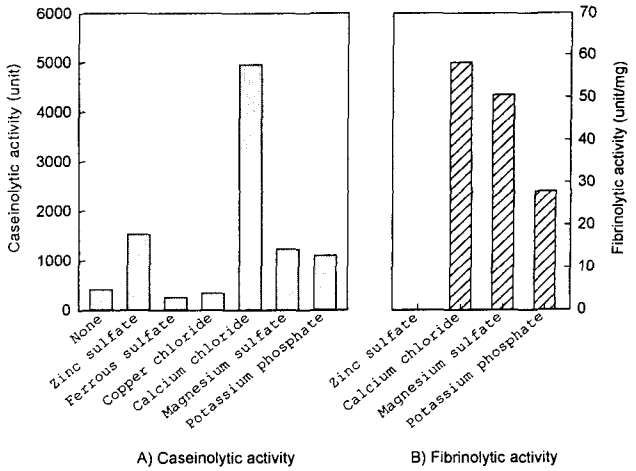

Fig. 3. Effect of different metal ion source on the production of fibrinolytic enzyme by *B. subtilis* KH-4.

ferent 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, $CaCl_2$ 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

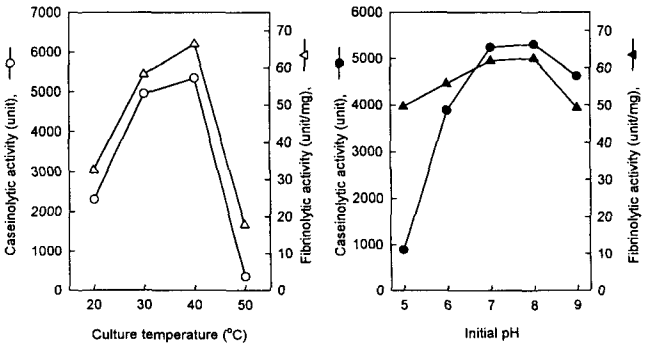

Fig. 4. Effect of culture temperature on the production of fibrinolytic enzyme by *B. subtilis* KH-4.

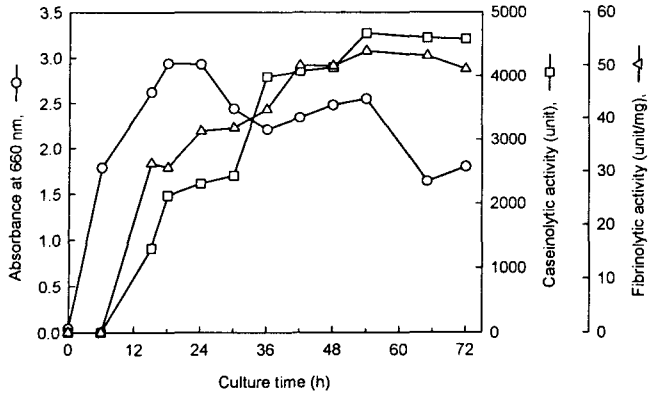

Fig. 5. Fibrinolytic and caseinolytic activities with relation to growth of *B. subtilis* KH-4.

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

1. Wun TC, Schleuning WD, Reich E. 1982. Isolation and characterization of urokinase from human plasmin. *J Biol Chem* 257: 3276-3283.
2. Pennica D, Holmes WE, Kohr WJ, Harkins RN, Vehar GA, Ward CA, Bennett WF, Yelverton E, Seeburg PH, Heyneker HL, Goeddel DV, Collen D. 1983. Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli*. *Nature* 301: 214-221.
3. El-Aassar SA. 1995. Production and properties of fibrinolytic enzyme in solid state culture of cultures of *Fusarium pallidoroseum*. *Biotechnol Lett* 17: 943-948.
4. Reed GL, Lin LF, Parhami-Seren B, Kussie P. 1995. Identification of plasminogen binding region in streptokinase that is necessary for the creation of functional streptokinase-plasminogen activator complex. *Biochemistry* 34: 10266-10271.
5. Lijnen HR, van Hoef B, de Cock F, Okada K, Ueshima S, Matsuo O, Collen D. 1991. On the mechanism of fibrin-specific plasminogen activation by staphylokinase. *J Biol Chem* 266: 11826-11832.
6. Medved LV, Solovjov DA, Ingham KC. 1996. Domain structure, stability and interactions in streptokinase. *Eur J Biochem* 239: 333-339.
7. Arah K, Mimuro J, Madoiwa S, Matsuda M, Sako T, Sakata Y. 1995. Effect of staphylokinase concentration of plasminogen activation. *Biochim Biophy Acta* 1245: 69-75.
8. Sumi H, Hamada H, Tsushima H, Mihara H, Muraki H. 1987. A nevel fibrinolytic enzyme in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet. *Experientia* 43: 1110-1111.
9. Nakamura T, Yamagata Y, Ichishima E. 1992. Nucleotide sequence of the *subtilis* NAT gene, *arpN*, of *Bacillus subtilis* (*natto*). *Biosci Biotechnol Biochem* 56: 1869-1871.
10. Sumi H, Hamada H, Nakanishi K, Hiratani H. 1990. Enhancement of the fibrinolytic activity in plasma by oral administration of NK. *Acta Haematol* 84: 139-143.
11. Fayek KI, El-Sayed ST. 1980. Fibrinolytic activity of an enzyme produced by *Bacillus subtilis*. *Z Ernaehrwiss* 19: 21-23.
12. Kim WK, Choi KH, Kim YT, Park HH, Choi JY, Lee YS, Oh HI, Kwon IB, Lee SY. 1996. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. Strain CK 11-4 screened from Chung-Kook-Jang. *Appl Environ Microbiol* 62: 2482-2488.
13. Kim HK, Kim GT, Kim DK, Choi WA, Park SH, Jeong YK, Kong IS. 1997. Purification and characterization of a novel fibrinolytic enzyme produced from *Bacillus* sp. KA 38 orginated from fermented fish. *J Ferment Bioeng* 84: 307-312.
14. Harerkate F, Traas DW. 1974. Dose response curves in the fibrin plate assay to determined the fibrinolytic activity of proteases. *Thromb Haemostasis* 32: 357-365.
15. Sumi H, Maruyama M, Yoneta T, Mihara H. 1983. Activation of plasma fibrinolysis after intrarectal administration of high molecular weight urokinase and its derivative. *Acta Haematol* 70: 289-295.
16. Longinova LG, Usaite IA, Seregina LM. 1980. Isolation of unpigmented producer of proteolytic enzymes from *Thermoactinomyces vulgaris*. *Appl Biochem Microbiol* 16: 24-30.
17. Mohamedin AH. 1999. Isolation, identification and some cultural conditions of a protease-producing thermophilic *Streptomyces* strain grown on chicken feather as a substrate. *Int Biodeter Biodegr* 43: 13-21.
18. Bascaran V, Hardisson C, Brana AF. 1990. Regulation of extra-cellular protease production in *Streptomyces clavuligenas*. *Appl Microbiol Biotechnol* 39: 208-213.
19. Chitte RR, Dey S. 2002. Production of a fibrinolytic enzyme by thermophilic *Streptomyces* species. *World J Microb Biotechnol* 18: 289-294.
20. Heo S, Lee SK, Joo HK. 1998. Isolation and identification of fibrinolytic bacteria from Korean traditional Chungkoojang. *Kor J Agr Chem Biotechnol* 41: 119-124.
21. Jang SA, Kim MH, Lee MS, Lee MJ, Jhee OH, Oh TK, Sohn CB. 1999. Isolation and identification of fibrinolytic enzyme producing strain from shrimp *Jeot-gal*, a tiny salted shrimps, and medium optimization for enzyme production. *Korean J Food Sci Technol* 31: 1648-1653.

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