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Optimization of the Production of Fibrinolytic Enzyme from *Bacillus firmus* NA-1 in Fermented Soybeans

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Abstract

***Bacillus* strains capable of producing fibrinolytic enzyme were isolated from traditional fermented Korean soybean paste and Japanese fermented soybean (Natto). Among the 16 strains, a selected *Bacillus* sp. was identified as *Bacillus firmus*, with 80.7% homology, by API kit analysis. Seed starter of *B. firmus* NA-1 was prepared with 5% soymilk prepared from micronized soybean powder. To produce fibrinolytic enzyme by *B. firmus* NA-1 the liquid culture was performed with NB broth (pH 7.0) fortified with 1% galactose, 0.1% tryptone, and 0.5% K_2HPO_4, by shaking with 180 rpm at 37°C. Fibrinolytic enzyme activity reached the highest value at 7.8 unit/mL (plasmin unit) after fermentation for 72 hr. The crude fibrinolytic enzyme showed higher relative activity in the range of pH 7.0~9.0. The activity of crude fibrinolytic enzyme was well maintained even after concentration by the vacuum evaporation at 50°C for 1 hr.**

Key words: fibrinolytic enzyme, *Bacillus*, liquid fermentation

INTRODUCTION

Fibrin is an insoluble protein component of blood clots. Fibrin is formed from the selective cleavage of fibrinogen by the action of thrombin, which is activated by a blood cascade mechanism for repairing injuries, followed by polymerization of fibrin monomers. Normally, the fibrin formed is subsequently dissolved by fibrinolytic enzymes such as plasmin within a few days after the wound has healed. However, fibrin may accumulate in blood vessels causing thrombosis leading to myocardial infarction, stroke, and other cardiovascular diseases resulting in serious morbidity or mortality (1). Treatment of thrombosis frequently utilizes synthetic compounds such as coumarin and warfarin, and hirudin (a compound obtained from leeches) have been used as thrombolytic agents (2-5). A urokinase purified from human and animal urine is sometimes administered intravenously, but its use is limited to acute thrombosis because of its cost (6-8). Among thrombolytic agents, the streptokinase obtained from *Streptococcus haemolyticus* has the advantage of a longer stability in the blood and is distributed more evenly than urokinase, but it functions as an activator of plasmin instead of dissolving fibrin directly, and has side-effects including hemorrhaging (9). The tissue-type plasminogen activator (tPA) derived from human melanoma cells is a potent fibrinolytic agent due to its high affinity with fibrin, but oral administration is impractical because of its high price and short shelf-life in the blood (10). Thrombolytic agents used for oral administration have been prepared from the dried powder of an earthworm (*Lumbricus lubellus*) that contained six types of fibrinolytic enzymes, and has been used in Korea and Japan in capsule forms (11,12). Urokinase has been shown to be absorbed in the intestinal tract and retain its fibrinolytic activity as it circulates in the blood (10). It has also been reported that orally administered urokinase is absorbed in the intestine and then transferred to the liver where it undergoes modifications that enhance it enzymatic activity and ability to dissolve fibrin (7,8). This suggests that fibrin in the blood can be effectively dissolved by oral administration of fibrinolytic enzymes.

Fibrinolytic enzymes from traditional fermented soybean (Natto) and a fermented fish product (shiokara) have been purified by Japanese scientists. It was reported that their oral administration enhanced the hydrolysis of fibrin in the blood (13-16). Natto has been known as a traditional functional food for dissolving fibrin and widely consumed as a healthy food in Japan. Traditional fermented foods in Korea have also been used as ingredients for the manufacture of health-promoting functional foods. Kil et al. (17) reported the optimal conditions for the production of fibrinolytic enzyme by a *Bacillus* strain isolated from Korean traditional fermented soybean paste. Effects of culture conditions and nutritional supplements on the production of fibrinolytic enzyme were evaluated in flask

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culture using *Bacillus subtilis* (18,19). A strain producing fibrinolytic enzyme was also isolated from fermented shrimp, and the culture conditions for the production of fibrinolytic enzyme were optimized (20). Recently, there has been intense research into the economical production of safe and effective fibrinolytic enzymes with fewer side-effects than currently used thrombolytic agents (1).

Cardiovascular disease is one of the most common and serious chronic diseases among elderly people. Many people believe that vascular disease can be prevented through the regular consumption of functional foods. The traditional fermented foods of Korea are widely accepted as health-promoting foods for preventing chronic disease and maintaining good health. In particular, *in vivo* studies have demonstrated that thrombosis can be improved by the consumption of Chungkukjang (17,19). Traditional fermented soybean foods have gained widespread acceptance as preventive medicines for curing or preventing vascular disease. If a fibrinolytic enzyme is produced efficiently by food-grade microorganisms in a fermented food, that food can be consumed daily to prevent thrombosis and other related diseases. In addition, newly isolated microorganisms may be used for the production of novel fibrinolytic enzymes.

The objectives of this study were to isolate *Bacillus* strains producing fibrinolytic enzyme from traditional fermented foods and to optimize the culture medium for the production of fibrinolytic enzyme.

MATERIALS AND METHODS

Materials

Traditional fermented soybean paste (Chungkukjang) was purchased from a local supermarket (Korea). Japanese fermented soybean (Natto) was obtained from a supermarket in Japan. Yeast extract and peptone were purchased from Difco Laboratories (Detroit, MI, USA). Bovine fibrinogen, thrombin and plasmin were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Phenol reagent was obtained from Junsei Chemical Co. (Tokyo, Japan). Micronized soybean powder was obtained from Perican Co. (Japan). Other chemicals including salts were analytical grade.

Isolation and identification of *Bacillus* sp.

To isolate the typical *Bacillus* strain, 1 g of fermented soybean paste was mixed with 9 mL of sterilized distilled water and then the serially diluted supernatant was inoculated on MRS agar plates. After incubating at 37°C for 24 hr, mucilage producing strains were isolated as candidates for the *Bacillus* strain. To isolate *Bacillus* strains that efficiently produce fibrinolytic enzyme, each *Bacillus* strain isolated was cultured in nutrient broth and then the fibrinolytic enzyme activity of the supernatant was evaluated by the fibrin plate method (21). To confirm the morphology and proteolytic activity of *Bacillus* strains producing fibrinolytic enzyme, each strain was inoculated on an NB agar plate containing 1% micronized soybean powder and MRS agar plate, and then incubated at 37°C for 24 hr. *Bacillus* strain that was grown on the MRS agar plate was analyzed with an Analytical Profile Index (API) kit.

Preparation of stater culture

To prepare the starter culture, *Bacillus* sp. NA-1 was grown on agar plate containing 1% soymilk at 42°C for 24 hr, and then transferred to 100 mL of 5% soymilk prepared with micronized soybean powder, and cultured in a 250 mL culture flask with 180 rpm shaking at 42°C for 24 hr.

Culture condition for production of fibrinolytic enzyme

To optimize the culture medium for production of fibrinolytic enzyme, nutrient broth (0.5% peptone, 0.3% beef extract, pH 7) was used for the basic culture medium. The medium composition, growth temperature and pH were manipulated. The nutrient broth was fortified with a 1% carbon source of either glucose, saccharose, mannose, galactose, maltose, lactose or soluble starch. Nitrogen sources evaluated were yeast extract, soy flour, tryptone, ammonium sulfate or glycine added at a 0.1% level. Inorganic salts including calcium chloride, magnesium sulfate, sodium chloride, sodium or potassium phosphates were supplemented at a 0.5% level in the nutrient medium. The starter was inoculated in 100 mL of liquid medium at a 1% level and then incubated with 180 rpm shaking at 37°C. After centrifugation of each culture medium at 22,000 × g for 10 min, the supernatant was analyzed for its fibrinolytic activity by the fibrin plate method.

Fibrinolytic enzyme activity

Fibrinolytic enzyme activity was determined by the modified method of Astrup and Müllertz (21). The enzyme substrate was a 0.5% bovine fibrinogen solution prepared in 0.067 M sodium phosphate buffer (pH 7.4). Thrombin enzyme was kept frozen at -20°C after dissolving in 0.067 M sodium phosphate buffer (pH 7.4) to a concentration of 100 NIH units/mL. To prepare the fresh fibrin plate, 10 mL of 0.5% fibrinogen solution was evenly distributed in a petri-dish (diameter 9 cm) and then was quickly mixed with 0.1 mL of thrombin enzyme (100 NIH units/mL). The fibrin plate for turbidity assay was solidified by standing for 30 min at room temperature. To measure the fibrinolytic enzyme activity, the supernatant

(20 μL) was spotted on the fibrin plate and then incubated at 37°C for 2 hr. The clear zone was measured and expressed as fibrinolytic activity. A standard curve was derived from clear zones produced by a standard plasmin enzyme at different concentrations. The plasmin enzyme was serially diluted with Tris-lysine buffer (pH 9.0) to activities of 0.6, 1.6, 2.6, 5 unit/mL and was applied with 20 μL.

Fibrinolytic enzyme activity (%) = (clear zone by culture broth / clear zone by plasmin) × 100

pH and thermal stability of enzyme

To determine the optimum pH for fibrinolytic enzyme activity, the pH of the crude extract containing fibrinolytic enzyme was varied by the addition of 1 M glycine-NaOH (pH 10), 1 M Tris-HCl (pH 7.0, 7.5, 8.0, 9.1), and 1 M sodium acetate (pH 5.2). The enzyme activity was determined by a modified method of Anson (22). A 0.6% (w/v) fibrin solution was prepared in 0.2 N NaOH and adjusted to pH 7.8. A mixture of 0.1 mL of crude enzyme extract and 0.1 mL of 1 M buffer was mixed with 0.35 mL of 0.6% fibrin solution, and then incubated at 40°C for 20 min. The reaction mixture was added to 0.45 mL of 0.44 M trichloroacetic acid and held at room temperature for 30 min. A 0.1 mL aliquot of the supernatant obtained after centrifugation at 22,000 × g for 10 min was reacted with 1 mL of 0.55 M Na_2CO_3 and 0.2 mL of phenol reagent (Folin-Ciocalte's reagent) for 30 min at room temperature. The absorbance was measured at 660 nm. One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 μg of tyrosine per min. To evaluate the thermal stability of the crude enzyme, the supernatant containing crude enzyme was heat evaporated in a vacuum evaporator (H-3000, Hanshin, Korea) at 50°C for 1 hr, and then the enzyme activity of concentrate was determined by the fibrin plate method.

RESULTS AND DISCUSSION

Identification and physiological properties of *Bacillus* sp.

Sixteen microorganisms were isolated from ten different types of traditional fermented soybean paste (Chungkukjang) and Japanese fermented soybeans (Natto). Each of the sixteen isolated cultures was characterized for colony type and mucilage production in different culture media such as NB agar, soymilk agar and MRS agar plates. Among the 16 candidates, a *Bacillus* sp. NA-1 isolated from Natto showed the highest fibrinolytic activity compared with the other strains, and was standardized for enzymatic activity (5 unit/mL). Among the 15 strains isolated from Korean fermented soybean paste, two candidates (CK-1, CK-2) also exhibited substantial fibrinolytic activity (Table 1). The *Bacillus* sp. NA-1 which produced the highest fibrinolytic activity was selected as the candidate for the production of fibrinolytic enzyme. A NA-1 strain grown on soymilk agar was confirmed to be a gram positive strain by gram staining and microscopic examination, and also hydrolyzed soy protein on the soymilk agar plate, resulting in the formation of clear zone. The optimal temperature for growth of NA-1 was 42°C, but the strain was capable of growth between 25°C and 50°C, but did not grow at 55°C. The identification characteristics from the API kit are shown in Table 2. The NA-1 strain could utilize glucose, saccharose and mannose as carbon sources, but not galactose or lactose. Since NA-1 was determined to be *B. firmus* with 80.7% homology based on the biochemical analysis, we designated it as *B. firmus* NA-1. *B. firmus* NA-1 forms a sticky colony with a rough surface when it grows on an MRS agar plate, but forms a spreading colony without stickiness when grown on NB agar plates (Fig. 1). Therefore, the *Bacillus* strain producing mucilage can be easily distinguished on MRS agar plates, since it grows with a sticky and rough morphology.

Effect of pH and temperature on the production of fibrinolytic enzyme

To determine the effect of pH on the production of fibrinolytic enzyme, the initial pH of the nutrient broth was adjusted to between 5 and 11. The fibrinolytic enzyme produced from *B. firmus* NA-1 showed the highest activity in culture medium at pH 7. When the pH of the culture medium was lowered, the relative activity was reduced compared with plasmin enzyme (Table 3). Fur-

Table 1. Fibrinolytic activity of the microorganisms isolated from Natto and Chungkukjang

Strains	Fibrinolytic activity (%)
NA-1	108
CK-1	83
CK-2	30
CK-3	73
CK-4	21
CK-5	42
CK-6	46
CK-7	85
CK-8	69
CK-9	53
CK-10	14
CK-11	39
CK-12	42
CK-13	39
CK-14	24
CK-15	34
Plasmin (5 unit/mL)	100

NA-1 and CK strains were isolated from Natto and Chungkukjang, respectively.

Table 2. Identification of *Bacillus* sp. NA-1 strain by API CHB kit

No.	Carbon sources	Growth	No.	Carbon sources	Growth
0	Control	-	25	Esculine	-
1	Glycerol	+	26	Salicine	-
2	Ertythritol	-	27	Cellobiose	-
3	D-Arabinose	-	28	Maltose	+
4	L-Arabinose	+	29	Lactose	-
5	Ribose	+	30	Melibiose	-
6	D-Xylose	-	31	Saccharose	+
7	L-Xylose	-	32	Trehalose	+
8	Adonitol	-	33	Inuline	+
9	β-Methyl-xyloside	-	34	Melezitose	-
10	Galactose	-	35	D-Raffinose	-
11	D-Glucose	+	36	Amodon	-
12	D-Fructose	+	37	Glycogene	-
13	D-Mannose	+	38	Xylitol	-
14	L-Sorbose	-	39	β-Gentiobiose	-
15	Rhamnose	-	40	D-Turanose	-
16	Dulcitol	-	41	D-Lyxose	-
17	Inositol	+	42	D-Tagatose	-
18	Mannitol	+	43	D-Fucose	-
19	Sorbitol	+	44	L-Fucose	-
20	α-Methyl-D-mannoside	-	45	D-Arabitol	-
21	α-Methyl-D-glucoside	-	46	L-Arabitol	-
22	*N*-Acetyl glucosamine	-	47	Gluconate	-
23	Amigdaline	-	48	2-Keto-gluconate	-
24	Arbutine	-	49	5-Keto-gluconate	-

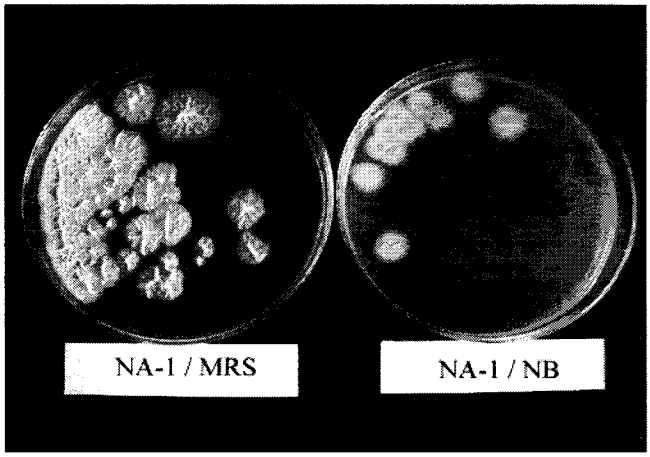

Fig. 1. Morphology of *Bacillus firmus* NA-1 strain grown on MRS and NB agar plates.

Table 3. Effect of initial pH on the relative activity of fibrinolytic enzyme from *Bacillus firmus* NA-1

pH	Relative activity (%)
5.0	64
6.0	80
7.0	108
8.0	83
9.0	73
10.0	0
11.0	0
Plasmin (5 unit/mL)	100

thermore, alkaline pH reduced fibrinolytic enzyme activity, and enzyme activity was not detected above pH 10. Heo et al. (23) reported that *Bacillus* sp. isolated from Chungkukjang showed the maximal enzyme production at pH 8.0. The fibrinolytic enzymes from *Bacillus* sp. KP-6048 and *B. subtilis* BK-17 were maximally produced at pH 8.0 and 9.0, respectively (17,18). Therefore *B. firmus* NA-1 optimally produces fibrinolytic enzyme at a neutral pH in contrast to other fibrinoltyc enzymes producing *Bacillus* sp.

In general, growth temperature is an important factor for the production of microbial metabolites. *B. firmus* NA-1 was grown in nutrient broth (pH 7.0) at different temperatures. The relative activities of fibrinolytic enzyme were 82.8%, 107.9%, and 94.9% at 30°C, 37°C and 42°C, respectively (unpublished results). This result was identical with the reports that *Bacillus* sp. isolated from Chungkukjang maximally produced fibrinolytic enzyme at 37°C (18,23). On the other hand, a thermophilic strain *Bacillus stearothermophilus* showed the maximal production of fibrinolytic enzyme at 50°C (24).

Effects of nitrogen, carbon and inorganic salt

To determine the ideal carbon source for production of the fibrinolytic enzyme, various carbon sources including monosaccharides, disaccharides and soluble starch were added to the nutrient medium at a 1% level. The addition of glucose, saccharose or mannose decreased the relative activity of fibrinolytic enzyme compared with a standard plasmin enzyme. Previously, biochemical analysis showed that glucose, saccharose and mannose were utilized by *B. firmus* NA-1. On the other hand, lactose and galactose,

which could not assimilated by the strain, enhanced the relative activity of the fibrinolytic enzyme. In particular, galactose addition resulted in the highest relative activity, even higher than the standard plasmin enzyme (Table 4). The soluble starch also enhanced the enzyme production, showing 97.9% relative activity. Choi (25) reported that *Bacillus* sp. isolated from a fermented soybean source efficiently produced enzyme in a medium containing soluble starch. These results imply that a deficiency in carbon source may enhance the production of fibrinolytic enzyme by *B. firmus* NA-1.

In general, it is known that the nitrogen source is a crucial factor for the production of extracellular enzyme (26). The relative fibrinolytic activity of NB was approximately the same with or without a nitrogen source (Table 4). The addition of tryptone enhanced the relative activity of fibrinolytic enzyme. Fibrin as a nitrogen source did not affect fibrinolytic enzyme production. These results suggest that *B. firmus* NA-1 shows just a lack of inducibility by nutrients. Kalebina et al. (27) reported that the fibrinolytic enzyme from *Bacillus brevis* was produced efficiently in medium containing yeast extract as the nitrogen source. Enzyme production from a *B. subtilis* strain isolated from fermented soybean was not enhanced in the presence of tryptone (18). Therefore, adding nitrogen sources to the nutrient broth only marginally affects the activity of fibrinolytic enzyme compared to the effects of carbon sources.

To determine the effect of inorganic salts on enzyme production, various salts were fortified at a 0.5% level in the nutrient broth. Most inorganic salts inhibited enzyme production, resulting in a lower relative enzyme activity compared with that of culture without inorganic salts. Only potassium phosphate enhanced the production of fibrinolytic enzyme, showing 112.9% of relative activity. On the other hand, calcium and magnesium decreased the relative activity by about 50%. Therefore, it turns out that inorganic salt concentration in nutrient broth is a very crucial factor for the production of fibrinolytic enzymes. Based on the results of this study, the optimum composition for the nutrient broth is shown in Table 5.

Table 4. Effect of carbon and nitrogen sources and inorganic salts on the relative activity of fibrinolytic enzyme from *Bacillus firmus* NA-1

Sources	Nutrient	Relative activity (%)
Carbon	Glucose	68
	Saccharose	55
	Maltose	78
	Soluble starch	98
	Mannose	48
	Galactose	116
	Lactose	90
Nitrogen	Fibrin	103
	Yeast extract	103
	Soy flour	92
	Tryptone	114
	Ammonium sulfate	105
	Glycine	92
Inorganic salt	$CaCl_2 \cdot 2H_2O$	56
	$MgSO_4 \cdot 7H_2O$	49
	NaCl	88
	Na_2HPO_4	95
	K_2HPO_4	113

The plasmin (5 units/mL) was used for standard enzyme and its relative activity was designated with 100%.

Effect of fermentation time

The effect of fermentation time on fibrinolytic enzyme production was evaluated in a batch fermentation in liquid medium with the optimal nutrient composition. As shown in Fig. 2, the relative activity of fibrinolytic enzyme was rapidly increased during the initial 36 hr of fermentation, and then gradually increased until 72 hr of fermentation time reaching a relative activity of 140% (7 plasmin units). After 72 hr, the relative enzyme activity decreased slightly. Kim et al. (28) reported that a bacterial strain isolated from Chungkookjang produced the highest fibrinolytic activity at 1.84 plasmin units when grown

Table 5. Optimal medium composition for the production of fibrinolytic enzyme from *Bacillus firmus* NA-1

Nutrient	Concentration (g/L)
Beef extract	3
Peptone	5
Galactose	10
Tryptone	5
K_2HPO_4	5
Distilled water	1 L
pH	7

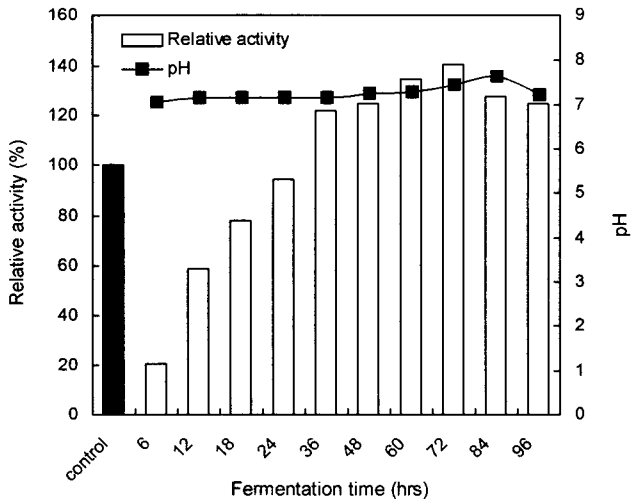

Fig. 2. Effects of fermentation time on the relative activity of fibrinolytic enzyme from *Bacillus firmus* NA-1.

in nutrient broth. Also, Jang et al. (29) reported that a fibrinolytic bacterial strain was isolated from Jeot-Gal, salt-fermented fish which produced 2.04 plasmin units. Therefore, the data suggest that the modified nutrient broth is the most efficient media for production of fibrinolytic enzyme from *B. firmus* NA-1. The pH of culture broth remained relatively constant, at pH 7.5, during fermentation for 72 hr. The relative enzyme activity during fermentation in liquid culture appeared to parallel the growth curve of the microorganism. The production of fibrinolytic enzyme by *B. firmus* NA-1 coincided with the cell growth, resulting in rapid increases during the initial 36 hr. In the stationary phase the production of enzyme gradually increased and then declined after fermentation for longer than 72 hr. This result agreed with the 72 hr optimal culture time previously determined for enzyme production by *Bacillus* sp. S19 (20). It was reported that the optimal culture time for enzyme production by other *Bacillus* strains was 48 hr (23). Therefore, the optimal culture time for production of fibrinolytic enzymes may be different according to the strain and culture medium, so that optimization is required. Based on our results, to produce fibrinolytic enzyme efficiently, the liquid fermentation should be completed within 3 days.

Effect of temperature and pH

Effects of temperature and pH on the activity of the fibrinolytic enzyme were evaluated. The optimal pH for activity of the fibrinolytic enzyme from *B. firmus* NA-1 is shown in Fig. 3. The relative activity of the fibrinolytic enzyme was well maintained in the range of pH 7.0 to pH 9.0, showing higher relative activity in the alkaline range, but under acidic conditions, the enzyme activity was greatly decreased, exhibiting 30% of relative activity. This result was different from that of the fibrinolytic

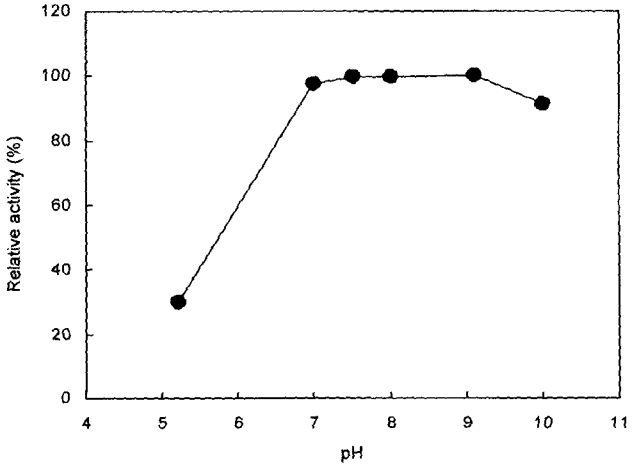

Fig. 3. Effect of pH on the relative activity of crude fibrinolytic enzyme obtained from the liquid culture of *Bacillus firmus* NA-1.

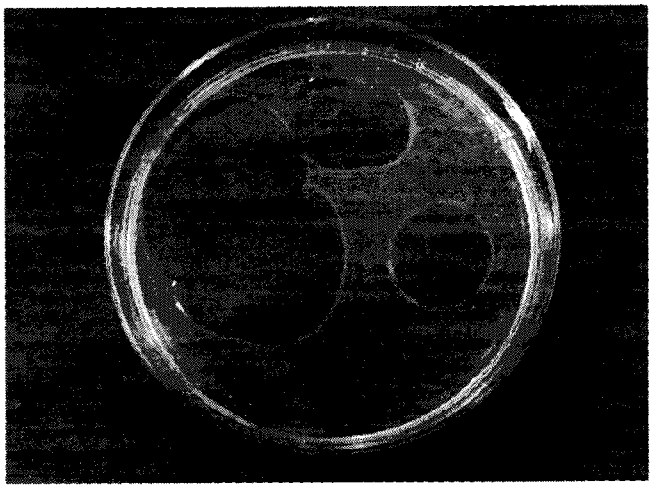

Fig. 4. Comparison in fibrinolytic enzyme activity of supernatant of liquid culture and its concentrate on the fibrin plate. A: concentrate, B: supernatant, C: diluted concentrate.

enzyme from *Bacillus* sp. KA38, which showed higher activity under acidic conditions, retaining 80% of relative activity (1). The activity of the crude fibrinolytic enzyme was mostly retained even after concentration by vacuum evaporation at 50°C for 1 hr. As shown in Fig. 4, the diluted concentrate reconstituted to original volume showed a similar pattern in the formation of a clear zone on the fibrin plate. However, the fibrinolytic enzyme concentrate formed the largest clear zone. It has been reported that the fibrinolytic enzyme from *Bacillus* sp. KA38 is stable up to 40°C, but its activity rapidly decreased at higher temperatures. As a result, about 70% of the relative fibrinolytic activity is lost at 50°C (1). In contrast, the fibrinolytic enzyme from *B. firmus* NA-1 in this study remained quite stable activity at that temperature. Therefore, vacuum evaporation can be used to obtain a concentrate with high activity from liquid culture, without the loss of activity.

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REFERENCES

1. Kim HK, Kim GT, Kim DK, Choi WA, Park SH, Jeong YK, Kong IS. 1997. Purification and characterization of a novel fibrinolytic enzyme from *Bacillus* sp. KA38 originated from fermented fish. *J Ferment Bioeng* 84: 307-312.
2. Lee SK, Son JH, Choi YS, Lee SK. 1993. Screening and purification of anticoagulant proteins from Korean leeches. *Korean J Biochem* 26: 228-234.
3. Electricwala A, Sawyer RT, Powell Jones C, Atkinson T.

1991. Isolation of thrombin inhibitor from the leech *Hirudinaria manillensis*. *Blood Coagul Fibrinolysis* 2: 83-91.
4. Krstenansky JL, Owen TJ, Yates MT, Mao SJT. 1990. The C-terminal binding domain of hirullin P18 antithrombin activity and comparison to hirudin peptides. *FEBS Lett* 269: 425-431.
5. Steiner V, Knecht R, Gruetter M. 1990. Isolation and purification of novel hirudins from the leech *Hirudinaria manillensis* by high-performance liquid chromatography. *J Chromatogr* 530: 273-282.
6. Abe T, Kazama M, Kinoshita T, Naito I, Ogushi T, Yoshimura Y, Teruya J, Shimizu N. 1982. Shift of fibrinolysis system at oral administration of urokinase in human subjects; Double blind cross over study. *Blood and Vessels* (in Japanese) 13: 472-479.
7. Sumi H, Maruyama M, Yoneta T, Mirhara H. 1983. Activation of plasma fibrinolysis after intrarectal administration of high molecular weight urokinase and its derivative. *Acta Haemat* 70: 289-295.
8. Sumi H, Sasaki K, Toki N, Robbins KC. 1980. Oral administration of urokinase. *Thromb Res* 20: 711-714.
9. Reed GL, Lin LF, Parhami-Seren B, Kussie P. 1995. Identification of plasminogen binding region in streptokinase that is necessary for the creation of a functional streptokinase-plasminogen activator complex. *Biochemistry* 34: 10266-10271.
10. Sumi H, Seiki M, Morimoto N, Tsushima H, Maruyama M, Mihara H. 1985. Plasma fibrinolysis after intraduodenal administration of urokinase in rats. *Enzyme* 33: 121-127.
11. Mihara H, Sumi H, Yoneta T, Misumoto H, Ikeda R, Seiki M, Maruyama M. 1991. A novel fibrinolytic emzyme extracted from the earthworm, *Lumbricus lubellus*. *Jpn J Physiol* 41: 461-472.
12. Nakajima N, Mihara H, Sumi H. 1993. Characterization of potent fibrinolytic enzyme in earthworm, *Lumbricus lubellus*. *Biosci Biotechnol Biochem* 57: 1726-1730.
13. Sumi H, Hamada H, Mihara H, Muraki H. 1987. A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet. *Experientia* 43: 1110-1111.
14. Sumi H, Nakajima N, Yatagai C. 1995. A unique strong fibrinolytic enzyme (katsuwokinase) in skipjack "Shiokara" a Japanese traditional fermented food. *Comp Biochem Physiol* 122B: 543-547.
15. Nakajima N, Taya N, Sumi H. 1993. Potent fibrinolytic enzyme from the lysate of *Katsuwonus pelamis* digestive tract (Shiokara): purification and characterization. *Biosci Biotechnol Biochem* 57: 1604-1605.
16. Sumi H, Hamada H, Nakanishi K, Hiratani H. 1990. Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. *Acta Hanematol* 84: 139-143.
17. Kil JO, Kim GN, Park IS. 1998. Production and characterization of fibrinolytic enzyme: Optimal condition for production of the enzyme from *Bacillus* sp. KP-6408 isolated from *Chungkook-jang*. *Korean J Food Sci Nutr* 27: 51-56.
18. Choi WA, Lee JW, Lee KH, Park SH. 1998. Effects of environmental and nutritional conditions on fibrinolytic enzyme production from *Bacillus subtilis* BK-17 in flask culture. *Korean J Biotechnol Bioeng* 3: 491-496.
19. Lee SK, Heo S, Bae DH, Choi KH. 1998. Medium optimization for fibrinolytic enzyme production by *Bacillus subtilis* KCK-7 isolated from Korean traditional *Chungkookjang*. *Korean J Appl Microbiol Biotechnol* 26: 226-231.
20. 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: 1648-1531.
21. Astrup T, Müllertz S. 1952. The fibrin plate method for estimating fibrinolytic activity. *Arch Biochem Biophys* 40: 346-351.
22. Anson ML. 1939. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J Gen Physiol* 22: 79-85.
23. Heo S, Lee SK, Joo HK. 1998. Isolation and identification of fibrinolytic bacteria from Korean traditional *Chungkookjang*. *Kroean J Agric Chem Biotech* 41: 119-124.
24. Yang HC, Lee TK, Whang JB, Kim SH. 1990. Production of cyclomaltodextrin from *Bacillus stearothermophilus*. *Korean J Appl Microbiol Biotechnol* 18: 578-584.
25. Choi KJ. 1995. Separation of *Bacillus* sp. and changes of NH-2N, NH-3N, and protease activity in Chongukchang meju adding with mugwort (*Artemisia asiatica* N.) extract. *MS Thesis*. Kon-Kuk University, Korea.
26. Bascaran V, Hardisson C, Brana AF. 1990. Regulation of extracellular protease production in *Streptomyces clavuligerus*. *Appl Microbiol Biotechnol* 34: 208-213.
27. Kalebina TS, Galina NR, Selyakh IO, Khodova OM, Kulaev IS. 1988. Serine proteinase from *Bacillus brevis*. *Appl Microbiol Biotechnol* 82: 531-537.
28. Kim YT, Kim WK, Oh HI. 1995. Screening and identification of the fibrinolytic bacterial strain from Chungkookjang. *Kor J Appl Microbiol Biotechnol* 23: 1-5.
29. Jang YR, Kim WK, Kwon IB, Lee HY. 1998. Screening and identification of the fibrinoltytic bacterial strain from Jeot-Gal, salt-fermented fish. *Korean J Food Sci Technol* 30: 655-659.

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