J. Microbiol. Biotechnol. (2002), **12**(4), 649–656

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Screening and Characterization of Microorganisms with Fibrinolytic Activity from Fermented Foods

YOON, SEON-JOO[1,2], MYEONG-AE YU[2], GWAN-SUB SIM[2], SEUNG-TAEK KWON[3], JAE-KWAN HWANG[2], JUNG-KUE SHIN[2], IK-HYUN YEO[3], AND YU-RANG PYUN[2]*

[1]*Graduate Program in Biomaterials Science and Engineering, Yonsei University, Seoul 120-749, Korea*
[2]*Department of Biotechnology and Bioproducts Research Center, Yonsei University, Seoul 120-749, Korea*
[3]*R&D Center, Pulmuone, Co. Ltd., Seoul 120-749, Korea*

Received: March 29, 2002
Accepted: July 10, 2002

Abstract Fibrinolytic microorganisms were screened from 42 samples of Korean fermented food (7 kinds of Chungook-jang, 14 kinds of commercial Doen-jang, 5 kinds of home-made Doen-jang, and 16 kinds of Jeot-gal), 15 samples of Japanese fermented food (5 kinds of home-made soybean paste, and 10 kinds of Natto), and 19 samples of Indonesian fermented food (Tempe) as well as starters of Meju (500 microflora from Korea, and 22 from China). Initially, 11 isolates with strong fibrinolytic activity were selected for further characterization. The fibrinolytic activity of the 11 isolates ranged from 89 to 199% of standard plasmin. Four strains, M51 from Korean fermented food (Meju), I 1-1, I 1-4, and I 5-1 from Indonesian fermented food (Tempe), were chosen based on the degree of activity and reproducibility, and identified as *Staphylococcus sciuri*, *Citrobacter* or *Enterobacter*, *Enterococcus faecalis*, and *Bacillus subtilis*, respectively. The first two isolates are pathogenic strains while the latter two are considered as GRAS (Generally Recognized As Safe). Fibrinolytic activity of *E. faecalis*, characterized and designated as BRCA-5, reached a maximum, when the producer was cultivated in M17 broth supplemented with 1.0% glucose for 5 h at 37°C with shaking at 180 rpm. Compared to commercial fibrinolytic enzymes, the cell-free culture supernatant of *E. faecalis* BRCA-5 showed stronger activity than plasmin and streptokinase, but similar degree of specific activity as nattokinase and urokinase, and it also demonstrated anticoagulant and antiplatelet activity *ex vivo*. These features of *E. faecalis* make it an attractive agent as a biomaterial for health-promoting foods.

Key words: Fibrinolysis, fermented foods, Tempe, *Enterococcus faecalis*, antiplatelet activity

*Corresponding author
Phone: 82-2-2123-3593; Fax: 82-2-312-6821;
E-mail: yrpyun@yonsei.ac.kr

Hemostasis is a highly elaborated and complicated system where blood coagulation and fibrinolysis are in fine balance and tightly controlled. Fibrin converted from fibrinogen during thrombosis events is an insoluble, polymeric meshwork and stabilized by thrombin-activated factor XIII through cross-linking [7]. It is critical to remove redundant fibrin deposits at the sites of tissue injury to restore full potency to the damaged vessel. The fibrinolytic system consisting of fibrinolytic enzymes including plasmin, tissue plasminogen activator (t-PA), plasminogen activator inhibitors (PAIs), and urokinase is the body's main defense mechanism against occlusive vascular diseases [21]. Accordingly, these agents have been developed as thrombolytic agents for treating thrombotic diseases. Application of these agents, however, accompanies several adverse effects including hemorrhagic complications and lack of fibrin specificity, as well as high cost. A safe and efficient thrombolytic agent should be relatively fibrin-specific to avoid systemic activation of the fibrinolytic pathway. Excess circulating plasmin, the serine protease, can also degrade fibrinogen and other coagulating factors, resulting in impaired blood coagulation and thus damaged hemostasis [31]. In this regard, search for new candidates as an effective thrombolytic agent has been an active research area, and at present, 8 commercial drugs are available including those prepared by recombinant technology [4].

During the past decades, microorganisms demonstrating fibrinolytic activity have been isolated from fermented foods [20, 35, 36, 38]. *Bacillus natto* producing nattokinase was isolated from a Japanese fermented food, Natto [35]. Sumi *et al.* [34] reported that nattokinase enhanced the plasma fibrinolysis *in vivo*, when administrated orally. At present, various health foods containing nattokinase have been developed commercially [33]. Several *Bacillus* strains, including strains CK11-4, DJ-4, and KA38, were isolated

from traditional Korean fermented foods, such as Chungook-jang, Doen-jang, Kamahi, and fermented fish products [14, 17, 19, 20, 27, 38]. The fibrinolytic enzymes produced by these food-grade microorganisms are of special interest in developing functional foods beneficial to public health.

In this study, naturally occurring microflora showing fibrinolytic activity, in addition to Meju strains belonging to the genus *Bacillus*, were screened from various Korean (42 samples) and international fermented foods (34 samples). Microorganisms were selected based on the extent of fibrinolytic activity as well as possibility to be developed as an alternative thrombolytic agent. Cultural and biochemical characteristics of the selected microorganisms were examined for taxonomic identification. Production of fibrinolytic activity of an isolate, *Enterococcus faecalis*, with strong activity was optimized by monitoring growth conditions.

Materials and Methods

Materials

Varieties of fermented foods were collected from Korea, Japan, and Indonesia (Table 1). Among the food samples collected from Korea were Chungkook-jang, Doen-jang (both commercial and home-made) and Jeot-gals. Natto and soybean pastes were purchased from a local Japanese market and Tempes from Indonesia. All chemicals were purchased from Sigma Chemical Co. (St. Louis, U.S.A.), unless stated otherwise. Growth media including nutrient broth, brain heart infusion (BHI), MRS, Luria-Bertani (LB), and M17 were purchased from Difco (Detroit, U.S.A.) and prepared according to the manufacturer's instruction.

Fibrinolytic Activity Assay

The fibrin plate method [2] was used with slight modification. Briefly, human fibrinogen was dissolved in 10 mM sodium phosphate buffer (pH 7.4) to a final concentration of 0.5% followed by adding thrombin (10 NIH units). The mixtures were solidified in petri dish for 1 h at room temperature prior to use. In order to examine fibrinolytic activity, aliquot of samples (20 μl) was spotted on fibrin plate followed by incubation at 37°C for 12 h. The diameter of cleared zone was measured and fibrinolytic activity (units/mg) was determined using a parallel standard curve based on the standard plasmin (4 units/mg).

Screening of Fibrinolytic Strains

Fermented food with fibrinolytic activity was screened by placing a small amount directly on fibrin plates. After selecting the fibrinolytic food samples, fibrinolytic microorganisms were isolated as follows. Briefly, a slurry of the selected samples (25 g) was homogenized in 225 ml of 10 mM sodium phosphate buffer (pH 7.5) using a Stomacher homogenizer. A loopful of each slurry was streaked on nutrient agar (NA), brain heart infusion agar (BHI), and M17 agar (duplicate each), and incubated at 37°C for 1–2 days. Single colonies were picked and cultivated in enrichment media whose supernatants were spotted on fibrin plate to assay the fibrinolytic activity. After incubating the fibrin plates at 37°C for 24 h, colonies with surrounding clear zones were selected and classified as fibrinolytic isolates. Isolates from Chungkook-jang were designated 'KC' while the strains from Indonesian fermented foods were assigned as 'I'. Starter cultures of Meju (over 500 Korean strains, M1-M500, and 22 strains from China, C1-C22) were tested for fibrinolytic activity.

Identification of Fibrinolytic Microorganisms

After enrichment, the cultural and biochemical characteristics of the 4 bacterial isolates selected based on the source and activity were analyzed according to Bergey's Manual of Systematic Bacteriology using API kits [10]. Analysis of the peptidoglycan and 16S rDNA of each isolate was carried out by DSMZ (Germany) in order to identify the isolates at the species level.

Table 1. Fermented foods used for screening and fibrinolytic isolates obtained from each food sample.

Geographic sources	Fermented foods				No. of isolates tested	Fibrinolytic isolates
	Name	Samples/ strains tested	Fibrinolytic samples/Strains	Samples/strains selected		
Korea	Chungkook-jang	7	6	2	17	7
	Commercial Doen-jang	14	7	ND[a]	ND	ND
	Home-made Doen-jang	5	0	ND	ND	ND
	Jeot-gal	16	0	ND	ND	ND
Japan	Home-made soybean paste	5	0	ND	ND	ND
	Natto	10	10	ND	ND	ND
Indonesia	Tempes	19	14		15	14
Starters for Meju	Korea	500	25	25	25	25
	China	22	2	2	2	2

[a]ND. Not determined.

Optimization of Culture Condition for Maximum Fibrinolytic Activity

Several rich media including NB, BHI, MRS, and M17 containing either glucose or lactose (final concentration of 1.0%), and LB were used to determine the media in which fibrinolytic microflora showed maximum fibrinolytic activity. Growth of the producer in M17 broth containing 1.0% glucose was monitored spectrophotometically at 600 nm while the fibrinolytic activity was simultaneously measured by the fibrin plate method as a function of time.

Animals

Sprague-Dawley (SD) male rats, weighing 200–250 g each, were used in the study. The rats were cared for following the guidelines of the National Institutes of Health [25]. Rats had free access to food and water until the beginning of experiments. After 24 h of fasting, the rats were used for analyzing the antiplatelet activity *ex vivo*. The rats were allowed to take water during the fasting period.

Determination of *Ex Vivo* Antiplatelet Activity of the Culture Supernatant of *E. faecalis*

Ex vivo antiplatelet activity of the cell-free culture supernatant of *E. faecalis* (CS) was investigated with a slightly modified method and compared to that of aspirin, a widely used antiplatelet agent [6]. Briefly, Sprague-Dawley (SD) male rats were divided into three groups, having 10 rats for each control, aspirin-, or the CS-treatment. Before administration of test samples to rats, 4.5 ml whole blood were drawn from the heart of anesthetized rats using syringes with 26G needle containing 0.5 ml of 3.8% sodium citrate. Aliquot of the whole blood (450 μl) was incubated at 37°C for 3 min followed by addition of 5 μl collagen to induce platelet aggregation. After 24 h fasting, the rats in each group were force-fed using Zonde filled with 50% DMSO or test samples. The CS of *E. faecalis* BRCA-5 was lyophilized and resolubilized in water prior to the treatment. Both aspirin dissolved in 50% DMSO and the CS solution were administrated at the dosage of 100 mg/kg body weight/day for 4 consecutive weeks prior to blood sampling for the antiplatelet activity. One hour after the last feeding, blood samples were drawn from the heart of anesthetized rats as described above. The platelet aggregation was monitored using the two-channel Chrono-Log platelet aggregometer (Chrono-Log, Co., Havertown, PA, U.S.A.) as described [3, 30, 39].

Analysis of Anticoagulant Activity Using Whole Blood

Blood samples obtained at 1 h after the last feeding were used to determine the whole blood clotting time. An aliquot (250 μl) of blood sample was transferred to a capped tube and prewarmed to 37°C. While rotating the capped tubes, an equal volume of 1.7% $CaCl_2$ was added in order to induce blood clotting, and the whole blood clotting time was monitored in seconds.

RESULTS AND DISCUSSION

Screening of Fibrinolytic Strains

Thrombosis is a major cause of death in the world, which has facilitated research in the field of thrombolytic therapy. Clinical, experimental, and pharmacological evidence suggest that fibrinogen and fibrin are involved in the growth and dissemination of malignant diseases, which further emphasize the importance of timely development of thrombolytic agents. At present eight commercial thrombolytic agents, which are clinically approved or under development, are available [4]. They are originated from either human plasma, microorganisms, or prepared using recombinant technology. Fibrinolytic agents of microbial origin include streptokinase, staphylokinase, and nattokinase recently isolated from a Japanese fermented food, Natto. Nattokinase is of special interest, since it is produced by *Bacillus natto*, a naturally occurring microorganism in fermented food. Application of nattokinase in commercial food products has promoted us to screen various fermented foods for a new fibrinolytic strain and its enzyme.

In this study, 42 Korean-, 15 Japanese-, and 19 Indonesian-fermented foods samples as well as the starters for Meju (500 Korean strains, 22 Chinese strains) were screened for microflora showing the fibrinolytic activity. A typical fibrin plate having fibrinolytic activity by isolates is shown in Fig. 1. Isolates like C, D, or E showing strong activity were selected from initial screening, while the other isolates such as A and B with weak or no activity were discarded. From the Korean samples of Chungkook-jang, a total of 7 isolates showing the fibrinolytic activity were obtained, and designated as KC series (Table 1). Although 7 out of 19 Doen-jangs were fibrinolytic, no effort

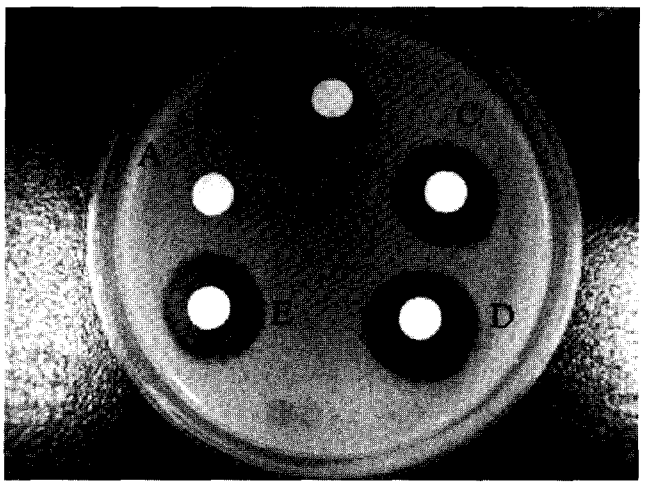

Fig. 1. Fibrin plate showing clear zones resulted from the fibrinolysis of isolates.
Cell-free culture supernatant of isolates DJ-2 (A), KC 1-1 (B), M51 (C), I 1-4 (D), and I 1-5 (E) were tested.

Table 2. Fibrinolytic activities and source of 11 isolates preliminary selected for the study.

Country	Microorganisms		Fibrinolytic activity (unit/mg)[c]
	Name[a]	Source[b]	
Korea	Plasmin		4.00
	KC 1-1	FF	3.56
	KC 4-7	FF	3.56
	M51	MJ	5.56
	M93	MJ	3.56
	M427	MJ	3.56
Indonesia	I 1-1	FF	7.96
	I 1-3	FF	4.36
	I 1-4	FF	7.24
	I 1-5	FF	7.12
	I 5-1	FF	3.56
	I 5-2	FF	3.56

[a]KC, strains isolated from Chungkook-jang; M, starters of Meju; I, Indonesian fermented foods.
[b]FF, Fermented Food; MJ, Meju.
[c]The fibrinolytic activity of isolates determined based on the fibrin plate method and expressed as unit/mg using a parallel standard curve based on plasmin standard (Sigma Chemical Co., U.S.A.).

was made to isolate microorganisms due to their relatively weak activity. The result was not in agreement with the reports that *Bacillus* species isolated from Doen-jang showed 3 to 4-fold higher fibrinolytic activity than other *Bacillus* species obtained from Chungkook-jang or Natto [15]. All Natto samples tested were fibrinolytic, but none of the Japanese soybean pastes hydrolyzed fibrin. Since fibrinolytic microorganism from Natto was well characterized, no further isolation was carried out for these samples. Fourteen isolates with strong fibrinolytic activity were collected from Indonesian fermented food, Tempe, and designated as I-strains. Out of 500 M-strains, 27 strains including 25 Korean strains and 2 Chinese strains were fibrinolytic and their activity was further examined.

Based on the extent of the activity and cultural characteristics, a total of 11 strains were chosen for further characterization (Table 2). Two KC-strains showed fibrinolytic activity that was approximately 89% of plasmin. Among the fibrinolytic Meju strains, the M51 strain showed the strongest activity (139% of plasmin). Three isolates from the Indonesian Tempes, I1-1, I 1-4, and I 1-5, had good fibrinolytic activity of 178-199% of plasmin.

Identification of Fibrinolytic Microorganisms

Out of 11 isolates, 4 strains, M51, I 1-1, I 1-4, and I 5-1, were chosen on the basis of the activity, reproducibility, and cultural characteristics for further characterization and strain identification (Table 3). Morphological, cultural, and biochemical tests for the 4 strains were carried out according to the Bergey's Manual of Systematic Bacteriology using the API kits. Two strains, M51 and

Table 3. Morphological, cultural, and biochemical characteristics[a] of four fibrinolytic isolates.

Strains	Morphology	Gram stain	Growth under aerobic/ anaerobic condition	Motility	Spore formation	Catalase	Urease	Fermentable sugars[c]	Peptidoglycan type[b]	16S rDNA sequence similarity[b]
M51	Coccus	+[c]	+/+w	–[c]	–	+	–	Glc, Frc, Man, Mal, Lac, Mann, Rib, Cel, Suc, NAG	A3α, L-Lys-L-Ala-Gly_4	100% to *Staphylococcus sciuri*
I 1-1	Short rods	–	+w/+	+	–	+	+	Glc, Frc, Man, Mal, Suc, Trh, Arb, Rhm, Gal, NAG	ND[c]	98.5 % to *Citrobacter* or *Enterobacter*
I 1-4	Coccus	+	+/+	–	–	–	–	Glc, Rib, Mann, Sorb, Lac, Trh, Suc, Mal	A3α, L-Lys-L-Ala_{2-3}	100% to *Enterococcus faecalis*
I 5-1	Rods	+	+/–	ND	+ (ellipsoid)	ND	ND	Glc, Arb, Xyl, Mann, Frc	ND	100% to *Bacillus subtilis*

[a]Morphology of each isolate was determined microscopically while biochemical tests were carried out using the API kit.
[b]Peptidoglycan type and 16S rDNA of each isolate were analyzed at the DSMZ.
[c]ND, not determined; +, growth or presence; –, no growth or absence. Glc, glucose; Frc, fructose; Man, mannose; Mal, maltose; Lac, lactose; Mann, mannitol; Rib, ribose; Cel, cellubiose; Suc, sucrose; Trh, trehalose; Arb, arabinose; Rhm, rhamnose; Gal, galactose; Sor, sorbitol; Xyl, xylose; NAG, N-acetylglucosamine.

Table 4. Identification of the four fibrinolytic microorganisms and their activity.

Isolates	Source	Strain identification	Pathogenicity[a]	Fibrinolytic activity (unit/mg)[b]	
				With plasminogen	Without plasminogen
M 51	Meju of Korea	*Staphylococcus sciuri*	Pathogen	6.12±0.30	5.44±0.26
I 1-1	Tempe of Indonesia	*Enterobacter* sp. or *Citrobacter* sp.	Pathogen	3.76±0.18	4.92±0.24
I 1-4	Tempe of Indonesia	*Enterococcus faecalis*	GRAS	4.92±0.24	7.20±0.36
I 5-1	Tempe of Indonesia	*Bacillus subtilis*	GRAS	3.84±0.28	3.84±0.18

[a]Pathogenicity of each strain was referred to literatures.
[b]The fibrinolytic activity of isolates determined was based on the fibrin plate method and expressed as unit/mg using a parallel standard curve based on plasmin standard (Sigma). Data are presented as means±SD. $p \leq 0.05$. When necessary, final concentration of plasminogen was composed of 1 mg/ml in fibrin plates.

I-4, were Gram-positive coccid and did not sporulate. Peptidoglycan types and 16S rDNA sequence analysis revealed that these cocci were 100% homologous to *Staphylococcus sciuri* (M51) and *Enterococcus faecalis* (I 1-4), respectively. Isolate I 1-1 was Gram-negative short rods with motility and showed 98.5% homology to *Citrobacter* or *Enterobacter* species, when analyzed for 16S rDNA sequence. The 4th strain, I 5-1, was Gram-positive, aerobic spore-formers, and identified as a *Bacillus subtilis* strain.

Table 4 summarizes the source, strain name, pathogenicity, and their fibrinolytic activity. When determined by the fibrin plates with or without plasminogen, the activity of I 1-1 and I 1-4 was not enhanced by the presence of plasminogen, indicating that the fibrinolytic agent was not plasminogen activator (PA) but fibrin-specific enzyme (Table 4). In contrast, the activity of M 51 and I 5-1 was higher on the plasminogen-containing plate than on the plate lacking the substance, suggesting that the active agent might be PA-like. These strains showed fibrinolytic activity on the plasminogen-lacking fibrin plate, suggestive of a fibrin-specific protein in addition to a PA-like enzyme. *S. sciuri* (M51) and *Citrobacter/Enterobacter* (I 1-1) were identified as pathogenic strains [11], while *E. faecalis* (I 1-4) and *B. subtilis* (I 5-1) were considered as GRAS [24, 35]. These findings made the latter two strains attractive candidates for developing as biomaterials in functional foods. Of the latter two strains, *E. faecalis* was of more interest, since the fibrinolytic enzyme acts directly on fibrin, not through plasminogen activation, whereas that of *B. subtilis* acts via a plasminogen activator. In addition, the fibrinolytic enzyme of *E. faecalis* has not been well characterized whereas that of *B. subtilis* has extensively been studied (e.g. CK 11-4, KA-38, KP-6408, DJ-4; [13, 14, 16, 18]). *E. faecalis*, formerly a Lancefield group D faecal *Streptococcus* species, is a member of the lactic acid bacteria and naturally present in the intestines of animals, Kimchi, or starter mixes, making the strain applicable to foods [10, 24]. *Streptococcus* species producing fibrinolytic enzyme, streptokinase, belong to the Lancefield groups A and E, which distinguishes them from *E. faecalis* [9, 10, 32]. Some strains of *E. faecalis* are reported to be pathogenic to humans, causing endocarditis or urinary infection [11, 12, 22]. However, since the late 1980s, *E. faecalis* and *E. faecium* have been investigated for their potential use as probiotics, and it was found that these strains, when administrated, supported the growth of other probiotic LABs and *Bifidobacterium* and inhibited the growth of *Salmonella* or yeasts in the intestines of animals [28, 29]. Taking all these observations into consideration, I 1-4 identified as *Enterococcus faecalis* was chosen for further characterization for the rest of this study and designated as BRCA-5.

Optimization of Culture Condition for Maximum Fibrinolytic Activity of Cell-Free Culture Supernatant of *E. faecalis* BRCA-5 (CS)

The culture condition of *E. faecalis* BRCA-5 was optimized. Optimum growth temperatures and pHs ranged 25–40°C and 4.5–8.0, respectively (data not shown). In order to optimize the culture media, several growth media were tested (Fig. 2). Among the growth media tested, M17 broth containing 1.0% glucose supported the highest growth (OD_{600}, 3.521) as well as relative fibrinolytic activity of 100%. When lactose instead of glucose was supplemented, the activity was only 80% compared to that of glucose

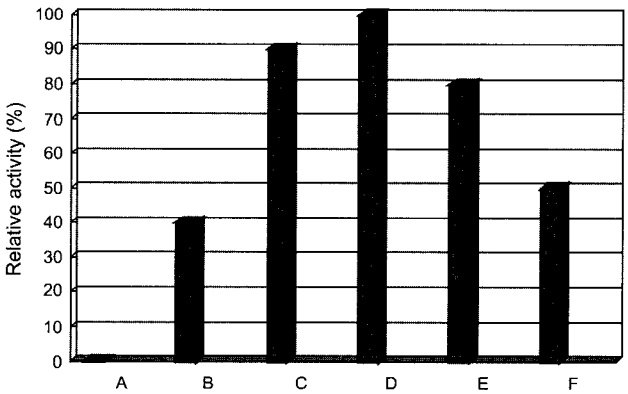

Fig. 2. Comparison of the fibrinolytic activity of *E. faecalis* BRCA-5 cultivated in several media broths.
Bars: A, NB; B, BHI; C, MRS; D, M17 broth+1% Glucose; E, M17 broth+1% Lactose; F, LB.

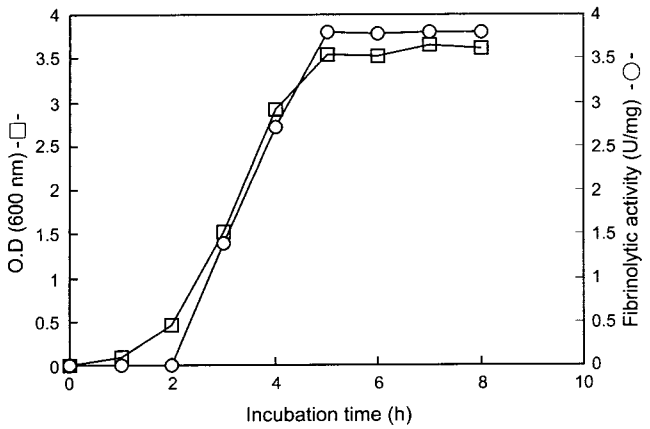

Fig. 3. Growth of *E. faecalis* BRCA-5 and fibrinolytic activity in M17 broth supplemented with 1.0% glucose at 37°C.
Legends: ■, growth; ○, fibrinolytic activity.

supplemented culture. Growth of *E. faecalis* BRCA-5 in nutrient broth or Luria Bertani (LB) broth led to no or weak activity (Fig. 2), and BHI broth was also a poor medium. Chemically defined media supplemented with different nutrients did not lead to the increased production of fibrinolytic activity by the bacteria (data not shown).

Fibrinolytic activity increased when *E. faecalis* BRCA-5 was cultivated at 37°C with shaking, reaching maximum at 180 rpm (data not shown). *E. faecalis* BRCA-5 reached stationary phase within 5 h under this condition when 1.0% overnight culture was seeded (Fig. 3). Fibrinolytic activity increased concurrently with the increase in cell mass under these conditions, reaching a maximum after 5 h incubation. Based on these results, optimum conditions for the growth and fibrinolytic activity were to cultivate *E. faecalis* BRCA-5 in M17 broth containing 1.0% glucose at 37°C with shaking (180 rpm) for 5 h.

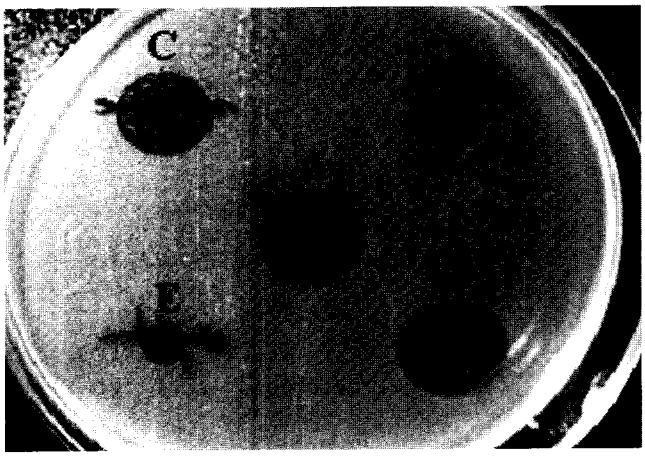

Fig. 4. Comparison of the fibrinolytic activity of the CS with commercial thrombolytic agents.
Legends: A, CS; B, plasmin; C, nattokinase; D, urokinase; E, streptokinase.

Comparison of Fibrinolytic Activity of Cell-Free Culture Supernatant of *E. faecalis* BRCA-5 (CS) with Other Thrombolytic Agents

The activity of various fibrinolytic enzymes was compared (Fig. 4). CS, urokinase, and nattokinase showed 1.5-3 fold higher activity than commercial plasmin or streptokinase, when determined by the sizes of clear zones. Nattokinase, a microbial fibrinolytic enzyme isolated from the Japanese fermented food, Natto, is currently used to prepare various health-promoting foods [33]. Likewise, *E. faecalis* BRCA-5 might be used for development of a thrombolytic agent that could be included in functional foods or administrated as clinical therapy against thrombotic diseases. However, further investigations, preclinical as well as clinical, should be carried out prior to use as a new biomaterial or drug.

Ex Vivo Antiplatelet and Anticoagulant Activity of Cell-Free Culture Supernatant of *E. faecalis* BRCA-5 (CS)

Clinical treatments for thrombotic diseases have been developed according to diverse mechanisms such as anticoagulant, antiplatelet action, or thrombolysis. Thrombosis and fibrinolysis are the pathways where both processes are closely linked and regulated [5, 23, 37]. It is, therefore, expected that changes in one process might result from the other process and vice versa. In the present study, we investigated whether *ex vivo* anticoagulant and/or antiplatelet activity of the CS would be observed when administrated to rats, using three groups of Sprague-Dawley (SD) male rats (Fig. 5). Thus, each group was treated with 50% DMSO (control), aspirin dissolved in 50% DMSO, and the CS, respectively, and the antiplatelet activity of aspirin and the CS were found to be 38.1% and 21.3%, respectively, compared to the control that showed normal platelet aggregation when induced by collagen. At the same dose of 100 mg/kg/day, the CS showed over 50% antiplatelet activity of aspirin, a widely used strong antiplatelet agent. It has been observed by SDS-PAGE that the fibrinolytic CS hydrolyzed not only fibrin but also fibrinogen with

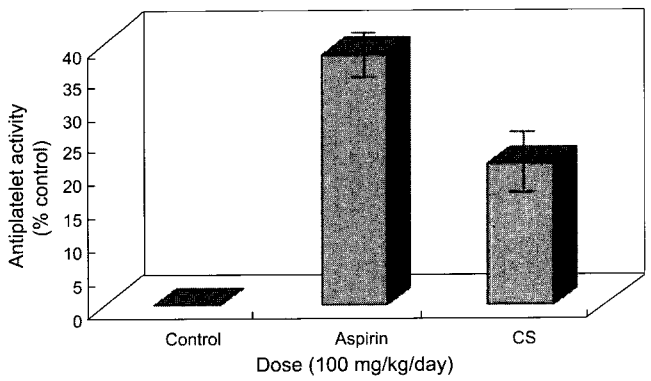

Fig. 5. *Ex vivo* antiplatelet activity of the CS and aspirin, a widely used antiplatelet agent.

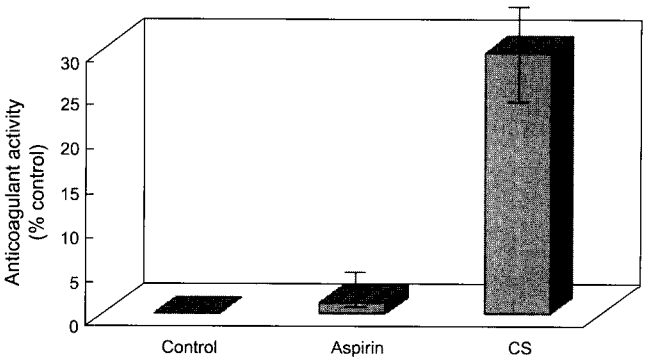

Fig. 6. Whole blood clotting time of the CS and aspirin, determined after 3-week treatment of rats.

high specificity (data not shown). Hydrolysis of fibrinogen by the CS might be attributed to the inhibition of platelet aggregation, because fibrinogen plays a key role in platelet aggregation by forming a complex with glycoprotein IIb-IIIa on the surface of platelets [1, 8, 26]. Many large molecular size proteins are readily inactivated *in vivo* when administrated orally; however, there are reports to show that oral administration of nattokinase resulted in the increased fibrinolysis *ex vivo*, which allowed the enzyme to be included in various health-promoting foods [34, 35]. It seemed, therefore, that the CS, like nattokinase, might have survived the gastric environment and exerted its fibrinolytic activity in rats.

The anticoagulant activity of the CS on the clotting time of whole blood was also determined *ex vivo* (Fig. 6). The blood clotting time of the rats fed with the CS was 289 seconds, which delayed by 30% of the control, while aspirin did not effectively inhibit the blood clotting. The observed anticoagulant activity of the CS might have resulted from the fact that fibrinogen, the substrate of thrombin, was hydrolyzed and thus lowered the formation of fibrin. These results may suggest that the anticoagulant and antiplatelet activity of the CS resulted via inhibition cascade of blood coagulation to influence thrombin-associated activity as well as fibrinogen-mediated platelet aggregation. Further investigation is necessary to confirm the hypothesis. It is, however, of great interest that the fibrinolytic CS induced the anticoagulant and antiplatelet activity *ex vivo*. These observations open the possibility of *E. faecalis* BRCA-5 or its metabolic products as an active component in functional foods.

Acknowledgments

This project was supported in part by grants from the Ministry of Science and Technology and from the Korea Science and Engineering Foundation through the Bioproducts Research Center at Yonsei University. The authors thank S. B. Kim and I. K. Wang at the R & D Center, Pulmuone, Co. Ltd. Helpful discussions with Dr. K. H. Chung at the Cardiovascular Center, Yonsei Medical Center are greatly appreciated.

REFERENCES

1. Asch, A. S., L. L. K. Leung, M. J. Polley, and R. L. Nachman. 1985. Platelet membrane topography: Colocalization of thrombospondin and fibrinogen with the glycoprotein IIb-IIIa complex. *Blood* **66:** 926–934.
2. Astrup, T. and S. Mullertz. 1952. The fibrin plate method for the estimation of fibrinolytic activity. *Arch. Biochem. Biophys.* **40:** 346–351.
3. Cardianl, D. C. and R. J. Flower. 1980. The electronic aggregometer: A novel device for assessing platelet behavior in blood. *J. Pharmacol.* **3:** 135–158.
4. Collen, D. and H. R. Lijnen. 2000. Recent developments in thrombolytic therapy. *Fibrinol. Proteol.* **14:** 66–72.
5. Davie, E. W. 1995. Biochemical and molecular aspects of the coagulation cascade. *Thromb. Haemost.* **74:** 1–6.
6. Diminno, G. and M. J. Silver. 1983. Mouse antithrombotic assay: A simple method for the evaluation of antithrombotic agents *in vivo*. Potentiation of antithrombotic activity by ethyl alcohol. *J. Pharmacol. Exp. Therap.* **225:** 57–60.
7. Furlan, M. (ed.). 1988. Structure of fibrinogen and fibrin, pp. 17–64. In J. L. Francis (ed.), *Fibrinogen, Fibrin Stabilization, and Fibrinolysis: Clinical, Biochemical and Laboratory Aspects.* Ellis Horwood, Ltd., New York, U.S.A.
8. Gogstad, G. O., F. Brosstad, M. B. Krutnes, I. Hagen, and N. O. Solum. 1982. Fibrinogen-binding properties of the human platelet glycoprotein IIb-IIIa complex: A study using crossed-radioimmunoelectrophoresis. *Blood* **60:** 664–671.
9. Holstrom, B. 1968. Production of streptokinase in continuous culture. *Appl. Microbiol.* **16:** 73–77.
10. Holt, J. G. 1984. *Bergey's Manual of Systematic Bacteriology.* Williams & Wilkins, Baltimore, MD, U.S.A.
11. Hricak, V. J., J. Kovacik, P. Marx, V. Fischer, and V. J. Kremery. 1998. Endocarditis due to *Enterococcus faecalis*: Risk factors and outcome in twenty-one cases from a five year national survey. *Scand. J. Infect. Dis.* **30:** 540–541.
12. Jacoby, G. A. 1995. Urinary tract infection with *Enterococcus faecalis*. *Ann. Intern. Med.* **122:** 238.
13. Kil, J. O., G. N. Kim, and I. Park. 1998. Production and characterization of fibrinolytic enzyme: Optimal condition for production of the enzyme from *Bacillus* sp. KP-6408 isolated from *Chungkook-jang*. *J. Korean Soc. Food Sci. Nutr.* **27:** 51–56.
14. Kim, H. K., G. T. Kim, D. K. Kim, W. A. Choi, S. H. Park, Y. K. Jeong, and I. S. Kong. 1997. Purification and characterization of a novel fibrinolytic enzyme from *Bacillus* sp. KA38 originated from fermented fish. *J. Ferm. Bioeng.* **84:** 307–312.
15. Kim, S. H. 1998. New trends of studying on potential activities of Doen-jang: Fibrinolytic activity. *Korea Soybean Digest* **15:** 8–15.

16. Kim, S. H. and N. S. Choi. 2000. Purification and characterization of subtilisin DJ-4 secreted by *Bacillus* sp. strain DJ-4 screened from *Doen-Jang. Biosci. Biotechnol. Biochem.* **64:** 1722–1725.
17. Kim, S. H., N. S. Choi, W. Y. Lee, J. W. Lee, and D. H. Kim. 1998. Isolation of *Bacillus* strains screening fibrinolytic enzymes from *Doenjang. Kor. J. Microbiol.* **34:** 87–90.
18. Kim, W. K., K. H. Choi, Y. T. Kim, H. H. Park, J. Y. Choi, Y. S. Lee, H. I. Oh, I. B. Kwon, and S. Y. Lee. 1996. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11-4 screened from Chungkook-Jang. *Appl. Environ. Microbiol.* **62:** 2482–2488.
19. Kim, Y. T., W. K. Kim, and H. I. Oh. 1995. Screening and identifications of fibrinolytic bacterial strains from *Chungkook-jang. Kor. J. Appl. Microbiol. Biotechnol.* **23:** 1–5.
20. Lee, S. K., S. Heo, D. H. Bae, and K. H. Choi. 1998. Medium optimization for fibrinolytic enzyme prodution by *Bacillus subtilis* KCK-7 isolated from Korean traditional Chungkookjang. *Kor. J. Appl. Microbiol. Biotechnol.* **26:** 226–231.
21. Lijnen, H. R. 2001. Elements of the fibrinolytic system. *Ann. NY Acad. Sci.* **936:** 226–236.
22. Milbrandt, E. 1998. A novel source of enterococcal endocarditis. *Clin. Cardiol.* **21:** 123–126.
23. Murano, G. 1978. The "Hageman" connection: interrelationships of blood coagulation, fibrino(geno)lysis, kinin generation, and complement activation. *Am. J. Hematol.* **4:** 409–417.
24. Naidu, A. S., W. R. Bidlack, and R. A. Clemens. 1999. Probiotic spectra of lactic acid bacteria (LAB). *Crit. Rev. Food Sci. Nutr.* **38:** 13–126.
25. National Institute of Health (NIH). 1985. *Guidelines for the Care and Use of Laboratory Animals.* U.S.A.
26. Nieuwenhuis, H. K., K. S. Sakariassen, W. P. M. Houdijk, P. M. Nievelstein, and J. J. Sixma. 1986. Deficiency of platelet membrane glycoprotein Ia associated with a decreased platelet adhesion to subendothelium: A defect in platelet spreading. *Blood* **68:** 692–698.
27. Noh, K. A., D. H. Kim, N. S. Choi, and S. H. Kim. 1999. Isolation of fibrinolytic enzyme producing strains from *kimchi. Korean J. Food Sci. Technol.* **31:** 219–223.
28. Ozawa, A., N. Ohnishi, S. Tazume, Y. Aiba, and K. Watanabe. 1986. Intestinal bacterial flora and host defense mechanisms. *Tokai J. Exp. Clin. Med.* **Suppl.:** 65–79.
29. Ozawa, K., K. Yabu-uchi, K. Yamanaka, Y. Yamashita, S. Nomura, and I. Oku. 1983. Effect of *Streptococcus faecalis* BIO-4R on intestinal flora of weanling piglets and calves. *Appl. Environ. Microbiol.* **45:** 1513–1518.
30. Ralf, K., N. Takamitsu, N. Jamamatsu, N. Yumiko, and T. Akikazu. 1995. Remmant-like lipoproteins stimulate whole blood platelet aggregation *in vitro. Thromb. Res.* **78:** 161–171.
31. Samama, M., G. Nguyen, E. Szwarcer, and J. Conard. 1985. Problems on t-PA specific inhibitor determination in human plasma. *Thromb. Haemost.* **154:** 726.
32. Smyth, C. J. and F. J. Fehrenbach. 1974. Isoelectric analysis of haemolysins and enzymes from streptococci of groups A, C and G. *Acta Pathol. Microbiol. Scand [B] Microbiol. Immunol.* **82:** 860–870.
33. Sumi, H. 1990. Development of nattokinase and healthy natto (in Japanese). *Bioindustry* **7:** 725–731.
34. Sumi, H., H. Hamada, K. Nakanishi, and H. Hiratani. 1990. Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. *Acta Haematol.* **84:** 139–143.
35. Sumi, H., H. Hamada, H. Tasushima, H. Mihara, and H. Muraki. 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.
36. Sumi, H., N. Nakajima, and C. Yatagai. 1995. A unique strong fibrinolytic enzyme (katsuwokinase) in skipjack "Shiokara", a Japanese traditional fermented food. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **112:** 543–547.
37. Sundsmo, J. S. and D. S. Fair. 1983. Relationships among the complement, kinin, coagulation, and fibrinolytic systems. *Springer Semin. Immunopathol.* **6:** 231–258.
38. Yoo, C. K., W. S. Seo, C. S. Lee, and S. M. Kang. 1998. Purification and characterization of fibrinolytic enzyme excreted by *Bacillus subtilis* K-54 isolated from Chung Guk Jang. *Kor. J. Appl. Microbiol. Biotechnol.* **26:** 507–514.
39. Zwierzina, W. D. and F. A. Kunz. 1985. A method of testing platelet aggregation in native whole blood. *Thromb. Res.* **38:** 91–100.