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Isolation and identification of an effective fibrinolytic strain *Bacillus subtilis* FR-33 from the Chinese *doufuru* and primary analysis of its fibrinolytic enzyme

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Fibrinolytic microorganisms were screened from the Chinese soy cheese *doufuru* as well as Japanese *natto* and Chinese *douchi*. Initially, 16 isolates with fibrinolytic activity were selected based on activity and cultural characteristics for further characterization. An exponential standard curve, covering 39 to 2500 IU/mL of urokinase activity, was well established with urokinase through the fibrin plate method. With fermentation and preliminary purification, the fibrinolytic activities of the crude enzymes of these isolates were estimated to range across 142 to 1833 IU/mL of urokinase through the standard curve using the thrombolytic area on the fibrin plate. The strain FR-33 from *doufuru* was chosen based on the maximum activity 1833 IU/mL and better reproducibility. Further purification of its crude enzyme through Sephadex G-50 gel filtration increased the enzyme activity to 2474 IU/mL, and the SDS-PAGE performance indicated that its molecular weight is approximately 30 kDa. The 16S rDNA of the isolate FR-33 was sequenced and analyzed, and the strain was identified as *Bacillus subtilis* based on the sequence combined with morphological, cultural, physiological and biochemical characteristics. However, in contrast to the typical *B. subtilis*, the strain FR-33 could grow in 9 and 12% NaCl, but could not secrete pyruvate dehydrogenase and urease, nor ferment lactose, sucrose and maltose. The crude enzyme of the isolate showed stronger activity than those reported from *natto* and *douchi*, and this makes it an attractive agent as a biomaterial for health-producing foods.

Key words: Doufuru, *Bacillus subtilis*, fibrinolytic enzyme, isolation, identification.

INTRODUCTION

Cardiovascular diseases, such as heart attack and stroke, are the leading causes of human death throughout the world. According to data provided by the World Health Organization in 2000, heart diseases are responsible for 29% of the total mortality rate in the world (World Health Organization, 2001). These cardiovascular diseases are mainly due to the accumulation of fibrin in the blood vessels. Fibrinolytic enzymes are agents that dissolve fibrin clots and have been discovered from both

food and non-food sources (Mine et al., 2005). Urokinase and tissue-type plasminogen activator (t-PA) are still widely used in thrombolytic therapy, but these agents have some undesirable side effects and are expensive (Wang et al., 2008).

A potent fibrinolytic enzyme (nattokinase, NK) was previously isolated from a traditional fermented food in Japan, *natto* (Sumi et al., 1987). This enzyme is an extracellular serine protease produced from *Bacillus*

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subtilis natto; it has been demonstrated that oral administration of *natto* or nattokinase capsules enhances fibrinolysis in canine plasma in an experimental thrombosis model (Sumi et al., 1990, 2004). As a result, fermented food products have been the focus of much research. During the past decades, several fibrinolytic enzymes were found in traditional fermented foods, such as Korean *Chungkook-jiang* soy sauce (Kim et al., 1996), edible honey mushrooms (Kim and Kim, 1999), and Chinese *douchi* (Peng et al., 2003; Wang et al., 2006).

The Chinese soy cheese *doufuru* is a popular fermented soybean food in China with over 1500 years of history. There has been little research for fibrinolytic microflora. In this study, the naturally occurring microflora of Chinese *doufuru*, together with Japanese *natto* and Chinese *douchi*, were screened for microorganisms based on the extent of fibrinolytic activity as well as its potential to be developed as an alternative thrombolytic agent. A strain screened from *doufuru*, FR-33, was found to have high fibrinolytic activity. This was identified as *Bacillus subtilis* on the basis of morphological and 16S rDNA sequence analysis although it has some unique physiological and biochemical characteristics. The fibrinolytic activity of the crude enzyme of the strain was equal to 1833 IU/mL of urokinase activity, and it reached 2474 IU/mL after Sephadex G-50 gel filtration. Its molecular mass was estimated to be approximately 30 kDa with SDS-PAGE.

MATERIALS AND METHODS

Three varieties of fermented foods, all purchased from local markets, were used for the screening of fibrinolytic enzyme-producing strains. The *doufuru* was manufactured by Zhongzhou Brewing Co., Ltd. (Zhongxian, Chongqing), *douchi* by Juxin Food Co., Ltd. (Jiangjin, Chongqing), and *natto* by Koishiya Food Co., Ltd. (Tochigi, Japan). Urokinase was produced by the Nanda Pharmaceutical Co., Ltd. (Nanjing, Jiangsu), lysozyme by Dingguo Biotechnology Co., Ltd. (Beijing), fibrinogen and thrombin by Sigma Chemical Co. (St. Louis, USA), Sephadex G-50 by GE Healthcare Co. (Sweden) and others, all in analytical grade and by local manufacturers in China.

Three kinds of bacterial growth media were prepared according to protocols (Sambrook and Russell, 2006). Casein medium: K_2HPO_4 0.1% (w/w), KH_2PO_4 0.05%, MgSO_4 0.01%, casein 0.2%, sucrose 0.1%, yeast extract 0.1%, agar 1.8 to 2.0%, CaCl_2 0.03%, pH 6.8 to 7.0. LB medium: yeast extract 0.5%, peptone 1.0%, NaCl 0.5%, pH 7.2 to 7.4. Fermentation medium: beef extract 0.3%, peptone 2.0%, NaCl 0.5%, pH 7.2 to 7.4.

Assay of fibrinolytic activity

The fibrinolytic activity was defined and expressed in units of urokinase activity (IU/mL). A standard curve of urokinase activity was plotted according to the fibrin plate method (Astrup and Mullertz, 1952) with slight modification for the estimation of fibrinolytic activity. In brief, 2 ml of fibrinogen solution (40 mg/ml) was mixed with 40 ml of 0.8% agar solution (w/v) in 20 mmol/L Tris-HCl buffer (pH 8.0) at 50°C, and 8 ml of this solution was poured into a Petri dish with 40 μl thrombin solution (80 NIH/ml thrombin in

20 mmol/L Tris-HCl, pH8.0). The dishes were allowed to stand for 30 min to form fibrin clots, and 5 holes (3 mm of diameter) were made on the fibrin dish.

Aliquots of 10 μl of urokinase at 39, 78, 156, 312, 624, 1250, 2500 and 5000 IU/mL were separately added in each hole and incubated at 37°C for 24 h, with two duplicates. The diameter of the cleared zone was measured and the thrombolytic circular area was calculated. The standard curve showing the relationship between urokinase activity (IU/mL) and thrombolytic circle area (mm^2) was established. The fibrinolytic activity of samples was determined using the curve through adding 10 μl of sample on each hole of the fibrin plate, incubating at 37°C for 24 h and measuring the lytic area.

Screening for fibrinolytic strains

About 1 g of sample was dissolved in 5 ml of sterilized normal saline (SS) and incubated at 200 rpm for 24 h at 37°C. 100 μl of supernatant was centrifuged at 10,000 rpm for 1 min and the precipitate was re-suspended in 1 ml of SS, followed by heating at 85°C for 5 min to remove undesired substances. 100 μl of supernatant was inoculated in 9.9 ml of SS, and an aliquot was separately diluted to 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} with SS. 100 μl of the aliquot for each concentration was then coated on casein medium and incubated at 37°C for 36 h. Single colonies with surrounding clear zones were considered fibrinolytic isolates, and those with a big fibrinolytic area were selected and cultivated separately on new casein medium. Six colonies of each sample food, with higher fibrinolytic activity, were confirmed and selected by repeating the process for several times and then preserving them in 70% glycerol.

The selected isolates were incubated at 180 rpm for 24 h at 37°C in 5 ml of LB medium, and 4% (v/v) of the medium was then inoculated to 100 ml of fermentation medium followed by incubation at 220 rpm for 36 h at 37°C. 10 μl of aliquot was spread on fibrin plate to assay the fibrinolytic activity. Strains from *natto* were designated "NT", *douchi* as "DC" and *doufuru* as "FR".

Purification and molecular weight estimation of fibrinolytic enzyme

Ammonium sulfate salting-out

The fermentation liquid was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was collected and adjusted to pH 7.0. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant containing the fibrinolytic enzyme to provide a 30% saturation. This mixture stood overnight at 4°C and was centrifuged at 10,000 rpm for 10 min at 4°C to remove particulate material. The supernatant was adjusted to pH 8.6 and 70% $(\text{NH}_4)_2\text{SO}_4$ saturation and held overnight at 4°C (Hao et al., 2002). The precipitate was collected by centrifugation at 10,000 rpm for 10 min at 4°C, and dissolved to 5 ml of PBS buffer (0.02 mol/l Na_2HPO_4 91.5 ml and 0.02 mol/l NaH_2PO_4 8.5 ml, pH 7.4). 10 μl of aliquot was loaded on a fibrin plate to assay the fibrinolytic activity.

Sephadex G-50 gel chromatography

1 ml of aliquot purified by ammonium sulfate salting-out was loaded onto a Biologic DuoFlow System column (BIO-RAD) equilibrated with PBS buffer, and then eluted with the same buffer at a flow rate of 0.2 ml/min (Yan and Xu, 2007). The active fraction was pooled and concentrated using a vacuum freeze dryer (Modulyo D-230) and the enzyme activity was measured and the recovery was calculated based on the description above (Astrup and Mullertz, 1952).

Figure 1. The standard curve showing the relationship between urokinase activity (Y) and thrombolytic circle area (X). A. Established based on the data of 39, 78, 156, 312 and 624 IU/mL of urokinase. B. Based on 39, 78, 156, 312, 624, 1250 and 2500 IU/mL.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed at room temperature for the concentrated enzyme to analyze purity and determine the molecular weight. A 12% polyacrylamide gel containing 1% SDS and bromophenol blue as a marker were used following the method of Laemmli (1970). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250.

Identification and characteristics of fibrinolytic strains

The morphological and cultural characteristics of the selected isolates were determined microscopically, while physiological and biochemical characteristics were tested according to Bergey's Manual of Systematic Bacteriology using API kits (Holt, 1984).

For molecular identification, genomic DNA was extracted from the cells of the selected isolate using a Sanprep extraction kit (Shenggong, China) according to the manufacturer's instructions. The 16S rDNA sequence was amplified by PCR using the forward primer 5'-CGTGGGCAACCTGCCTGTATGACA-3' and the reverse primer 5'-GCGTGGTCAAGCCAATCCCAT-3', which were designed based on homologous sequences collected from NCBI. This amplification produced 1444 bp of fragment and the amplified PCR product was analyzed on an agarose gel. The target fragments in gel were recovered and purified using a Sanprep recovery kit (Shenggong, China). The 16S rDNA was subsequently ligated to the vector pMD19-T (TaKaRa, Dalian, China), followed by the transformation of the *E. coli* JM109 with the recombinant pMD19-T. The 16S rDNA in the transformed cells was sequenced in both directions using the ABI PRISM 3100 Avant (Applied Biosystem) with general primers.

This sequence was deposited into GenBank and compared with its relatives in the database using BLAST through the NCBI server. A neighbor-joining tree was constructed using PAUP 4.0 (Swofford, 2001) and drawn using TreeView X. Node support was assessed using 1000 bootstrap pseudo-replicates. A species in the genus *Geobacillus*, *Geobacillus toebii* served as the outgroup due to sequence availability and affinity (Fritze, 2004).

RESULTS

The standard curve of urokinase activity

The applications of 10 µl of urokinase, at 39, 78, 156, 312, 624, 1250, 2500 and 5000 IU/mL, all produced clear fibrinolytic zones in the fibrin plates. The urokinase activity (IU/mL) and the lytic circle area (mm²), averaged with two duplicates, were highly linearly correlated in the range of 39 to 624 IU/mL ($R^2 = 0.9985$, $P < 0.001$) (Figure 1A). However, in the range of 39 to 2500 IU/mL, the activity and the area (mm²) were highly exponentially correlated ($R^2 = 0.9990$, $P < 0.001$) (Figure 1B). The later standard curve (Figure 1B) can be confidentially used for the estimation of fibrinolytic activity of samples. The data for 5000 IU/mL substantially deviated from the exponential curve; this indicated that the activity higher than 2500 IU/mL could not be measured.

Screening of fibrinolytic strains

In this study, 35, 28 and 19 colonies were preliminarily screened through casein medium from three representatives of fermented foods, *doufuru*, *douchi* and *natto*, respectively. All of them showed fibrinolytic activity. Based on the extent of the activity and cultural characteristics, a total of 18 strains with 6 for each kind of food, were chosen for further characterization (Table 1). With the fermentation of the strains and protein precipitation by ammonium sulfate, the fibrinolytic activity was estimated through the standard curve using the thrombolytic area on a fibrin plate (Table 1). A typical fibrin plate showing fibrinolytic activity by isolates is

Table 1. Selected isolates and their fibrinolytic activity.

Food source	Isolate	Fibrinolytic area (mm ²)	Fibrinolytic activity (IU/mL)
<i>natto</i>	NT-11	389	142
	NT-12	580	755
	NT-13	408	184
	NT-14	390	143
	NT-15	479	377
	NT-16	498	439
	Average	457	340
<i>douchi</i>	DC-21	641	1038
	DC-22	566	693
	DC-23	694	1317
	DC-24	457	312
	DC-25	407	182
	DC-26	436	255
	Average	534	633
<i>doufuru</i>	FR-31	511	486
	FR-32	687	1280
	FR-33	779	1833
	FR-34	715	1440
	FR-35	627	968
	FR-36	609	884
	Average	655	1148

presented in Figure 2. The 6 FR-strains selected showed the largest average fibrinolytic area of 655 mm² (Table 1) with the average fibrinolytic activity estimated to be 1148 IU/mL based on the standard curve of urokinase activity (Figure 1B). Following the FR-strains are the DC-strains, whose average fibrinolytic area was 534 mm², and the average fibrinolytic activity was estimated to be 633 IU/mL. The 6 NT-strains had the lowest average fibrinolytic area of 457 mm² that was equivalent to 340 IU/mL of urokinase. Among the six FR-strains, FR-33 produced the largest fibrinolytic area of 779 mm² and highest fibrinolytic activity at 1833 IU/mL. DC-23 produced the largest fibrinolytic area of 694 mm² and highest fibrinolytic activity of 1317 IU/mL among the 6 DC-strains. NT-12 produced the largest fibrinolytic area of 580 mm² and highest fibrinolytic activity at 755 IU/mL among the 6 NT-strains. As far as a single strain was concerned, FR-33 had the largest fibrinolytic activity among all investigated strains, followed by FR-34 (area 715 mm² and activity 1440 IU/mL) and DC-23 (area 694 mm² and activity 1317 IU/mL) (Table 1). The isolate FR-33 was chosen for further study.

Purification and molecular weight estimation of fibrinolytic enzyme

Sephadex G-50 gel chromatography produced by elution of the FR-33 crude enzyme is shown in Figure 3. Four

eluted protein peaks were observed on the chromatography, but only the fourth peak (the highest peak) (Figure 3) was found to have the thrombolytic activity on the fibrin plate while the others showed no activity. The fourth peak occurred at the elution time of 360 to 600 min, and was distinguished in subunit molecular weight from the other three enzymes.

The activity of the recovered fibrinolytic enzyme increased to 2474 IU/mL with a 1.35 time of purification and 66.33% recovery rate on the basis of the content in the crude enzyme (Table 2). The recovery protein of the purified fibrinolytic enzyme was subjected to SDS-PAGE and showed only one band. The molecular weight was estimated to be approximately 30 kDa (Figure 4).

Identification and characteristics of fibrinolytic strains

The morphological and cultural characteristics

The colonies of the strain FR-33 were oyster white, opaque, nearly circular and non-pigmented with the surface wrinkled, the edge irregular, and a diameter of 1.1 to 1.3 mm (Figure 5A). The single cell was a short rod with both ends rounded, spore-producing when observed with staining, the spore round or oval, mesic and with the sporangium not inflated (Figure 5B). These characteristics are basically consistent with Gram-

Figure 2. Fibrin plate showing clear zones resulting from the fibrinolysis of isolates: FR-33 (A), FR-34 (B), FR-35 (C), FR-36 (D) and negative control (E).

Figure 3. Sephadex G-50 gel filtration of the FR-33 plasminogen after salting-out with ammonium sulfate.

positive bacteria and further match *B. subtilis* based on Bergey's Manual of Systematic Bacteriology (Holt, 1984).

The physiological and biochemical characteristics

A comparison of characteristics is shown in Table 3. Generally, the strain FR-33 and *B. subtilis* share most of the physiological and biochemical characteristics investigated, but some differences do exist. Mainly, *B.*

subtilis can secrete pyruvate dehydrogenase and urease, and ferment lactose, sucrose and maltose to produce acid, but the strain FR-33 does not. On the other hand, the strain FR-33 could grow in 9 and 15% NaCl solution but *B. subtilis* cannot (Table 3). Molecular identification: The 16S rDNA of the strain FR-33 was successfully sequenced. It is 1444 bp long and has been deposited into GenBank with the accession number HQ009804. Using the 16S rDNA sequence of FR-33 as query, 100 sequences were retrieved from GenBank through BLAST

Table 2. Separation results of the fibrinolytic enzyme Sephadex G-50 gel chromatography.

Parameter	Crude enzyme	Eluent enzyme
Specific activity	1833 IU/mL	2474 IU/mL
Total quality	30 mg	19 mg
Recovery rate		63.33%
Purified time	1	1.35

Figure 4. SDS-PAGE of the purified fibrinolytic enzyme from *B. subtilis* FR-33. Left lane, protein molecular mass markers; right lane, eluted fibrinolytic enzyme through Sephadex G-50 gel filtration.

Figure 5. The morphology of colony (A) and single bacterium (B) of the strain FR-33.

using the NCBI server. All of these sequences have higher than a 90% identity with the queried sequence. 94 out of the 100 sequences were originally identified by the authors to belong to *B. subtilis*, while 4 belonged to *Bacillus licheniformis* (GQ93333.1, AY971527.2, DQ167473.1 and GQ926884.1), 1 to *Bacillus axarquiensis* (DQ993670.1) and 1 to *Pseudomonas* sp. (AM913904.1). The later 6 sequences were tentatively

compared with the 16S rDNA sequence collection of *B. licheniformis*, *B. axarquiensis* and *Pseudomonas* sp., respectively, and they were found not to be sourced from these species but from *B. subtilis*. A neighbor-joining tree was constructed with *Geobacillus toebii* as the outgroup to show the relationship of the strain FR-33 and its close relative isolates based on 1444 bp of 16S rDNA sequences (Figure 6). Among the known strains

Table 3. Physiological and biochemical characteristics of the strain FR-33 compared with *Bacillus subtilis*.

Experiment	Species or strain		Experiment	Species or strain	
	<i>Bacillus subtilis</i>	FR-33		<i>Bacillus subtilis</i>	FR-33
Gram stain	+	+	Urease	+	-
Aerobic	+	+	Motility	+	+
Casein hydrolysis	+	+	Citrate test	+	+
Indole test	+	+	NaCl 7%	+	+
V-P experiment	+	- ^a	9%	-	+
MR test	-	-	12%	-	+
H ₂ S	+	+	15%	-	-
NO ₃ ⁻ -NO ₂ ⁻	+	+	Lactose ^b	+	-
Gelatin liquefaction	+	+	Sucrose ^b	+	-
Litmus milk reduction	+	+	Maltose ^b	+	-
Starch hydrolysis	+	+	Mannitol ^b	+	+
Spore	mesic	mesic	Glucose ^b	+	+
Catalase test	+	+	Glucose ^c	-	-

^a+, yes, growth, presence or positive; -, No, no growth, absent or negative; ^bSugars fermentable to produce acid; ^cSugars fermentable to produce gas.

Figure 6. Neighbor-joining tree showing the relationship of the strain FR-33 and its close relatives inferred from the 16S rDNA (1444 bp). Bootstrap percentages of 1000 replicates are shown above the branches where they exceed 50%. Branch lengths are not arbitrary.

with 16S rDNA sequences, FR-33 has the closest affinity to *B. subtilis* strain N391 (DQ906100.1) with a 99.9% identity match for the 16S rDNA sequence.

DISCUSSION

Thrombosis is a major cause of death in the world, which has necessitated research in the field of thrombolytic therapy. Microorganisms are important sources to search for these thrombolytic agents with streptokinase, staphylokinase and nattokinase having been isolated from microorganisms as fibrinolytic agents. Nattokinase is of special interest, since it is produced by *B. subtilis natto*, a naturally occurring microorganism in fermented foods. Application of nattokinase in commercial food products has promoted scientists to screen various fermented foods for a new fibrinolytic strain and its enzyme. A number of investigations have been done for this purpose, and indeed some new fibrinolytic strains and their enzymes have been identified (Wang et al., 2008; Yoon et al., 2002). Different kinds of fermented foods and even different varieties of raw materials for fermented foods produce different activity from fibrinolytic enzymes (Luo et al., 2010).

The Chinese soy cheese *doufuru* is a traditional soybean-fermented food with a long history; however, there is little research on its fibrinolytic strains. Through first screening with casein medium, and further screening with fermentation and enzyme purification, the 6 strains from *doufuru* showed a much higher average fibrinolytic activity (1148 IU/mL of urokinase) than those from *douchi* (633 IU/mL) and *natto* (340 IU/mL). The “Zhongxian *doufuru*” is a very pleasing, popular and famous food in China, especially in Southwest China, and the *doufuru* is steeped in fermented sauce with salted condiments. This fermented sauce may be effective for cultivating strains with more effective fibrinolytic activity compared with *douchi* and *natto* used in the present study, which are consumed dry.

The highest fibrinolytic activity of strain isolated from *doufuru*, FR-33, has an activity equivalent to 1833 IU/mL of urokinase, whereas the highest activity of strains from *douchi* (DC-23) and *natto* (NT-12) are equivalent to 1317 and 755 IU/mL, respectively. Earlier, Bao et al. (2002), Yang and Zhang (2007) and Ma et al. (2007) each reported a strain from *natto* with a fibrinolytic activity of 786, 1033 and 1722.4 IU/mL, respectively (Wang et al., 2008; Yoon et al., 2002). Different kinds of fermented foods and even different varieties of raw materials for fermented foods produce different activity from fibrinolytic enzymes (Luo et al., 2010). Tan et al. (2009) isolated a strain from *douchi* with a fibrinolytic activity of 1225.5 IU/mL. The strain FR-33 isolated in the present study appears more effective than those reported based on fibrinolytic activity.

The Sephadex G-50 gel chromatography shows that the purification with ammonium sulfate is quite effective

for the enzyme produced from the fermentation of the strain FR-33. The further purification using Sephadex G-50 gel filtration can increase the enzyme activity to 2474 IU/mL. A single protein band with 30 kDa of molecular weight was detected on the SDS-PAGE, which indicates that the Sephadex G-50 purification is very good. The molecular weights of fibrinolytic enzymes have been reported to range from 20, 27, 28, 31 to 36 kDa (Yan and Tong, 2000; Li and Ran, 1999; Han and Zhang, 2000). The fibrinolytic enzyme of the strain FR-33 fits in the middle of the range reported.

Morphological, cultural and molecular characteristics all identified the strain FR-33 as *B. subtilis*. However, our strain showed unique properties. Unlike typical *B. subtilis*, the strain FR-33 can grow in 9 and 125% of NaCl solution, but can neither secrete pyruvate dehydrogenase and urease, nor ferment lactose, sucrose and maltose to produce acid. This may stem from a long history of adaptation and evolution in the high salted *doufuru*.

The crude enzyme of the isolate FR-33 showed a stronger activity than those reported from *natto* and *douchi*, and this makes it an attractive agent as a biomaterial for health-producing foods. Recently, *doufuru* is still mostly processed by the traditional natural fermentation at a small scale of production. The finding of the fibrinolytic strain *B. subtilis* FR-33 can help to improve the food through increasing its fibrinolytic activity as a functional *doufuru* food. The further study on the purification, characterization, expression and function of the enzyme from the strain FR-33 is underway in our laboratory.

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