JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Production of Fibrinolytic Enzyme from *Bacillus amyloliquefaciens* by Fermentation of Chickpeas, with the Evaluation of the Anticoagulant and Antioxidant Properties of Chickpeas

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ABSTRACT: To develop safe and cheap thrombolytic agents, a fibrinolytic enzyme productive strain of LSSE-62 was isolated from Chinese soybean paste. This strain was identified as *Bacillus amyloliquefaciens* by 16S rDNA sequence analysis. Nucleotide and amino acid sequence analysis showed that this fibrinolytic enzyme was identical to subtilisin DJ-4. Chickpeas were used as the substrate for fibrinolytic enzyme production from *B. amyloliquefaciens* in solid-state fermentation. Under the optimized conditions (34 °C and 50% initial moisture content), the fibrinolytic activity of fermented chickpeas reached 39.28 fibrin degradation units (FU)/g. Additionally, the fermented chickpeas showed anticoagulant activity, and the purified anticoagulant component showed higher anticoagulant activity than heparin sodium. After fermentation, the total phenolic and total flavonoid contents increased by 222 and 71%, respectively, and then the antioxidant activities were improved significantly. This study provided a novel method for the preparation of multifunctional food of chickpeas or raw materials for the preparation of functional food additives and potential drugs.

KEYWORDS: *Bacillus amyloliquefaciens*, chickpeas, fibrinolytic enzyme, anticoagulation, antioxidant

■ INTRODUCTION

Typical thrombolytic agents, including urokinase, streprokinase, and tissue plasminogen activator (t-PA), have been widely applied in clinical therapy. However, these drugs are expensive and have a short half-life after intravenous administration, and there is an excessive risk of hemorrhagic complication.[1,2] Therefore, a safer and cheaper fibrinolytic enzyme has been attracting more interests during these past few decades.

Several fibrinolytic enzymes are found from different food-grade microorganisms, such as nattokinase from *Bacillus subtilis*[3] and subtilisin DJ-4[4] and subtilisin DFE from *Bacillus amyloliquefaciens*.[5] These fibrinolytic enzymes show promise in the prevention and treatment of thrombosis for their advantages of safety and low price when used as functional food additives and potential drugs.[1] *In vivo*, nattokinase is effectively absorbed from the intestinal tract and induces fibrinolysis in plasma.[6] Oral administration of natto or nattokinase can enhance the fibrinolytic activity in plasma.[7] Natto produced by solid-state fermentation has been consumed as a functional food for a long time, and nattokinase extracted from natto has been applied as a functional ingredient, with the potential to be developed as a drug.[1] *B. amyloliquefaciens* species have been reported to produce enzymes with potent fibrinolytic activity.[5] However, the fibrinolytic enzyme related to functional food production by *B. amyloliquefaciens* in solid-state fermentation has not been reported, and much work needs to be performed extensively, such as the development of functional food and potential drugs.

Chickpea (*Cicer arietinum* L.) is one of the most important legumes in the world. Chickpea seeds are a cheap and good source of protein, carbohydrates, and minerals and are accepted as an important food for human and domestic animals in south Asia. Moreover, chickpeas have been applied as a Uighur traditional medicine in the Xinjiang province of China for a long time.[8] Additionally, chickpeas are rich in phenolic compounds, which have shown broad biological activities, especially antioxidant activity.[9] The intake of antioxidant-containing food is associated with a decreasing incidence of thrombus and cardiovascular diseases.[10,11] From fermentation with *B. subtilis*, the black soybeans showed the enhanced antioxidant activity and total phenolic content (TPC) and total flavonoid content (TFC).[11] *B. amyloliquefaciens* is a close relative to *B. subtilis* as a food-grade microorganism,[12] but no study has focused on enhancing antioxidants by fermentation with *B. amyloliquefaciens*. Herein, a fibrinolytic enzyme-producing strain was isolated from Chinese soybean paste and identified as *B. amyloliquefaciens* LSSE-62. Solid-state fermentation of chickpeas with *B. amyloliquefaciens* was investigated for the first time. The fibrinloytic enzyme production was optimized, and the anticoagulant activities as well as the antioxidant activities were evaluated.

■ MATERIALS AND METHODS

Samples and Chemicals. The chickpeas were purchased from Wushi Cereals, Oils Co., Ltd., Xinjiang, China. The soybean paste samples were collected from several farmers' markets in Beijing, China.

Received: December 26, 2010
Revised: February 26, 2011
Accepted: February 27, 2011
Published: March 10, 2011

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Fibrinogen and thrombin were purchased from Merck KGaA, Darmstadt, Germany. Heparin sodium was bought from BioDee BioTech Corporation, Ltd., Beijing, China. 1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Isolation and Identification of the Bacterium. The soybean paste samples were boiled for 10 min in distilled water (80 °C), and the suspension was spread out onto the fibrin plate solid medium containing 10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, and 12 g/L fibrin at pH 7.2. After incubation at 37 °C for 24 h, the colonies surrounded by a broad transparent zone were picked up, inoculated into liquid fermentation medium (10 g/L maltose, 8.28 g/L soy peptone, 0.74 g/L yeast extract, 0.64 g/L $CaCl_2 \cdot 2H_2O$, 1 g/L $K_2HPO_4 \cdot 3H_2O$, and 0.5 g/L $MgSO_4 \cdot 7H_2O$ at pH 7.2), and then cultured at 37 °C and 180 rpm for 48 h. The fibrinolytic activity was measured by the fibrin degradation assay in the following text. The highest productive strain was identified by 16S rDNA sequence analysis methods. Genomic DNA was prepared by the TIANamp Bacteria DNA kit (Tiangen Biotech, Beijing, China). The 16S rDNA was amplified using primers (27f, AGAGTTTGATCCTGGCTCAG) and (1492r, GGTTACCTTGTTACGACTT).[13] The polymerase chain reaction (PCR) was performed under the following conditions: 94 °C for 5 min, followed by 28 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1.5 min, and then final extension at 72 °C for 10 min. The nucleotide sequences were determined on an ABI Prism 370 automatic sequencer with 27f and 1492r primers (Applied Biosystems, Foster City, CA). The sequence was compared to the Genebank DNA database by the Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed with MEGA 4.0 software.

Cloning of the Fibrinolytic Enzyme Gene. The partial gene (open-reading frame sequence of fibrinolytic enzyme gene) was amplified using primer aprF (CCGTGAGAGGCAAAAAGGTATGGATCA) and primer aprR (ATTTACTGAGCTGCCGCCTGTACGTTG). The PCR was performed at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 45 s, 72 °C for 1 min, and a final extension step of 72 °C for 10 min. The amplified fragment was ligated into pUC19 vector and transformed into *Escherichia coli* DH5α. The nucleotide sequences were determined using M13F and M13R universal sequencing primers with an ABI Prism 370 automatic sequencer (Applied Biosystems, Foster City, CA).

Optimization of Fibrinolytic Enzyme Production Conditions. Prior to fermentation, the chickpeas were washed and soaked in tap water at room temperature for 10 h. After the water was decanted, the chickpeas (20 g) in 250 mL flasks were autoclaved at 115 °C for 30 min. The *B. amyloliquefaciens* LSSE-62 cells were inoculated into 10 mL of Luria-Bertani (LB) medium. After cultivation at 37 °C at 200 rpm for 12 h, the cells (5%, v/v) were inoculated into the steamed chickpea seeds for static fermentation. First, the fermentation was conducted at different temperatures (31, 34, 37, and 40 °C) to select the optimal temperature. Because the optimal fibrinolytic enzyme production was obtained at 34 °C, the effect of the initial moisture content [50, 55, 60, 65, and 70% (w/w)] was investigated at 34 °C.

Determination of Fibrinolytic Activity. The fermented chickpeas were extracted with distilled water (1:10, w/v) by shaking at 200 revolutions/min for 1 h, and the supernatant was used as an enzyme sample after centrifugation at 4000*g* for 20 min. Fibrinolytic activity was determined by the fibrin degradation assay adapted by the Japan NattoKinase Association and the Japan Health Food and Nutrition Food Association (http://j-nattokinase.org/jnka_nk_english.html). A total of 1.3 mL of Tris-HCl (50 mM, pH 7.8) and 0.4 mL of fibrinogen solution (0.72%, w/v) were placed in a test tube and incubated at 37 °C for 5 min. Then, 0.1 mL of thrombin solution (20 units/mL) was added and kept at 37 °C for 10 min. A total of 0.1 mL of enzyme sample was added, incubated at 37 °C for 60 min, and mixed at 20 and 40 min. At 60 min, the reaction was ended by the addition of 2 mL of trichloroacetic acid (0.2 M). After centrifugation of the reaction mixture at 4000*g* for 20 min, the absorbance of the supernatant was measured at 275 nm. A total of 1 unit (fibrin degradation unit, FU) of enzyme activity is defined as the amount of the enzyme producing a 0.01 min^{-1} increase in absorbance at 275 nm.

Determination of Viable Cells. Aliquots of samples were drawn and diluted at preset time intervals, and the dilution was spread out onto the LB solid medium containing 10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl at pH 7.2.

Measurement of the Anticoagulant Activities. First, fermented chickpea and non-fermented steamed chickpea samples were homogenized and extracted with distilled water (1:10, w/v) by shaking at 20 °C for 12 h. The extracts were filtered, vacuum-concentrated, and freeze-dried, as were the water extracts. Strain LSSE-62 was also cultured in conventional LB medium at 180 rpm for 48 h, and the culture supernatants were collected after centrifugation at 4000*g* for 20 min and freeze-dried for the determination of the anticoagulant activities. To compare the anticoagulant component of fermented chickpeas to the common anticoagulant drug heparin sodium, the anticoagulant component was purified by $(NH_4)_2SO_4$ fractional precipitation. The unbroken fermented chickpeas were extracted with distilled water (1:10, w/v) at 200 revolutions/min for 1 h. After centrifugation at 4000*g* for 20 min, the liquid supernatant was added to 24% (w/v) $(NH_4)_2SO_4$ and kept at 4 °C for 1 h. After separation of the precipitate at 4000*g* for 20 min, another 16% (w/v) $(NH_4)_2SO_4$ was added to the supernatant and the mixture was kept at 4 °C for 1 h. Then, the precipitate was collected by centrifugation at 4000*g* for 20 min. The precipitate was dialyzed (molecular weight cutoff of 3500) and freeze-dried as the purified anticoagulant component. The qualitative analysis of protein extracts was performed by the biuret reaction.[14]

The anticoagulant activities were evaluated by determining the inhibitory ability on coagulation of fibrinogen, with minor modification.[15] Tris-HCl buffer (0.05 M, pH 7.2) was used as the solvent for fibrinogen, thrombin, and the extract samples. First, 1.5 mL of fibrinogen solution (0.1%) and 1 mL of extract sample solution were mixed in cuvettes. Then, 0.1 mL of thrombin solution (20 international units/mL) was added to the cuvettes. After incubation at 37 °C for 20 min, the absorbance at 405 nm was read. The inhibitory effects were calculated according to the following formula:

$$\text{scavenging effect } (\%) = [1 - \text{absorbance of sample/absorbance of control}] \times 100\%$$

Determination of the TPC and TFC. The fermented and non-fermented steamed chickpeas were homogenized and extracted twice with 80% methanol (1:10, w/v) by shaking at 30 °C for 24 h. The extracts were filtered, vacuum-concentrated, and freeze-dried. The TPC was determined according to a reported method, with minor modification.[11] A total of 0.2 mL of extract was mixed with 0.2 mL of Folin−Ciocalteau phenol reagent (1 N) (Sigma Aldrich Co., St. Louis, MO) and reacted for 3 min, and then 0.4 mL of Na_2CO_3 (1 N) was added. After incubation for 90 min at room temperature, 2 mL of deionized water was added and absorbance was measured at 725 nm. The results were expressed as milligrams of gallic acid equivalent per gram of dry chickpea weight (mg of gallic acid/g). The TFC was determined using ultraviolet (UV) spectrophotometry, and rutin was used as a standard.[16] Extracts were diluted with distilled water and the absorbance was measured at 258 nm. The results were expressed as milligrams of rutin equivalent per gram of dry chickpea weight (mg of rutin/g).

Determination of Antioxidative Activity. The antioxidant activity of chickpea extracts was evaluated by measuring DPPH free-radical-scavenging activity.[11] A total of 2.5 mL of 75 μM DPPH solution and 0.5 mL of sample with different concentrations were mixed. After incubation at ambient temperature for 90 min, the absorbance was measured at 517 nm. The DPPH free-radical-scavenging activity was

calculated as follows:

$$\text{scavenging effect }(\%) = [1 - \text{absorbance of sample/absorbance of control}] \times 100\%$$

Statistical Analyses. Each experiment was performed at least in triplicate. The *t* test was used to analyze the difference in means at the 95% confidence level. All statistical analyses were performed using Statistica 6.0 software package.

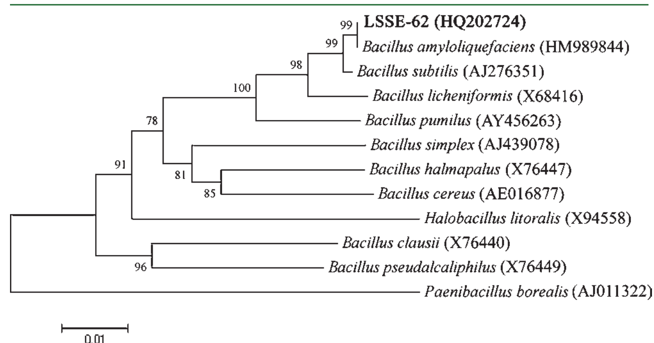

Figure 1. 16S rDNA-based phylogenetic trees showing the position of strain LSSE-62 (HQ202724) among its closely related organisms. Numbers in parentheses are accession numbers of published sequences. The scale bar represents 0.01 nucleotide substitution per position.

RESULTS AND DISCUSSION

Isolation and Identification of the Bacterium. Typical thrombolytic agents (urokinase and tissue plasminogen activator) lead to undesirable side effects when orally administrated, such as excessive risk of hemorrhagic complication within the intestinal tract.[1] Traditional fermented food, such as Japanese natto, has been consumed for a long time, and nattokinase produced by *B. subtilis* isolated from natto has been proven to be safe for oral administration (without hemorrhagic complication).[1] Accordingly, present fibrinolytic enzyme productive strains were mainly isolated from traditional fermented foods, such as Japanese natto,[3] Korean doen-jang,[4] and Chinese douchi.[5] In the present study, another Chinese traditional fermented food, soybean paste, was collected as the new food source for screening of the fibrinolytic enzyme-producing bacterium.

After the transparent zone plate screening, nine strains with large transparent zone were used for liquid fermentation. The most productive strain LSSE-62 showed fibrinolytic activity of 2.53 FU/mL, which was as high as the newly reported fibrinolytic enzyme productive strain, *Pseudomonas* sp. TKU015.[17] The 16S rDNA sequence of strain LSSE-62 [1414 base pairs (bp)] was submitted to the GeneBank database (HQ202724). It showed 99.9% similarity to that of *B. amyloliquefaciens* BFE 5322

Figure 2. Nucleotide (upper line) and deduced amino acid (lower line) sequences of the fibrinolytic enzyme from *B. amyloliquefaciens* LSSE-62. The predicted signal peptide, pro-peptide, and mature peptide (mature) were marked with arrows.

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              Pre                                  Pro
LSSE-62   MRGKKVWISL LFALALIFTM AFGSTSPAQA AGKSNGEKKY IVGFKQTMST MSAAKKKDVI -47
DJ-4      .......... .......... .......... .......... .......... ..........
DFE       .......... .......... .......... .......... .......... ..........
NAT       ..S..L.... ....T..... ..SNM.-... ....ST.... .........A ..S.......
BSF1      ..S..L.... ....T..... ..SNM.-... ....ST.... .........A ..S.......
                                                            Mature
LSSE-62   SEKGGKVQKQ FKYVDAASAT LNEKAVKELK KDPSVAYVEE DHVAQAYAQS VPYGVSQIKA 13
DJ-4      .......... .......... .......... .......... .......... ..........
DFE       .......... .......... .......... .......... .......... ..........
NAT       .......... ....N..A.. .D........ .......... ..I.HE.... ....I.....
BSF1      ......I... ....N..T.. .......... Q......... ..I.HE.... ....I.....

LSSE-62   PALHSQGFTG SNVKVAVIDS GIDSSHPDLK VAGGASMVPS ETNPFQDNNS HGTHVAGTVA 73
DJ-4      .......... .......... .......... .......... .......... ..........
DFE       .......... .......... .......... .......... .......... ..........
NAT       .......Y.. .......... .........N .R...F.... ....Y..GS. ........I.
BSF1      .......Y.. .......... .........N .R...F.... ....Y..GS. ........I.

LSSE-62   ALNNSVGVLG VAPSASLYAV KVLGADGSGQ YSWIINGIEW AIANNMDVIN MSLGGPSGSA 133
DJ-4      .......... .......... .......... .......... .......... ..........
DFE       .......... .......... .......... .......... .......... ..........
NAT       .....I.... .......... ...DST.... .......... ..S....... ......T..T
BSF1      .....I.... ...N...... ...DST.... .......... ..S....... .........T

LSSE-62   ALKAAVDKAV ASGVVVVAAA GNEGTSGGSS TVGYPGKYPS VIAVGAVNSS NQRASFSSVG 193
DJ-4      .......... .......... .......... .......... .......... ..........
DFE       .......... ...I...... .......S.. .......... .......... ..........
NAT       ...TV..... S..I.A.... ....S..ST. .....A.... T......... ..........
BSF1      ...TV..... SN.I.A.... ....S..ST. .....A.... T......... ........A.

LSSE-62   SELDVMAPGV SIQSTLPGNK YGAYNGTSMA SPHVAGAAAL ILSKHPNWTN TQVRSSLENT 253
DJ-4      .......... .......... .......... .......... .......... ..........
DFE       .......... .......... .......... .......... .......... ..........
NAT       .......... ........GT .......... T......... ......T... A...DR..S.
BSF1      P......... ........GT .......... T......... ......T... A...DR..S.

LSSE-62   TTKLGDAFYY GKGLINVQAA AQ 275
DJ-4      .......... .......... ..
DFE       .......... .......... ..
NAT       A.Y..NS... .......... ..
BSF1      A.Y..NS... .......... ..
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Figure 3. Amino acid sequence alignment of the fibrinolytic enzyme from *B. amyloliquefaciens* LSSE-62, with subtilisin (DJ-4, DFE) from *B. amyloliquefaciens* and subtilisin (NAT, BSF1) from *B. subtilis*. The signal peptide, pro-peptide, and mature peptide (mature) were indicated with arrows. "." indicated the same residue as the first sequence. The catalytic center residues (Asp-32, His-64, and Ser-221) were boxed. The initial amino acid of the mature peptide was numbered as +1.

(GU250445). The subsequently constructed phylogenetic tree showed that the strain LSSE-62 was tightly clustered with *B. amyloliquefaciens* among its closely related bacteria (Figure 1). From these results, the strain was identified as *B. amyloliquefaciens*. The strain was deposited at the China General Microbiological Culture Collection Center (CGMCC, 4157).

Identification of the Fibrinolytic Enzyme. To identify the fibrinolytic enzyme of *B. amyloliquefaciens* LSSE-62, the partial fibrinolytic enzyme gene was characterized according to the previous report.[18] First, a pair of primers was designed on the basis of the open-reading frame sequence of subtilisin DJ-4 gene (AY627764) and subtilisin DFE gene (DQ132806) from *B. amyloliquefaciens*.[19,20] Then, the gene was cloned and sequenced (1149 bp). On the basis of sequence alignment by the Blast program, the gene sequence (HQ419279) showed 99.7% similarity to that of subtilisin DJ-4, 99.4% similarity to that of subtilisin DFE, and 79.8% similarity to that of subtilisin NAT (S51909) and fibrinolytic enzyme BSF1 (FJ517584) from *B. subtilis*.[19−22]

The gene sequence was translated into an amino acid sequence (382 amino acids) by BioEdit software. When this amino acid sequence was compared to other reported sequences, the amino acid sequence showed a predicted signal peptide of 30 amino acids (pre-sequence), a pro-peptide of 77 amino acids, and a mature peptide of 275 amino acids (Figure 2), which were identical to that of subtilisin DJ-4 and subtilisin DFE and different from subtilisin NAT and fibrinolytic enzyme BSF1 (pre-sequence of 29 amino acids, pro-peptide of 77 amino acids, and mature peptide of 275 amino acids). As shown in Figure 3, the amino acid sequence of LSSE-62 showed 100% identity to subtilisin DJ-4, 99.5% identity to subtilisin DFE, 85.3% identity to subtilisin NAT, and 84.3% identity to fibrinolytic enzyme BSF1.[19−22] The mature peptides of these sequences contained the same conserved catalytic triad of Asp-32, His-64, and Ser-221 (Figure 3), suggesting that these fibrinolytic enzymes were the members of the subtilisin family of serine proteases.[23]

Fibrinolytic Enzyme Production. The culture temperature and initial moisture are the key factors for solid-state fermentation. First, the fibrinolytic enzyme production by solid-state fermentation of chickpeas was carried out at different culture temperatures with an initial moisture content of 55%. Figure 4a showed that the maximum fibrinolytic activity reached 35.98 FU/g at 34 °C. Then, we investigated the effect of the initial moisture content on the fibrinolytic eynzyme production at 34 °C. The fibrinolytic activity decreased as the initial moisture increased; therefore, the optimal initial moisture content was 50% (Figure 4b). Effects of additional nutrients (such as maltose, soy peptone, yeast extract, and calcium chloride) on fibrinolytic enzyme production were also evaluated, but there was no marked difference with various concentrations (data not shown). Chickpeas were rich in protein, carbohydrates, and minerals, and the above results showed that the nutrients were adequate for fibrinolytic enzyme production.

Under the optimized fermentation conditions (34 °C and 50% moisture content), the time profile of cell growth and fibrinolytic enzyme production was investigated. Correlation analysis

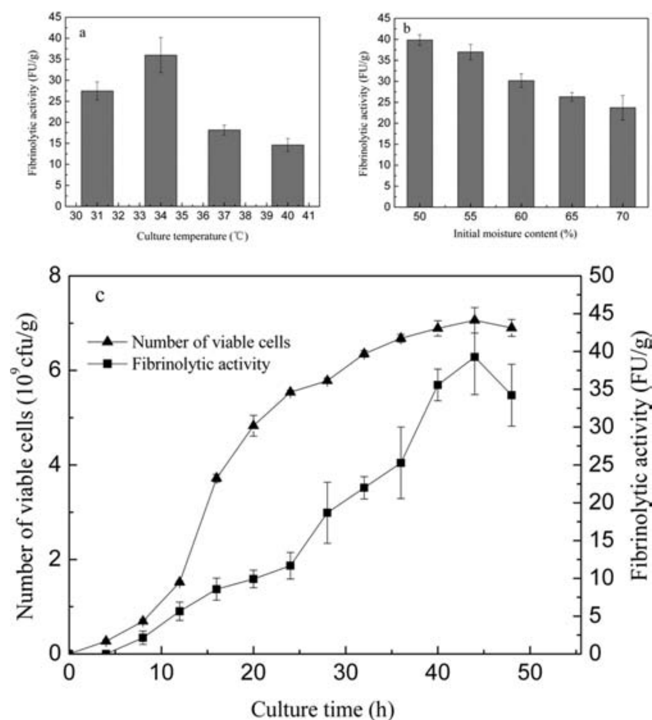

Figure 4. Fibrinolytic enzyme production. (a) Effect of the culture temperature on fibrinolytic activity. (b) Effect of the initial moisture content on fibrinolytic activity. (c) The time profile of cell growth and fibrinolytic activity during fermentation.

(R = 0.89; p < 0.01) showed that the fibrinolytic enzyme was biosynthesized along with the cell growth. The highest fibrinolytic enzyme activity (39.28 FU/g) was attained when the maximum viable cell number reached 7.06 × 10^9 colony-forming units (cfu)/g (Figure 4c). The above results showed that the fibrinolytic enzyme production was growth-associated, which is in accordance with the fibrinolytic enzyme production by *B. subtilis*.[24] In liquid fermentation with *Pseudomonas* sp. TKU015 using shrimp shells as substrate, the maximum fibrinolytic enzyme (nattokinase) activity reached 2.3 FU/mL.[17] Generally, commercially available fibrinolytic enzyme (nattokinase) functional food showed fibrinolytic activity of 20−40 FU/g.[25] Therefore, our results showed that solid-state fermentation of chickpeas with *B. amyloliquefaciens* LSSE-62 was effective for fibrinolytic enzyme production and the fibrinolytic enzyme activity of fermented chickpeas achieved the commercial level of natto food.

Anticoagulant Activities. Thrombin-catalyzed coagulation of fibrinogen (thrombus) consists of three steps: fibrinogen releases fibrin monomers; fibrin monomers form intermediate polymers; and intermediate polymers form the fibrin. Thrombin is involved in the first step, which is a limited proteolysis to release fibrinopeptides to form fibrin monomers.[26] The inhibitory effect of extracts on thrombin-catalyzed coagulation of fibrinogen was investigated to evaluate the anticoagulant activities. First, the anticoagulant activities of the water extracts from unfermented and fermented chickpeas were measured. The water extracts of fermented chickpeas obviously inhibit the coagulation of fibrinogen, and the inhibitory ability was enhanced as the extract content increased in a dose-dependent manner (Figure 5a). On the contrary, the water extracts of unfermented chickpeas with various concentrations showed no inhibitory effects. Meanwhile, the culture supernatants from LSSE-62 in LB medium also showed potent anticoagulant effects in a dose-dependent manner (Figure 5a). These results indicated that the anticoagulant component was produced by *B. amyloliquefaciens* LSSE-62 and not the modification or release of some product from chickpeas. To the best of our knowledge, this is the first report that *B. amyloliquefaciens* can biosynthesize the anticoagulant component.

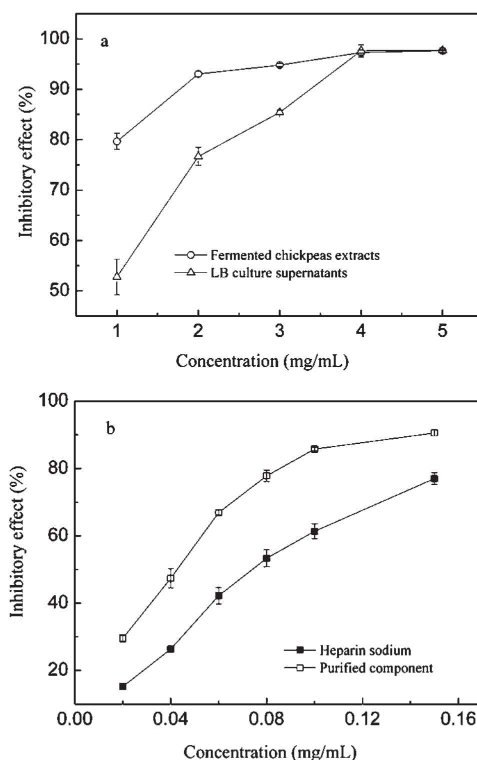

Figure 5. Anticoagulant activity. (a) Inhibitory effect of water extracts of fermented chickpeas and LB culture supernatants on fibrinogen coagulation. (b) Inhibitory effect of purified component and heparin on fibrinogen coagulation.

Afterward, the anticoagulant component was initially purified to compare to the anticoagulant drug of heparin sodium. The purified component showed a higher inhibitory effect compared to heparin sodium (Figure 5b) (t = 9.6192; p = 0.0002). The half-inhibition concentration (IC_{50}), which was the efficient concentration required to result in a 50% inhibitory effect of coagulation of fibrinogen, was calculated according to the data shown in Figure 5b. Under our measurement conditions, the IC_{50} of the purified anticoagulant component reached 0.043 ± 0.002 mg/mL, which was significantly lower than that of heparin sodium (0.074 ± 0.004 mg/mL) by t test analysis (t = 12.4276; p = 0.0002). In clinical practice, all of the anticoagulant agents (such as heparin sodium, warfarin, and dicumarol) were not safe regarding the adverse effect of major hemorrhage.[2] The NKCP protein extracts, derived from *B. subtilis* natto fermentation, possessed anticoagulant and fibrinolytic activity.[27] This protein was proven to be safe after toxicity tests and preliminary clinical trials with human volunteers. Therefore, the anticoagulant substance derived from food microorganism *B. amyloliquefaciens* LSSE-62 could be safe.

Table 1. TPC and TFC of Non-fermented and Fermented Chickpeas

samples	TPC (mg of gallic acid/g)	TFC (mg of rutin/g)
non-fermented chickpea	0.85 ± 0.02	4.86 ± 0.10
fermented chickpea	2.74 ± 0.02	8.33 ± 0.32

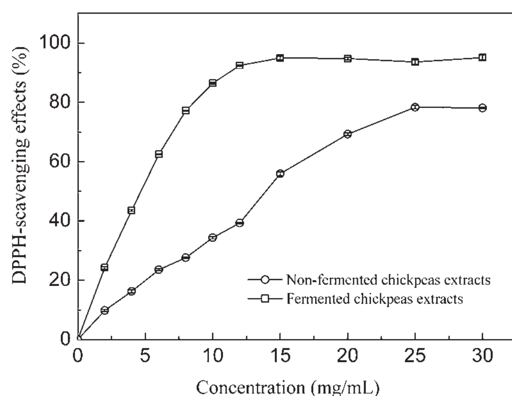

Figure 6. DPPH radical-scavenging effect of the various concentration extracts of non-fermented and fermented chickpeas.

The anticoagulant component was purified by $(NH_4)_2SO_4$ fractional precipitation, which was the universal method for protein extraction. As a close relative to *B. subtilis*, the anticoagulant component from *B. amyloliquefaciens* may be a protein, and the positive reaction in the biuret reaction also indicated that this anticoagulant component contained protein. These results indicated that this anticoagulant component might be a protein, and the further characterization of this anticoagulant agent is underway. Herein, a new method to enrich the fermented chickpeas food with anticoagulant activity was developed, and the anticoagulant activity will enhance the bioactive effect of fermented chickpeas as functional food for the prevention or treatment of thrombus diseases.

TPC and TFC. As shown in Table 1, the TPC of non-fermented chickpeas reached 0.85 mg of gallic acid/g, which agreed with those results reported for different chickpeas.[9,28] Fernandez-Orozco et al.[29] found that chickpea fermentation with *Lactobacillus plantarum* or with natural fermentation increased TPC by about 200−311%. After fermentation with *B. amyloliquefaciens* LSSE-62, the chickpea showed a TPC of 2.74 mg of gallic acid/g, an increase of 222%. Flavonoids are the predominant components of phenolics. Fermentation with *B. amyloliquefaciens* LSSE-62 also resulted in 71% enhancement of the TFC, ranging from 4.86 to 8.33 mg of rutin/g. Our results showed that fermentation with *B. amyloliquefaciens* LSSE-62 was an effective method for enhancement of TPC and TFC. In plant materials, phenolics (total phenolic and total flavonoid) usually exist in conjugated forms with sugar and glycosides through hydroxyl groups.[30] In black soybean fermentation with *B. subtilis* or fungi, β-glucosidase secreted by microorganisms is considered to hydrolyze the conjugated forms for the release of the total phenolics and flavonoids.[11,31] *B. amyloliquefaciens* is a close relative to *B. subtilis*, and the improved TPC and TFC in the present study may be due to the release of phenolics from chickpeas by β-glucosidase.[11]

In the human diet, the phenolic compounds are the major antioxidants, which possess diverse biological activities, such as cardiovascular protection, antithrombotic activity, anti-atherosclerosis, anti-aging, and anticancer.[32] It can be concluded that administration of fermented chickpeas will contribute to the prevention or treatment of these diseases.

DPPH Radical-Scavenging Effect. Measurement of the DPPH free-radical-scavenging ability has been broadly used in evaluating the antioxidant activity in a short time. Figure 6 showed the dose−response curves for DPPH radical-scavenging effects of various concentration extracts of unfermented and fermented chickpeas. For each extract, the DPPH-scavenging effect was enhanced as the extract content increased until it reached a certain threshold, and the extract from fermented chickpeas showed significantly higher scavenging ability compared to that of unfermented chickpeas at the same dosage. The maximum scavenging effect reached 94.92% at 15 mg/mL of extract of fermented chickpeas, while the highest value was just 78.33% at 25 mg/mL of extract of unfermented chickpeas.

By linear regression analysis of the data of Figure 6, the half-inhibition concentration (IC_{50}), which is the efficient concentration required to result in a 50% decrease of the initial DPPH concentration,[31] was also calculated. The IC_{50} (4.79 ± 0.02 mg/mL) of the fermented chickpea extracts was significantly lower than that of the unfermented chickpea extracts (15.20 ± 0.35 mg/mL) by t test analysis (t = 51.4088; p = 0.0004), indicating that the antioxidant activity of chickpeas was enhanced by fermentation with *B. amyloliquefaciens* LSSE-62. Similar results were also reported in other legume fermentations with *B. subtilis* and some fungi.[11,31] The TPC and TFC of food is directly associated with the antioxidant activity.[9] Therefore, the increased antioxidative activity of chickpeas may be due to the enhancement of TPC and TFC coming from fermentation with *B. amyloliquefaciens* LSSE-62.

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Funding Sources

This work was funded by the Innovative Research Group Science Fund (Y020308133).

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