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Fibrinolytic characteristics of *Bacillus subtilis* G8 isolated from natto

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Due to the high prevalence of vascular obstructive diseases, discovering potent, safe, and affordable fibrinolytic agents is of importance. There is particular interest concerning the use of functional foods that have a fibrinolytic activity, such as natto, a Japanese fermented soy-based product made with *Bacillus subtilis* (natto) strain BEST195. We recently isolated another bacterial strain from natto commercialized in Indonesia, *B. subtilis* G8, which has proven to exert fibrinolytic activity. Herein, a further characterization of *B. subtilis* G8 was assessed through a comparison with commercialized nattokinase, the major fibrinolytic enzyme of *B. subtilis*, by utilizing various *in vitro* fibrinolytic assays, namely whole blood clot lysis, euglobulin clot lysis, the fibrin plate method, and zymography. Both nattokinase and *B. subtilis* G8 were able to dissolve both whole blood and euglobulin clots. Furthermore, both nattokinase and *B. subtilis* G8 were able to lyse blood clots, presumably due to their ability to directly lyse fibrin. Finally, a crude extract of *B. subtilis* G8 displayed six zymogram bands of approximately 42.0, 35.5, 30.8, 26.7, 20.0, and 13.7 kDa, with the strongest activity observed at 20.0 kDa. This indicates that *B. subtilis* G8 contained several fibrinolytic enzymes, which might have comprised nattokinase and other fibrinolytic enzymes. In summary, we demonstrated that a crude extract of *B. subtilis* G8 has potent fibrinolytic activity and that the activity was mediated by various fibrinolytic enzymes.

Key words: *Bacillus subtilis* G8, nattokinase, fibrinolysis, vascular obstructive disease

INTRODUCTION

Unwanted thrombus formation is the root cause of various lethal vascular obstructive diseases, including venous thrombosis, obstructive coronary artery disease, and ischemic stroke. In healthy homeostasis, blood clotting events are kept in check by fibrinolytic activity, which prevents it from obstructing blood flow. Excessive or prolonged blood coagulation or disruption in the fibrinolytic system, however, results in unwanted thrombosis [1]. This emphasizes the importance of fibrinolytic agents that could be used for pharmacologic dissolution of a thrombus as a cure for such vascular obstructive diseases [1].

In the search for safe and cost-effective fibrinolytic agents, functional foods made of microbes with fibrinolytic activity are of interest. Among them, natto, a Japanese cheese-like traditional food made of soybeans that are fermented with *Bacillus subtilis* (natto) strain BEST195 [2], is of interest, as it is widely consumed in Asia [3] and is associated with a reduced risk of cardiovascular diseases [4]. Nattokinase, an enzyme secreted by *B. subtilis*, has been proven to exhibit fibrinolytic activity, in addition to its other beneficial effects, like anti-coagulation, anti-atherosclerosis, anti-hypertension, and neuroprotection

[5, 6]. Several published studies support the plausibility of dietary proteins within the intestinal lumen being absorbed with their functional structures undisturbed/intact. Two proposed mechanisms for this are microfold/M cell-mediated transcytosis [7] and goblet cell-associated antigen passage [8]. In the case of nattokinase, a pilot human study demonstrated that upon ingestion of a single capsule of nattokinase containing 2,000 FU, the highest detectable levels of this enzyme in the blood were noticed at approximately 13 hours post-consumption [9]. It has also been reported that those subjects who ingested capsules of nattokinase experienced certain nattokinase-associated health benefits, including control of blood pressure [10] and activation of fibrinolysis [11].

Other strains of *B. subtilis* have been used in various healthy, fermented soy-based products, such as *B. subtilis* DC33 in Chinese douchi [12] and *B. subtilis* CK 11-4 in Korean chungkook-jang [13]. Of note, the fibrinolytic activity of those strains is not mediated by nattokinase, suggesting that there might be other microbial enzymes that could be screened as candidates for fibrinolytic agents. In line with this notion, we recently isolated another strain of *B. subtilis* from natto commercialized in Indonesia, named *B. subtilis* G8 [14]. A phylogenetic tree

analysis, based on the 16s rRNA sequences, suggested that *B. subtilis* G8 has 99% similarity to *B. subtilis* (natto) strain BEST195 [14]. Importantly, this strain has been demonstrated to exert fibrinolytic activity as well [14].

In order to better characterize the fibrinolytic activity of *B. subtilis* G8, this study compared a crude extract of *B. subtilis* G8 with a well-described, commercialized nattokinase in standardized assays comprising whole blood clot lysis, euglobulin clot lysis, the fibrin plate method, and zymography. We report that the crude extract of *B. subtilis* G8 indeed had potent fibrinolytic activity and that the activity was mediated by various fibrinolytic enzymes.

MATERIALS AND METHODS

B. subtilis G8 strain was isolated from Japanese fermented soybean natto commercialized in Indonesia [14]. The strain was subsequently grown in nutrient broth at 37°C with agitation. After 24 hours of incubation, the culture was centrifuged at 5,000 × g for 5 min, and the supernatant was collected as a crude enzyme extract. A commercial nattokinase with 2,000 fibrinolytic units (FU) per capsule was purchased from Doctor's Best (Tustin, CA, USA). Chicken blood was purchased from a local traditional market. Phosphate-buffered saline (PBS) was prepared in house. Nutrient broth (NB), sodium chloride (NaCl), calcium chloride ($CaCl_2$), acetone, acetic acid, acrylamide and bis-acrylamide, human fibrinogen, and bovine thrombin were purchased from MilliporeSigma (St. Louis, MO, USA). Agarose and broad range protein molecular weight marker were purchased from Promega (Madison, WI, USA). Coomassie brilliant blue R250 was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Whole blood clot lysis test

Pieces of chicken blood clot were weighed, and a 0.15-gram piece was transferred to each microcentrifuge tube. Subsequently, the blood clots were washed with 0.9% NaCl 2–3 times. Each tube containing a blood clot was reweighed to determine the initial weight of the clot. Then 1 milliliter of crude enzyme extract of *B. subtilis* G8, 1 mL of NB (as the negative control), or 50 FU of nattokinase was added into each tube. The mixtures were subsequently incubated at 37°C for 6 hours [14]. After incubation, the dissolved clots were analyzed qualitatively (by visual inspection) and quantitatively (by performing a wavelength scan (350–700 nm) with a spectrophotometer and by counting numbers of released red blood cells with haemocytometer. The remaining clots were dried at 80°C for 20 min and reweighed to obtain their final weights. The percentage of blood clot degradation was calculated based on dry weight of the clot using the following formula:

Percentage of degradation (%) =

$$\frac{initial\ weight\ of\ blood\ clots - final\ weight\ of\ blood\ clots}{initial\ weight\ of\ blood\ clots}$$

$$\times\ 100\%.$$

Euglobulin clot lysis test

One milliliter of chicken blood plasma was diluted with 9 mL of distilled water at 4°C. The pH was adjusted to 4.5 by adding 100 µL of 1% acetic acid. Subsequently, 1.5 mL of the mixture was transferred to each microcentrifuge tube and centrifuged at 3,000 rpm for 10 min. After centrifugation, the euglobulin fraction was precipitated. After discarding the supernatant, the precipitate was resuspended with 20 µL of PBS. Forty microliters of the suspension was mixed with 40 µL of 25 mM $CaCl_2$ in order to form euglobulin clots. Subsequently, 1 mL of crude enzyme extracts of *B. subtilis* G8, 1 mL of NB, or 50 FU of nattokinase was added into each respective tube and incubated at 37°C for 2 hr [15]. The dissolved euglobulin clot's fraction was analyzed qualitatively (by visual inspection) and quantitatively (by performing a wavelength scan at 200–500 nm with a spectrophotometer and by subjecting it to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Fibrin plate method

The crude enzyme extract of *B. subtilis* G8 was precipitated with acetone at a ratio of 1:3. The mixtures were incubated at −30°C for 60 min and centrifuged at 10,000 rpm for 5 min. The precipitates were air-dried until the acetone fully evaporated. The precipitates were subsequently resuspended with 20 µL of PBS. A fibrin plate was created by mixing 0.4% of fibrinogen in 7 mL of 0.9% NaCl with 7 mL of 2% agarose and 80 µL of 100 NIH units/mL thrombin [14]. Wells were created on the fibrin plate and filled with either 20 µL of PBS, protein precipitates of crude enzyme extracts of *B. subtilis* G8 (1:3 ratio), or various concentrations of nattokinase (2, 5, 10, 25, and 50 FU). The plate was subsequently incubated at 37°C for 8 hours. After incubation, the resultant clear zone was measured (diameter length). A standard curve was created based on the fibrinolytic activities of nattokinase at different concentrations and was used for determining the fibrinolytic activity [16].

SDS-PAGE and zymography

SDS-PAGE was performed by using 12% of gel polyacrylamide [14]. Fibrin zymography of samples was performed by incorporating 0.0006% (w/v) of fibrinogen and 50 µL of 100 NIH units/mL thrombin into the polyacrylamide solution [14]. Ten microliters of each sample was loaded into the gels, and the electrophoresis was performed at 50 millivolts for the first hour and at 100 millivolts for the following hour. The gels were subsequently stained with Coomassie brilliant blue R250.

Statistical analysis

Statistical analysis was performed on the results of the whole blood clot lysis test. Data are presented as mean ± SD values. Statistical significances were determined by one-way analysis of variance followed by Tukey's method, with a 95% confidence interval. IBM SPSS Statistics for Windows version 20.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses.

RESULTS

A comparison of fibrinolytic activity between a crude extract of *B. subtilis* G8 and commercialized nattokinase was performed by utilizing the following *in vitro* tests: the whole blood clot lysis test, euglobulin clot lysis test, fibrin plate method, and
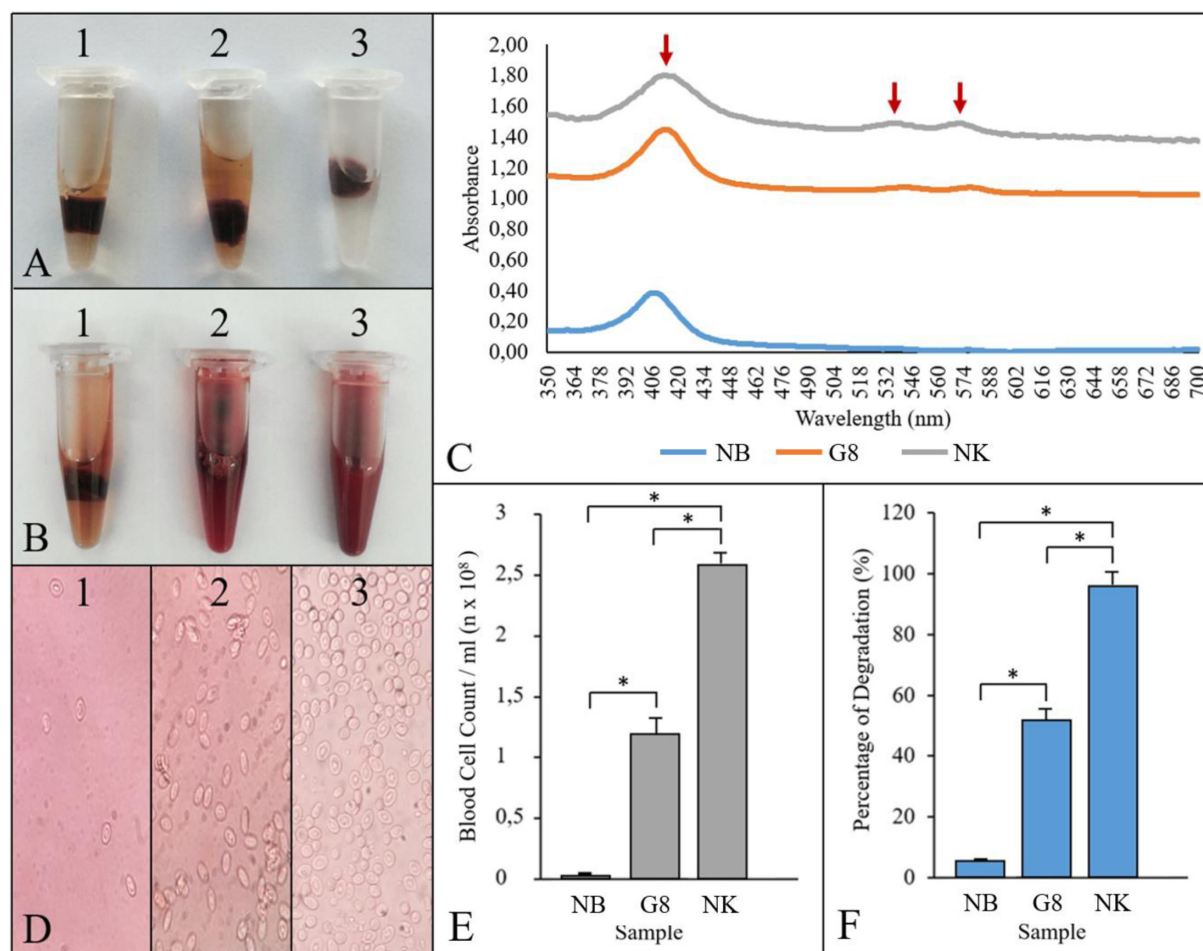
Fig. 1. Crude extract of *B. subtilis* G8 lysed whole blood clots. Representative visual results of whole blood clots treated with (1) nutrient broth/NB, (2) crude extract of *B. subtilis* G8, or (3) nattokinase/NK 50 FU at (A) zero hours and (B) after 6 hours of incubation (n=3). (C) Representative spectrophotometry results after 6 hours of incubation. Arrows indicated observed peaks upon incubation. Released red blood cells after 6 hours of incubation with (1) NB, (2) crude extract of *B. subtilis* G8, or (3) nattokinase/NK 50 FU, via (D) inspection, (E) cell count, and (F) percentage of clot degradation. Each bar represents mean value with standard deviation (n=3). *Statistical significance at $p<0.05$.

zymography.

B. subtilis *G8 lysed whole blood clots*

The whole blood clot lysis test was performed in order to assess fibrinolytic activity of a particular substance (e.g., nattokinase) in the presence of whole blood components [17]. A previous study of *B. subtilis* G8 showed that its crude extract induced extensive lysis of whole chicken blood clots at the 6 hour time point, based on visual inspection [14]. As shown in Fig. 1A and B, this finding was reconfirmed, as *B. subtilis* G8 and nattokinase extensively dissolved clots, as compared with NB. Furthermore, quantitative analyses were performed as well to reinforce the reliability of this test. Figure 1E and F show significant degradation of blood clots, and hence higher counts of erythrocytes, when clots were incubated with *B. subtilis* G8 (to a lesser degree) or nattokinase, compared with those incubated with NB. These findings were supported by visual inspection in a hemocytometer (Fig. 1D). The spectrophotometry readings in Fig. 1C reinforce the above findings. Absorbance peaks were observed at 412, 540, and 580 nm, in which higher numbers of free erythrocytes correlated with higher absorbance readings at

these wavelengths. Unsurprisingly, the blood clots treated with commercialized nattokinase displayed a higher absorbance than those treated with the crude extract of *B. subtilis* G8. Taken together, both the qualitative and quantitative assessments in the whole blood clot lysis test corroborated each other, suggesting that *B. subtilis* G8 has potent fibrinolytic activity.

B. subtilis *G8 lysed euglobulin clots*

The euglobulin clot lysis test is classically utilized to screen for intrinsic fibrinolytic activity, as the euglobulin fraction of plasma contains preserved levels of fibrinolytic factors (e.g., plasminogen and plasminogen activators) but with much reduced levels of plasminogen activator inhibitor-1 [15]. Arguably, this assay could be used to reconfirm results from the whole blood clot lysis test, due to low levels of anti-fibrinolytic agents in the euglobulin fraction of plasma. The qualitative results of the test are shown in Fig. 2, and *B. subtilis* G8 and nattokinase were able to dissolve the euglobulin clots. The results were subsequently analyzed quantitatively via spectrophotometry readings at wavelengths of 200–500 nm. Despite the fact that treatment with *B. subtilis* G8 or nattokinase resulted in different
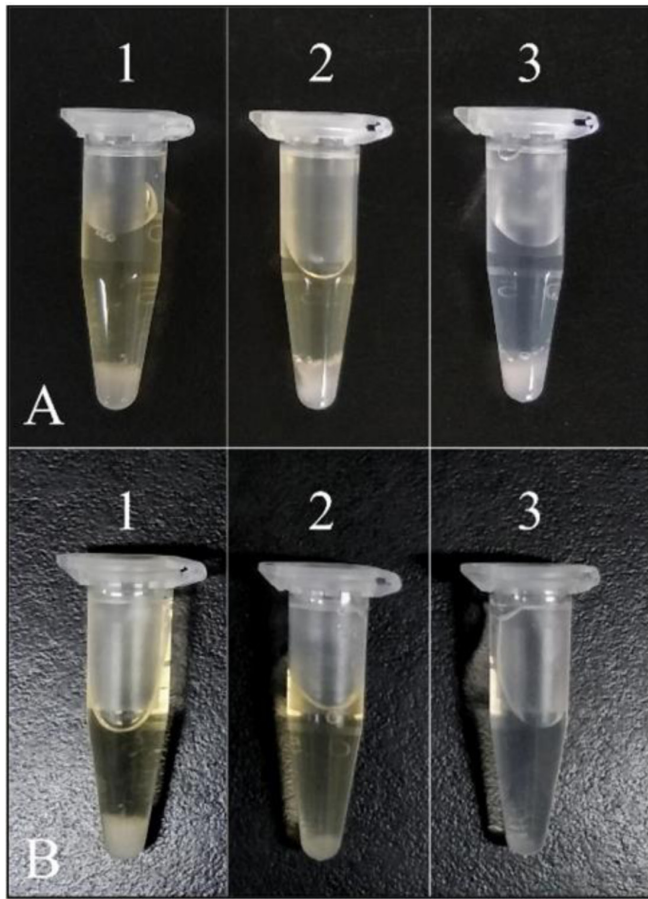
Fig. 2. Crude extract of *B. subtilis* G8 lysed euglobulin clots. Representative visual results of euglobulin clots treated with (1) nutrient broth/NB, (2) crude extract of *B. subtilis* G8, or (3) nattokinase 50 FU at (A) zero hours and (B) after 6 hours of incubation at 37°C (n=3).

Fig. 3. SDS-PAGE of euglobulin clots lysed by crude extract of *B. subtilis* G8 and nattokinase. Representative SDS-PAGE results of euglobulin clots incubated with (A) crude extract of *B. subtilis* G8 and (B) nattokinase 50 FU, as shown in lane 3. Lanes 1 and 2 refer to the protein marker and untreated euglobulin clot, respectively.

peak absorbances than that obtained with NB, intriguingly, the treatment with the commercialized nattokinase resulted in a much lower reading (data not shown). One possible explanation was that nattokinase, as a potent fibrinolytic enzyme, repeatedly degraded the euglobulin clot during the 2-hr incubation. This could result in a much lower concentration of protein or peptide.

Another approach was subsequently used for the quantitative assessment, i.e., analysis of the lysed samples via SDS-PAGE. Figure 3A demonstrates that *B. subtilis* G8 degraded non-crosslinked (i.e., alpha, beta, and gamma chains) as well as crosslinked fibrin forms (i.e., alpha-dimer, gamma-dimer, and gamma-alpha dimer), which were found in the euglobulin clot, as reported previously [18]. This indicated that a fibrinolytic enzyme(s) of *B. subtilis* G8 was able to degrade fibrin directly. Figure 3A also shows that *B. subtilis* G8 was able to cleave plasminogen and hence could act as a plasminogen activator as well. Collectively, this supports the notion that a fibrinolytic enzyme(s) of *B. subtilis* G8 degraded fibrin directly as well as indirectly by acting as a plasminogen activator [19].
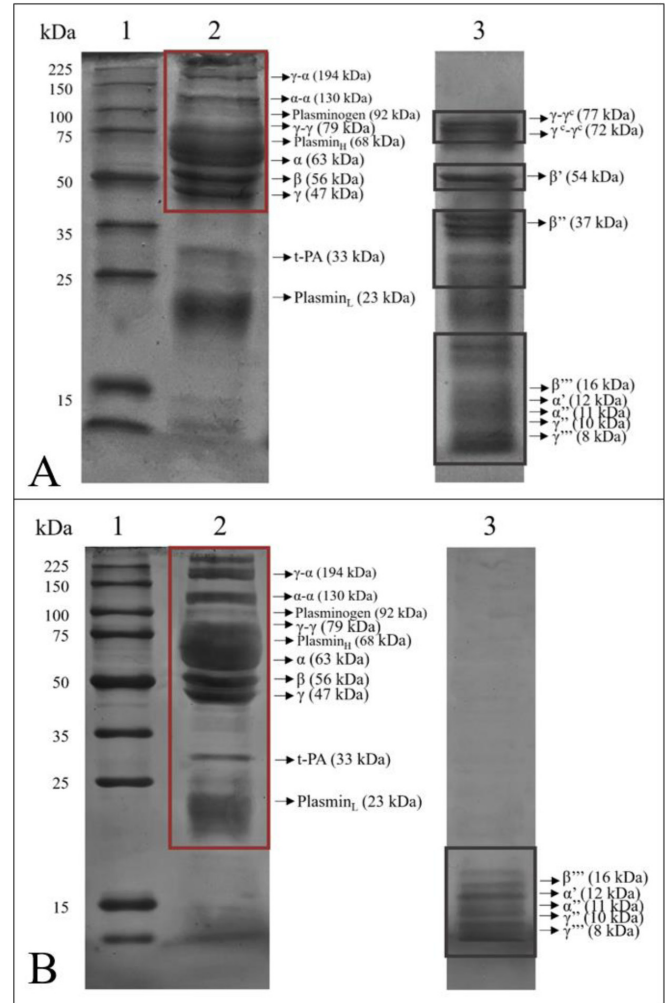
Next, important results after treatment with nattokinase were confirmed, as shown in Fig. 3B. Treatment with nattokinase completely degraded the fibrin forms as well as cleaved plasminogen. Indeed, only degraded products of individual alpha, beta, and gamma chains were observed in this treatment. Taken together, this indicated that visual inspection and SDS-PAGE could be used in tandem as assessment tools for euglobulin clot lysis test. Furthermore, these findings supported the existence of fibrinolytic activity of *B. subtilis* G8.

B. subtilis *G8 exerted direct fibrinolytic effects*

A previous study also demonstrated that a crude extract of *B. subtilis* G8 degraded fibrin plates, as indicated by the presence of a clear zone [14]. In order to determine degree of direct fibrinolytic activity of *B. subtilis* G8, various concentrations of nattokinase were tested to construct a standard curve. The results are displayed in Fig. 4 and demonstrate that nattokinase was able to degrade fibrin at its lowest concentration (2 FU). Based on the constructed standard curve (data not shown), the fibrinolytic

activity of *B. subtilis* G8 was estimated to be as high as 22.8 FU. In sum, this finding provided confirmation of direct fibrinolytic activity of *B. subtilis* G8.

B. subtilis *G8 contained fibrinolytic enzymes*

To identify putative fibrinolytic enzymes of *B. subtilis* G8, crude extracts of *B. subtilis* G8 were partially purified using acetone precipitation and analyzed by SDS-PAGE and zymography [14]. There were some differences between the current results, shown in Fig. 5, and those reported previously [14], as 6 bands were identified in contrast to the previously identified 4 bands. The bands with weaker signals were of 42.0, 35.5, 30.8, 26.7, and 13.7 kDa, which differed from the previously identified weak bands [14]. The differences could be partly attributed to the acetone precipitation, as it could incompletely recover proteins with low concentrations [20]. Nonetheless, the current results identified a thick band (presumably a strong fibrinolytic enzyme) with a molecular weight of approximately 20.0 kDa, which arguably corresponded to the previously identified thick band of 19.1 kDa [14]. In addition, the crude extract of *B. subtilis* G8 might have contained nattokinase, as the 26.7 kDa band might have corresponded to its reported size (27.7 kDa). Collectively, several fibrinolytic enzymes of *B. subtilis* G8 with various molecular sizes were identified.

DISCUSSION

We herein reported further characterization of the fibrinolytic activity of *B. subtilis* G8 isolated from a Japanese traditional food, natto, commercialized in Indonesia. Building on our previous results [14], we uncovered several new findings. First, by using both whole blood and euglobulin clot lysis assays, we showed that a crude extract of *B. subtilis* G8 was indeed able to dissolve blood clots, as compared with commercialized nattokinase and NB as the positive and negative controls, respectively. The clot lysis results were not merely based on a qualitative parameter

(i.e., visual inspection), as various quantitative parameters (i.e., erythrocyte count, spectrophotometer readings, and SDS-PAGE) corroborated these results. Our SDS PAGE results (Fig. 3A) interestingly indicated that the crude extract of *B. subtilis* G8 could degrade various fibrin forms and could act as a plasminogen activator. This suggests that the crude extract of G8 behaved in a manner similar to the reported nattokinase [19].

Second, we reconfirmed the finding of our previously published study [14] that a crude extract of *B. subtilis* G8 was able to directly lyse fibrin, as observed in experiments using euglobulin clots and fibrin plates (Figs. 3A and 4). This reconfirms the earlier mentioned inference that *B. subtilis* G8 has direct fibrinolytic activity. Furthermore, by using known units of fibrinolytic activity of commercialized nattokinase to construct a standard curve, we measured the fibrinolytic activity of *B. subtilis* G8 (Fig. 4). A limitation of this experiment was that the crude extract of *B. subtilis* G8 used might have contained multiple fibrinolytic enzymes, and hence the obtained units of *B. subtilis* G8 might have been a summation of the activities of various enzymes. Nonetheless, the results obtained from the fibrin plate method supported the direct fibrinolytic activity of *B. subtilis* G8. Finally, we observed that the crude extract of *B. subtilis* G8 contained several fibrinolytic enzymes, as suggested by the presence of 6 zymogram bands (Fig. 5). As further purification and characterization of those bands was not performed, we relied on published *B. subtilis*-derived fibrinolytic enzymes to predict the identity of most of the zymogram bands. The band measuring 26.7 kDa arguably corresponded to the size of nattokinase or subtilisin NAT [21], and the bands measuring 30.8 and 20.0 kDa correlated to sizes of other types of subtilisin [12, 22]; on the other hand, the band measuring 42 kDa appears to be similar to the size of metalloprotease [23]. Collectively, our findings support the presence and functionality of various fibrinolytic enzymes that might be secreted by *B. subtilis* G8.

A commercialized nattokinase was used as the reference in this study, as we did not have access to a pure, research-grade
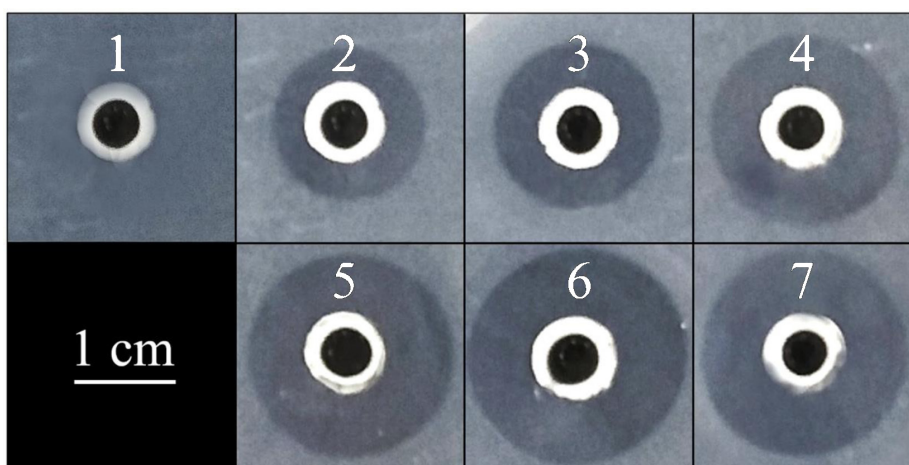

Fig. 4. Crude extract of *B. subtilis* G8 directly degraded fibrin. Representative fibrin plate results (calculated as the diameter of the clear zone) upon treatment with (1) nutrient broth/NB [0 cm], (2) nattokinase 2 FU [0.55 cm], (3) nattokinase 5 FU [0.60 cm], (4) nattokinase 10 FU [0.70 cm], (5) nattokinase 25 FU [0.89 cm], (6) nattokinase 50 FU [1.21 cm], and (7) crude extract of *B. subtilis* G8 [0.85 cm]. The provided values in the square brackets are the average diameters of the clear zones from triplicate experiments. The plates were incubated at 37°C for 8 hours.
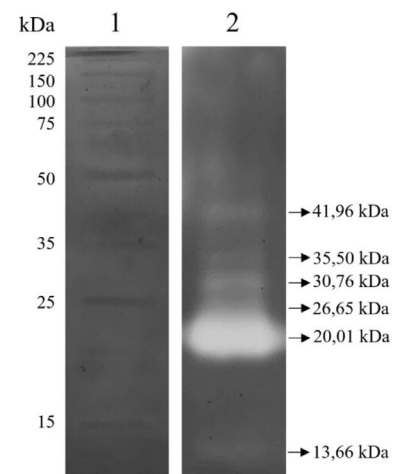

Fig. 5. Crude extract of *B. subtilis* G8 displayed several zymogram bands. Representative zymography results for the crude extract of *B. subtilis* G8 (lane 2). Lane 1 refers to the protein marker.

nattokinase. Nonetheless, this commercialized nattokinase acted as a potent fibrinolytic agent in this study. The results of the whole blood and euglobulin clot lysis tests demonstrated that the commercialized nattokinase exerted stronger fibrinolytic activity at 50 FU than the crude extract of *B. subtilis* G8, partly due to the strength of the crude extract of *B. subtilis* G8 only reaching 22.8 FU. Next, despite the reported size of nattokinase being approximately 27.7 kDa [21], we intriguingly observed its size as 12.4 kDa via zymography (data not shown). As very limited information was provided by the manufacturer, we are tempted to speculate that this commercialized nattokinase was a truncated version of the original serine protease but retained a high degree of or complete fibrinolytic activity.

As many groups have determined the fibrinolytic activity of substances primarily based on the fibrin plate method [24–26], we would like to note that this method only provides a partial assessment of fibrinolytic activity. It does not take into consideration the fact that cellular components of blood also play an important role in fibrinolysis [24], which can be assessed by the whole blood clot lysis test instead. In addition, a tested substance could be interpreted as having a direct fibrinolytic activity when a clear zone is observed in the fibrin plate method [25]. It is worth noting, however, that the fibrin plate method might contain a residual quantity of plasminogen due to the presence of plasminogen in commercially purchased fibrinogen [26, 27]. Therefore, a resultant clear zone in the presence of a substance might also bepartly due to the activity of plasmin (i.e., an indirect fibrinolytic activity). This pitfall can be circumvented by either comparing heat-treated and non-heat-treated fibrin plate methods [26, 27] or by running the lysed fraction of euglobulin clots through SDS-PAGE (Fig. 3). This supports our hypothesis in favor of performing various assays to assess the fibrinolytic activity of *B. subtilis* G8.

In summary, a detailed characterization of the fibrinolytic activity of a crude extract from *B. subtilis* G8 was reported in this study based on various *in vitro* fibrinolytic assays. Our results suggest that *B. subtilis* G8 has potent fibrinolytic activity and that its activity presumably is mediated by various enzymes. Further studies to isolate and elucidate each of the fibrinolytic enzymes are warranted to support the discovery of potent, safe, and affordable fibrinolytic agents that could have potential clinical applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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