Prev. Nutr. Food Sci. 2014;19(3):213-219
http://dx.doi.org/10.3746/pnf.2014.19.3.213
pISSN 2287-1098 · eISSN 2287-8602

Purification and Characterization of a Fibrinolytic Enzyme from *Bacillus pumilus* 2.g Isolated from *Gembus*, an Indonesian Fermented Food

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ABSTRACT: *Bacillus pumilus* 2.g isolated from *gembus*, an Indonesian fermented soybean cake, secretes several proteases that have strong fibrinolytic activities. A fibrinolytic enzyme with an apparent molecular weight of 20 kDa was purified from the culture supernatant of *B. pumilus* 2.g by sequential application of ammonium sulfate precipitation, ion-exchange chromatography, and hydrophobic chromatography. The partially purified enzyme was stable between pH 5 and pH 9 and temperature of less than 60°C. Fibrinolytic activity was increased by 5 mM $MgCl_2$ and 5 mM $CaCl_2$ but inhibited by 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium dodecyl sulfate (SDS), and 1 mM ethylenediaminetetraacetic acid (EDTA). The partially purified enzyme quickly degraded the α and β chains of fibrinogen but was unable to degrade the γ chain.

Keywords: fibrinolytic enzyme, *Bacillus pumilus*, fermented food, *gembus*

INTRODUCTION

Cardiovascular diseases are the number one cause of death globally. According to a report published by the World Health Organization (WHO) in 2011, an estimated 17.3 million people died from cardiovascular diseases in 2008, representing 30% of all global deaths (1). The number of people who die from cardiovascular diseases is expected to increase to 23.3 million people by 2030. Heart disease and stroke are projected to remain the leading causes of death throughout this period (2).

Intravascular thrombosis (i.e., the clotting of blood in blood vessels), is one of the major causes of cardiovascular diseases. Clots formed from insoluble fibrin restrict the smooth flow of blood in blood vessels, leading to thrombosis and heart attacks. Insoluble fibrin is the major protein component of blood clots, which are formed from fibrinogen by thrombin (3). This insoluble fibrin could be hydrolyzed into fibrin degradation products by plasmin, which is generated from plasminogen by plasminogen activators (4). The basis of fibrinolytic therapy is intravenous administration of an exogenous plasminogen activator, that lyses the thrombus and restores blood flow to the area of ischemia. The three fibrinolytic agents that are currently being used for this purpose are urokinase, streptokinase, and genetically engineered tissue plasminogen activator (t-PA). However, these enzymes are expensive, thermolabile and can produce undesirable side effects such as gastrointestinal bleeding, allergic reactions, and resistance to repercussion (5,6).

Recently, potent fibrinolytic enzymes were discovered in various fermented food products, including Japanese *natto* (7-9), skipjack *shiokara* (10), Korean *cheonggukjang* (11-13), *doenjang* (14), a traditional Asian fermented shrimp paste seasoning (15), and a traditional Chinese soybean food, *douchi* (16). Nattokinase, an extracellular fibrinolysin produced by *Bacillus subtilis* natto, can directly hydrolyze fibrin in blood clots and promote the production of t-PA, which activates plasminogen into active plasmin to hydrolyze fibrin. Oral administration of natto or nattokinase effectively enhances the release of

Received 13 May 2014; Accepted 5 August 2014; Published online 30 September 2014
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an endogenous plasminogen activator in animal models and in human subjects (17).

Microbial fibrinolytic enzymes from food-grade microorganisms have the potential to be developed as additives for functional foods and as drugs to prevent or cure cardiovascular diseases. *Tempeh* is an Indonesian fermented soybean food that can be made from a variety of raw materials. *Gembus* is a variety of *tempeh*. *Gembus* is different from *tempeh* in that it is made from the solid soybean waste of tofu (18). Previous work indicates that *Bacillus pumilus* 2.g can be isolated from *gembus* and has high proteolytic and fibrinolytic activities (19). In this paper, a fibrinolytic enzyme was purified from the supernatant of a *B. pumilus* 2.g culture, and the properties of the partially purified enzyme were studied.

MATERIALS AND METHODS

Bacterial strains and culture conditions

To compare media, *B. pumilus* 2.g was grown in Luria-Bertani broth (LB; Becton, Dickinson and Company, Sparks, MD, USA), tryptic soy broth (TSB), nutrient broth (NB), and brain heart infusion (BHI) at 37°C with vigorous shaking.

Assay of fibrinolytic activity

The fibrinolytic activities of the supernatants of *B. pumilus* 2.g cultures was determined using the fibrin plate method (20). Briefly, 7 mL of 0.3% (w/v) fibrinogen (MP Biomedicals, Santa Ana, CA, USA) solution in 1 M phosphate-buffered saline (PBS) was mixed with an equal volume of 2% (w/v) agarose solution and 0.1 mL of thrombin solution (100 NIH units/mL; MP Biomedicals) in a petri dish. The petri dish was left at room temperature for 1 h to allow a fibrin clot layer to form, a glass capillary tube was used to make a hole in the fibrin plate. Next, 20 μL of sample was dropped into the hole, and the plate was incubated at 37°C for 8 h. The size of the clear zone that formed was converted into plasmin units (U) by comparison to zones formed by known quantities of plasmin. Protein concentration was determined by the Bradford method (21) using bovine serum albumin (BSA) as the standard. All measurements were performed in triplicate and the average values are shown.

Partial purification of fibrinolytic enzymes

Fibrinolytic enzymes secreted by *B. pumilus* 2.g were purified from NB cultures that had been incubated for 72 h. The supernatant was isolated by centrifugation (12,000 *g*, 10 min, 4°C), filtered through a disposable filter unit (0.45 μm; Sarstedt AG & Co., Nümbrecht, Germany) and subjected to ammonium sulfate precipitation (80% saturation, w/v) overnight at 4°C. The precipitate was resuspended in buffer A (20 mM Tris-HCl, pH 7.0) and then dialyzed against 20 volumes of the same buffer for 24 h at 4°C with four buffer changes. After dialysis, the sample was freeze-dried and resuspended in buffer A. The resuspended sample was loaded onto a 2.5×8 cm CM-Sephadex column (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and proteins were eluted by sequential application of 6×100 mL of buffer A containing increasing concentration of NaCl; NaCl concentrations increased from 0 M to 1 M, in a stepwise manner (0.2 M increments). The fibrinolytic activity of each fraction was measured with the fibrin plate method and fractions with fibrinolytic activity were pooled. Pooled samples were dialyzed against buffer A, lyophilized, and then resuspended in 20 mL of buffer A.

Hydrophobic interaction chromatography with Phenyl Sepharose 6 Fast Flow resin (Amersham Pharmacia Biotech AB) was used for further purification of fibrinolytic enzymes. Briefly, each sample was loaded onto a column (2.5×12 cm) that had been pre-equilibrated with buffer A containing 1 M $(NH_4)_2SO_4$. Proteins were eluted by sequential application of 50 mL of buffer A containing decreasing concentration of $(NH_4)_2SO_4$; $(NH_4)_2SO_4$ concentrations decreased from 1 M to 0 M in a stepwise manner (0.2 M decrements). The protein content and fibrinolytic activity of each fraction were measured. Fractions with activity were pooled, dialyzed against buffer A, and lyophilized. Sodium dedocyl sulfate (SDS)-PAGE was conducted by Laemmli's method (22), and the fibrin zymography was done as previously described (20). For fibrin zymography, the separating gel solution (12%, w/v) was prepared in the presence of fibrinogen (0.02%, w/v) and 100 μL of thrombin (10 NIH units/mL).

Properties of the partially purified fibrinolytic enzyme

To determine the effect of pH on the fibrinolytic enzyme activity, a 0.02 μg sample of partially purified fibrinolytic enzyme was incubated in either 50 mM citrate-NaOH buffer (pH 3.0~4.0), 50 mM sodium phosphate buffer (pH 5.0~6.0), 50 mM Tris-HCl (pH 7.0~8.0), or 50 mM glycine-NaOH buffer (pH 9.0~10.0) for 2 h at 37°C. Following incubation, the fibrinolytic enzyme activity of each mixture was measured by the fibrin plate method.

For thermal stability measurements, 0.02 μg of partially purified fibrinolytic enzymes were suspended in 50 mM Tris-HCl buffer (pH 7.0) and incubated in a water bath for 30 min at varying temperatures (37~80°C). After incubation at each temperature, the fibrinolytic enzyme activity of each mixture was measured by the fibrin plate method.

To determine the effect of metal ions and inhibitors on the activity of the partially purified fibrinolytic enzyme, 0.02 μg samples were incubated in Tris-HCl buffers (pH 7.0) containing 5 mM metal ions (i.e., KCl, $MgCl_2$, $CaCl_2$,

$CuSO_4$, $MnCl_2$, or $ZnCl_2$) or 1 mM inhibitors [i.e., phenylmethylsulfonyl fluoride (PMSF), SDS, ethylenediaminetetraacetic acid (EDTA), cantharidic acid, pepstatin A, bestatin hydrochloride, or E-64] for 30 min at 37°C.

The hydrolysis of fibrinogen by the partially purified fibrinolytic enzyme was measured. A total of 2 mg of fibrinogen was mixed with 0.02 μg of partially purified fibrinolytic enzyme in 500 μL of 20 mM Tris-HCl (pH 7.0), and the mixture was incubated at 37°C for up to 12 h. At each interval, 20 μL of sample was removed, mixed with 5×SDS sample buffer, boiled for 5 min, and then analyzed by SDS-PAGE using a 15% acrylamide gel.

Amidolytic activities

Amidolytic activity was determined according to the method of Jo et al. (23). Briefly, a 500 μL mixture containing 50 μL of 10 mM substrate, 10 μL of enzyme (10 μg), and 440 μL of 50 mM Tris-HCl buffer (pH 7.0) was incubated at 37°C for 10 min. Then, 500 μL of citrate-NaOH buffer (pH 3.0) was added to stop the reaction. The resulting mixture was immedietely placed on ice and the centrifuged at 12,000 *g* for 5 min. The $OD_{410\ nm}$ of the supernatant was measured, and the degree of hydrolysis was calculated from the absorbance values and the molar extinction coefficient value of *p*-nitroanilide (*p*-NA) (8,800 $M^{-1}cm^{-1}$).

RESULTS AND DISCUSSION

Fibrinolytic activity of *Bacillus pumilus* 2.g

Previously, bacilli strains with fibrinolytic activities were isolated from *gembus* prepared by traditional methods in Semarang, Central Java, Indonesia (18,19). Among the isolated strains, the 2.g strain had the highest fibrinolytic activity. The strain was identified as *B. pumilus* by 16S rRNA gene sequencing (97% homology), and confirmed by API 50 CHB kit (bioMérieux SA, Marcy-l'Étoile, France) with apiweb™ software (99.7% identity). Among the microorganisms that produce fibrinolytic enzymes, bacilli from Asian traditional fermented foods are the most important (9-12,14). *B. subtilis*, *B. amyloliquefaciens*, and *B. licheniformis* are the most common bacilli species isolated from fermented foods, and the fibrinolytic enzymes found in these species have been reported (9,11,12). However, to the best of our knowledge, the fibrinolytic enzyme present in *B. pumilus* has not been reported. The isolation of diverse bacilli species and the characterization of their fibrinolytic enzymes are important for understanding the fibrinolytic capacities of these organisms and for developing functional foods and therapeutic agents for the prevention of cardiovascular diseases.

Growth and fibrinolytic activity of *B. pumilus* 2.g

The effects of four different media on the growth and fibrinolytic activity of *B. pumilus* 2.g were compared (Fig. 1). Each medium was inoculated with an overnight culture (2%, v/v) and incubated for up to 96 h at 37°C with shaking. SDS-PAGE and zymography revealed that the culture in NB had the highest fibrinolytic activity (187 U/mg protein) at 72 h, and the fibrinolytic activity of this culture was stable until 96 h (Fig. 2).

The highest fibrinolytic activity values were observed in the stationary phase culture. Cultures in LB, BHI, and TSB had much lower fibrinolytic activities, i.e., 29 U/mg protein for the culture in LB (96 h), 11 U/mg protein for the culture in BHI (96 h), and 74 U/mg protein for the culture in TSB (96 h). Previous work indicates that TSB is the best medium and NB is the second best medium for a 50 h incubation of *B. amyloliquefaciens* CH51 (11). In addition, LB is the best medium for the incubation of *B. licheniformis* CH3-17 (12). As these results indicate, the best medium for the measurement of fibrinolytic activity is variable, depending upon the specific organism of interest. In addition, growth environment greatly affects the fibrinolytic enzyme yield of a given organism.

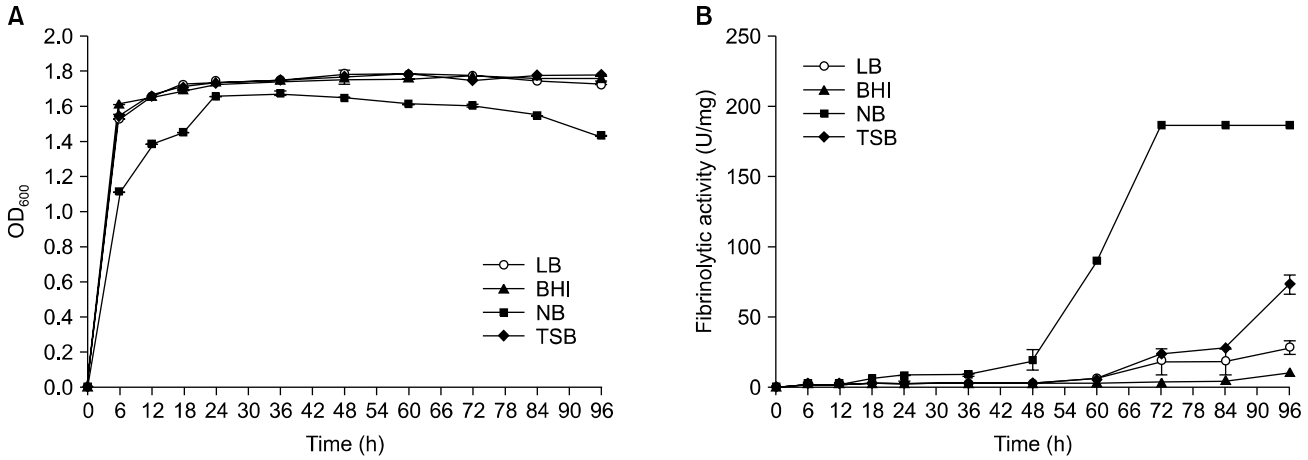

Fig. 1. Changes in the growth (A) and fibrinolytic activities (B) of *B. pumilus* 2.g cultured in different growth media.

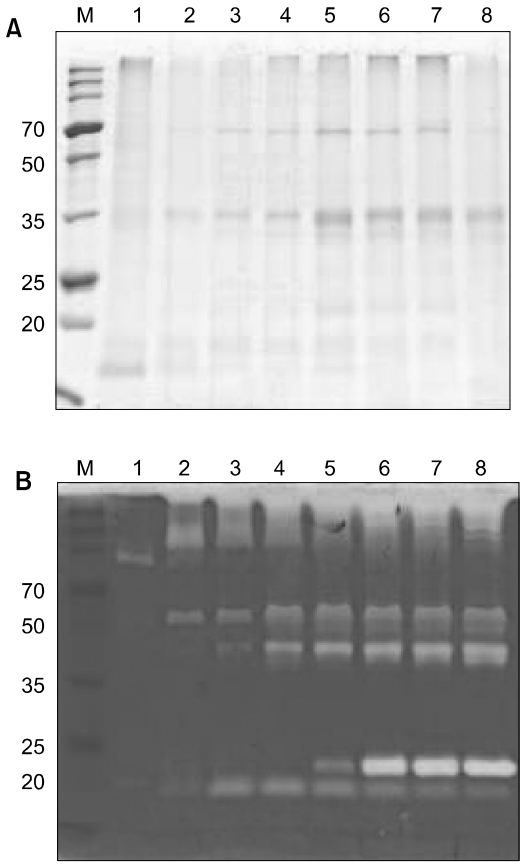

Fig. 2. SDS-PAGE (A) and fibrin zymography (B) of culture supernatant from *B. pumilus* 2.g incubated in NB medium. Lane M, broad-range size marker (Dokdo-Mark™, ElpisBiotech, Daejon, Korea); lanes 1~8: culture supernatant from 12 h (1), 24 h (2), 36 h (3), 48 h (4), 60 h (5), 72 h (6), 84 h (7), and 96 h (8) of incubation.

Partial purification of a fibrinolytic enzyme

A fibrinolytic enzyme produced by *B. pumilus* 2.g was partially purified from 3 L of culture supernatant. The results are summarized in Table 1. After the Phenyl-Sepharose column chromatography step, the final purification fold was 16.0, and the yield was 25%. The partially purified enzyme was analyzed by SDS-PAGE and zymography. The results of these analyses confirmed that most (Fig. 3), but not all (Fig. 4A), of the other proteins were removed by the purification process. Fibrin zymography of the purified sample revealed a single band with a molecular mass of 20 kDa. Previous studies have reported different molecular masses for different fibrinolytic enzymes: 29 kDa for *B. subtilis* natto B-12 (9), 28.2 kDa for *Bacillus* sp. CK 11-4 (10), 27 kDa for *B. licheniformis* CH3-17 (12), 28 kDa for *B. amyloliquefaciens* DC-4 (16), 37 kDa for *B. licheniformis* KJ-31 (24), 41 kDa for *Bacillus* sp. KA38 (25), and 35 kDa for *Streptomyces* sp. CS684 (26).

Properties of a partially purified enzyme

The activity of partially purified fibrinolytic enzyme from *B. pumilus* 2.g was highest at pH 7.0 (Fig. 5A). The enzyme was not stable under acidic conditions but was relatively stable at basic pHs (pH 7.0~9.0). No fibrinolytic activity was detected at pH 10. At pH 7.0, the purified enzyme was stable at temperature up to 50°C, but fibrinolytic activity rapidly decreased at temperatures greater than or equal to 60°C (Fig. 5B). The results of this study indicate that the partially purified enzyme is

Table 1. Summary of the steps performed to partially purify a fibrinolytic enzyme from *B. pumilus* 2.g

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold	Yield (%)
Supernatant	13,873.0	153.5	90.4	1	100
Ammonium sulfate precipitation	11,936.4	59.7	200.1	2.2	86
CM-Sephadex	8,252.7	16.1	512.2	5.7	59
Phenyl Sepharose 6-FF	3,421.4	2.4	1,442.3	16.0	25

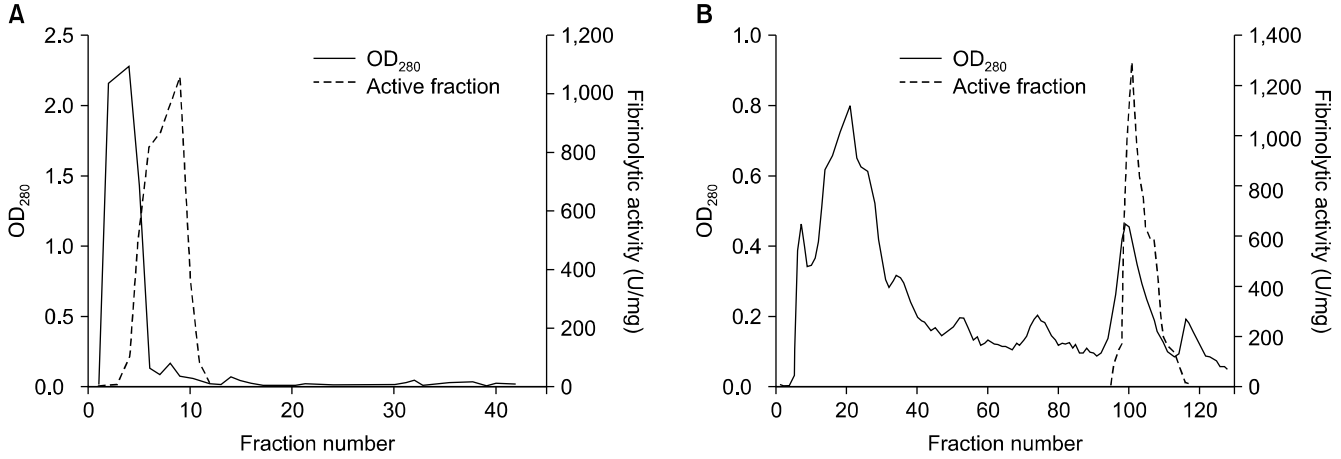

Fig. 3. Elution profile of a fibrinolytic enzyme from *B. pumilus* 2.g through a CM-Sephadex column (A) and a Phenyl Sepharose 6-FF column (B).

not thermotolerant. Thus, products containing this enzyme should not be subjected to heat treatment above 50°C.

The activity of the partially purified enzyme from *B. pumilus* 2.g was reduced with exposure to K^+ (87.39%), Mn^{2+} (80.64%), and Zn^{2+} (89.77%) but slightly increased with exposure to Mg^{2+} (105.70%) and Ca^{2+} (102.85%). Cu^{2+} caused the strongest inhibition (100% inhibition) (Table 2). Copper (Cu) is one of the heavy metals that has a high affinity for organic compounds, including serine, glycine and cycloserine. Cu can bind with sulfhydryl groups, which causes the enzymes containing this group to become inactive. The effect of metal ions on the fibrinolytic activity depends on the origin of the enzyme. For example, the activity of AprE5-41 from *B. amyloliquefaciens* MJ5-41, a strain isolated from *meju*, is sligthly reduced by K^+ and Mg^{2+} but is increased by Ca^{2+} (23). In addition, Nattokinase from *B. subtilis* YJ1 is completely inactivated by Zn^{2+} and severely inhibited by Cu^{2+} (27).

In this study, the partially purified enzyme from *B. pumilus* 2.g was completely inhibited by 1 mM PMSF and 1 mM EDTA. PMSF is known to sulphonate the essential serine residue in the active site of a protease, resulting in a total loss of enzyme activity (28). Several different types of inhibitors were examined in this study, including cantharidic acid (an inhibitor of protein phosphatases), pepstatin A (an inhibitor of acid proteases), bestatin hydrochloride (an inhibitor of aminopeptidases), and E-64 (an inhibitor of cysteine proteases). All of the inhibitors tested inhibited the partially purified enzyme

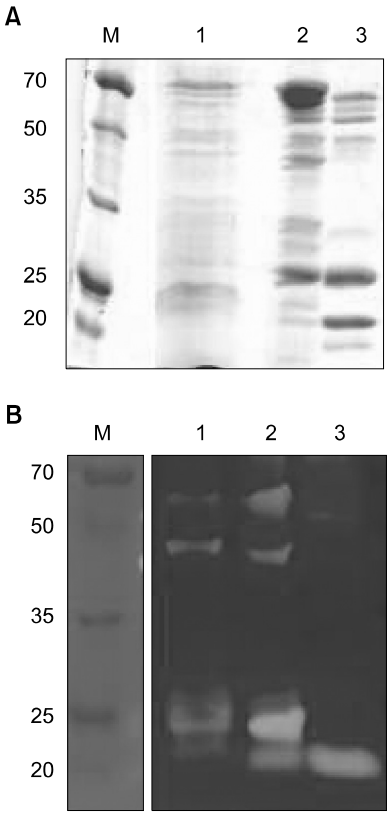

Fig. 4. SDS-PAGE (A) and fibrin zymography (B) of protein samples at different purification stages. Lane M, size marker (Dokdo-Mark™); lanes 1~3: sample after 80% ammonium sulfate precipitation (1), sample after CM-Sephadex purification (2), sample after Phenyl Sepharose 6-FF purification (3).

Table 2. Effect of metal ions and inhibitors on the activity of a partially purified fibrinolytic enzyme from *B. pumilus* 2.g

Metal ions (5 mM)/Inhibitors (1 mM)	Relative activity (%)
None	100
KCl	87.39±3.40
$MgCl_2$	105.70±4.03
$CaCl_2$	102.85±4.03
$CuSO_4$	0
$MnCl_2$	80.64±3.03
$ZnCl_2$	89.77±3.42
PMSF	30.72±1.17
SDS	0
EDTA	0
Cantharidic acid	47.91±1.83
Pepstatin A	78.45±0.07
Bestatin hydrochloric	76.44±2.91
E-64	89.77±3.42

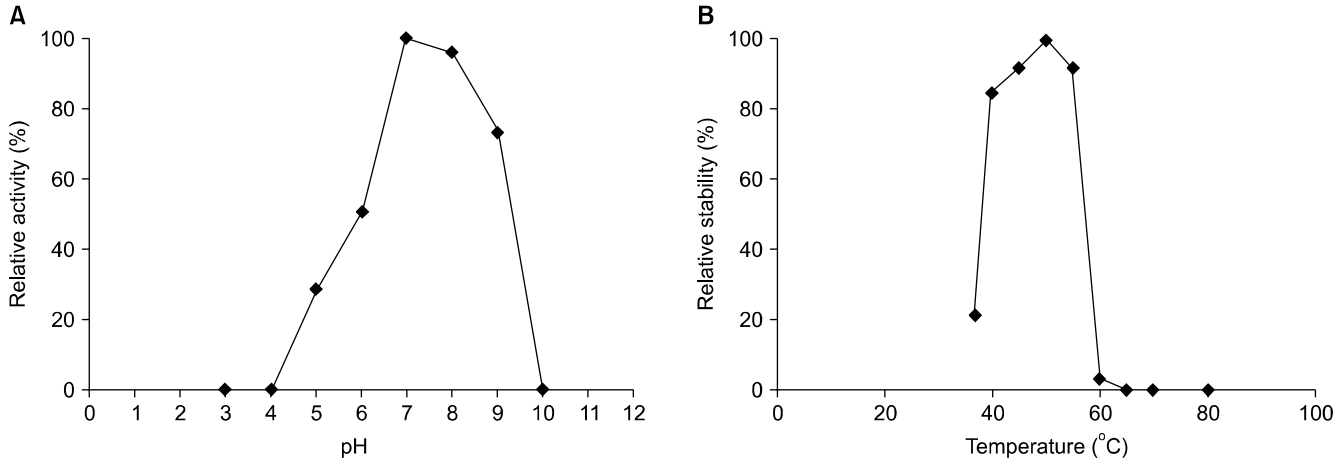

Fig. 5. Effect of pH (A) and temperature (B) on the stability of a partially purified fibrinolytic enzyme from *B. pumilus* 2.g.

from *B. pumilus* 2.g to some degree. These results suggest that the purified enzyme is a serine protease.

Amidolytic activities

Among the synthetic substrates tested, N-Succinyl-Ala-Ala-Pro-Phe-*p*NA, a substrate for subtilisin and chymotrypsin, was the most efficient (Table 3). The fibrinogen hydrolysis assay revealed that the partially purified enzyme from *B. pumilus* 2.g degraded the α chain of fibrinogen in 10 min and the β chain of fibrinogen in 4 h but could not degrade the γ chain of fibrinogen, even after 12 h (Fig. 6). This degradation pattern is similar to that of AprE3-17, the major fibrinolytic protease of *B. licheniformis* CH3-17 (12), and AprE5-41, the major fibrinolytic protease of *B. amyloliquefaciens* MJ5-41 (23), but different from that of a fibrinolytic enzyme from *B. subtilis*, which degrades the β chain first (29). The degradation pattern of fibrinogen could vary by fibrinolytic enzyme, and thus each enzyme should be individually checked.

Bacillus strains with desirable properties, especially for the pH stability and temperature stability, could be used for the production of functional foods or medicines to treat cardiovascular diseases in the near future. The results of this study demonstrate that *B. pumilus* 2.g may be used as a novel fibrinolytic enzyme source.

Table 3. Amidolytic activity of a partially purified fibrinolytic enzyme from *B. pumilus* 2.g

Synthetic protease substrate (10 mM)	Substrate hydrolysis (mM/min/mg)
N-Succinyl-Ala-Ala-Pro-Phe-*p*NA	61.34
N-Benzoyl-Phe-Val-Arg-*p*NA hydrochloride	4.41
N-Benzoyl-Pro-Val-Arg-*p*NA hydrochloride	0.24
N-(*p*-tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt	0.88

*p*NA: *p*-nitroanilide.

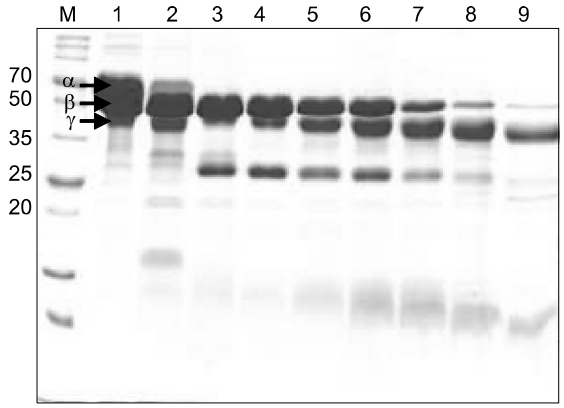

Fig. 6. Fibrinogen hydrolysis by a partially purified enzyme from *B. pumilus* 2.g. Lane M, size marker (Dokdo-Mark™); lane 1: control (no enzyme treatment); lanes 2~9: fibrinogen after treatment with purified enzyme for 0 min (2), 10 min (3), 30 min (4), 1 h (5), 2 h (6),4 h (7), 8 h (8), and 12 h (9).

ACKNOWLEDGEMENTS

This work was supported by the Directorate General of Higher Education, Ministry of Education and Culture, Republic of Indonesia and Diponegoro University.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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