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RESEARCH ARTICLE

Fibrinolytic activity and molecular identification of PB-12 isolate from *Papuma Coastal* at Jember regency

Evi Umayah Ulfa [1], Sattya Arimurti [2]

[1] Faculty of Pharmacy, University of Jember, Jember, East Java, Indonesia
[2] Faculty of Mathematics and Natural Sciences, University of Jember, Jember, East Java, Indonesia

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Correspondence
Evi Umayah Ulfa
Department of Pharmaceutical Biology
Faculty of Pharmacy
University of Jember
East Java
Indonesia
evi.farmasi@unej.ac.id

Abstract

Background: Coastal areas have a high bacterial diversity, which can be used as a source of pharmaceutical substances, including the fibrinolytic enzyme. Fibrinolytic enzymes can lyse blood clots by degrading the fibrin. These enzymes are important for treating circulatory disorders, e.g., pulmonary embolism, deep vein thrombosis, and myocardial infarction. **Objective:** This study aimed to determine the fibrinolytic activity of crude protein extract and precipitate protein of fibrinolytic bacteria PB-12 isolated from Papuma coastal, Jember district, Indonesia. **Method:** The molecular weight of fibrinolytic enzymes was determined by fibrin zymography, while 16s rDNA sequencing was performed to identify the PB-12 isolate. **Result:** The fibrinolytic activity result showed CPE at 24 hours had the highest fibrinolytic activity with a lysis diameter of 5.23 ± 0.68 mm. The fibrinolytic activity of this bacterium was contributed with ~114 kDa protein. PB-12 was identified as *Bacillus aryabhattai* based on 16S rRNA gene sequence. **Conclusion:** This is the first to report *Bacillus aryabhattai* as a potential fibrinolytic enzyme producer.

Introduction

Cardiovascular diseases (CVDs) such as myocardial infarction, deep vein thrombosis, and pulmonary embolism are dominant causes of mortality worldwide (Roth et al., 2020). The World Health Organization (WHO) reported that 17.9 million people die every year from CVDs (WHO, 2017). A primary attribute of various CVDs is a blockage of blood vessels by excessive fibrin accumulation. Anticoagulants and antiplatelet drugs have long been used to treat cardiovascular disease. However, it was unable to eliminate existing thrombi (Sharma, Osmolovskiy, & Singh, 2021). Fibrinolytic enzymes are becoming more important in treating CVD due to their ability to dissolve fibrin clots within blood vessels (Sharma, Osmolovskiy, & Singh, 2021).

Streptokinase (SK) and Tissue plasminogen activator (TPA) are commonly used in thrombosis treatment. However, these enzymes are expensive, have a short half-life, and still have haemorrhagic side effects (Kunamneni, Abdelghani, & Ellaiah, 2007). Therefore, the search for new fibrinolytic enzymes from different sources is paramount. Fibrinolytic enzymes have been discovered from various sources, including microorganisms, plants, and animals (Barzkar, Jahromi, & Vianel, 2022). The most useful source for these enzymes are microorganisms due to cost-effective production, fewer to no side effects, broad biochemical diversity, and allow for genetic manipulation. Marine microorganisms have long been recognised as good sources of pharmaceutical products and cosmetics (Barzkar et al., 2005). High or low temperatures, alkaline water, and limited substance in the sea lead marine microorganisms to produce various compounds to tolerate their environment. These distinctive characteristics have attracted many researchers, and lead to discoveries of multiple compounds (Zibin, Ming & Wenhui, 2017).

In order to explore the potential of coastal microorganisms, fibrinolytic bacteria were isolated from Papuma Coastal, Jember Regency, Indonesia. The isolate PB-12 showed fibrinolytic activity on the fibrin plate assay. This work was carried out to determine the molecular weight of fibrinolytic enzyme-producing isolate PB-12 and identified isolate PB-12.

Methods

Microorganisms

The bacterial isolate PB-12 was collected from Papuma Coastal-Jember, Indonesia. The isolate PB-12 was taken from the glycerol stock and revived using nutrient agar plates at 37 °C for 48 hours. A single colony of the bacterial isolate was used for further studies.

Crude protein extracts production

The medium used for cultivation of the bacterial isolate and enzyme production was Luria Bertani medium which consisted of 1% (w/v) tryptone, 1% (w/v) Sodium chloride, and 0.5% (w/v) yeast extract. Seed cultures were obtained by cultivating a single colony of isolate PB-12 into 5 mL LB medium at 37 ° C in a shaker incubator for 24 hours. 5% (v/v) of seed culture was then transferred to basal LB-medium and incubated at 37 ° C by shaking. Bacterial cultures were harvested after incubating 12, 24, 36, and 48 hours by centrifugation at 10.000 rpm, 4 ° C, and 15 minutes. The crude protein extract (CPE) was concentrated by the addition of chilled acetone (5:1) and incubated at 20 °C for 2h until a protein precipitate (PP) was formed. The protein precipitate was collected by centrifugation and dissolved in phosphate buffer saline pH 7.4. The CPE and PP were used for the fibrinolytic assay.

Fibrinolytic assay

Fibrinolytic activity was determined using the fibrin plate assay method. Plates diameter of 50 mm containing fibrinogen (0.1%; 10 mL) in phosphate-buffered saline, 0.2 mL bovine thrombin (10 NIH U/mL) in 0.5 M CaCl2, and 1.2% agarose were allowed to stand for 30 min at room temperature to form the fibrin clot layer (Astrup & Mullertz, 1952). Ten microliters of CPE and PP respectively were dropped into wells previously made on the fibrin media plate. Plates were incubated for 15 hours at 37 °C, and the potency of fibrinolytic activity was scored by measuring the diameter of clear zones.

SDS–PAGE and zymogram analysis

SDS-PAGE was performed using 12.5 % polyacrylamide gels as separating gel and 4% polyacrylamide gels as stacking gel. After running, the gel was stained with Coomassie Brilliant Blue. For fibrin zymography, the gel was prepared by copolymerized fibrin substrate into 12.5% separating gel. A total of 10 mL of separating gel was mixed with 1 mL of 0.25% fibrinogen and 0.2 mL of 0.1% thrombin. After electrophoresis, the gel was denatured using 2.5 % Tween 20 (v/v) by agitation for 1 hour and then digested for 30 min at 60 ° C in 0.05 M phosphate buffer pH 8.0. The gel was stained with Coomassie Brilliant Blue. The presence of fibrinolytic enzyme was shown as a clear zone on the blue background.

Identification of the bacterial isolate

Isolate PB-12 was identified using biochemical tests, morphology, and 16S rRNA. The total genomic DNA of PB-12 was isolated using the Wizard Genomic DNA Purification kit (Promega Corporation, USA). The PCR amplification of the 16S rRNA gene was carried out using two primers 27F (27F (5’ AGA GTT TGA TCM TGG CTC AG 3’)- 907R (5’ CCG TCA ATT CMT TTG AGT TT 3’), and 533F (5’ GTG CCA GCM GCC GCG GTA A 3’)-1492R 1492R (5’ GGT TAC CTT GTT ACG ACT T 3’) (Sri Pananjung et al., 2016). The 16S rRNA gene sequences were similar using the BLASTN programme. The phylogenetic tree was constructed by applying the neighbour-joining method using the MEGA5.05 programme with 1000 replicates of bootstrap values.

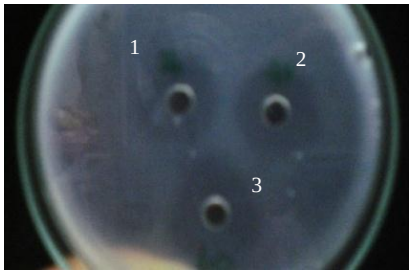
Results

Fibrinolytic assay

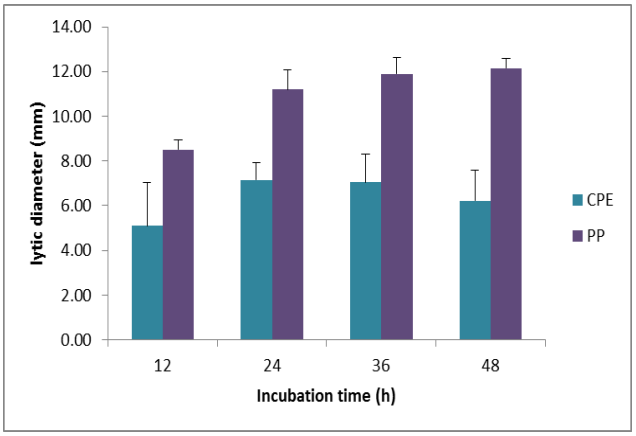
Fibrinolytic activities were evaluated in CPE and PP of PB-12 isolate after being grown in LB broth and harvested at hours 12, 24, 36, and 48 (Figure 1A). Both CPE and PP can degrade the fibrin substrate. This result indicated PB-2 isolate producing extracellular fibrinolytic enzyme. The fibrinolytic activity result showed CPE at 24 h had the highest fibrinolytic activity with a lysis diameter of 5.23 ± 0.68 mm. The lysis diameter of PP is greater than the CPE lysis diameter (Figure 1B).

SDS-PAGE and zymography analysis

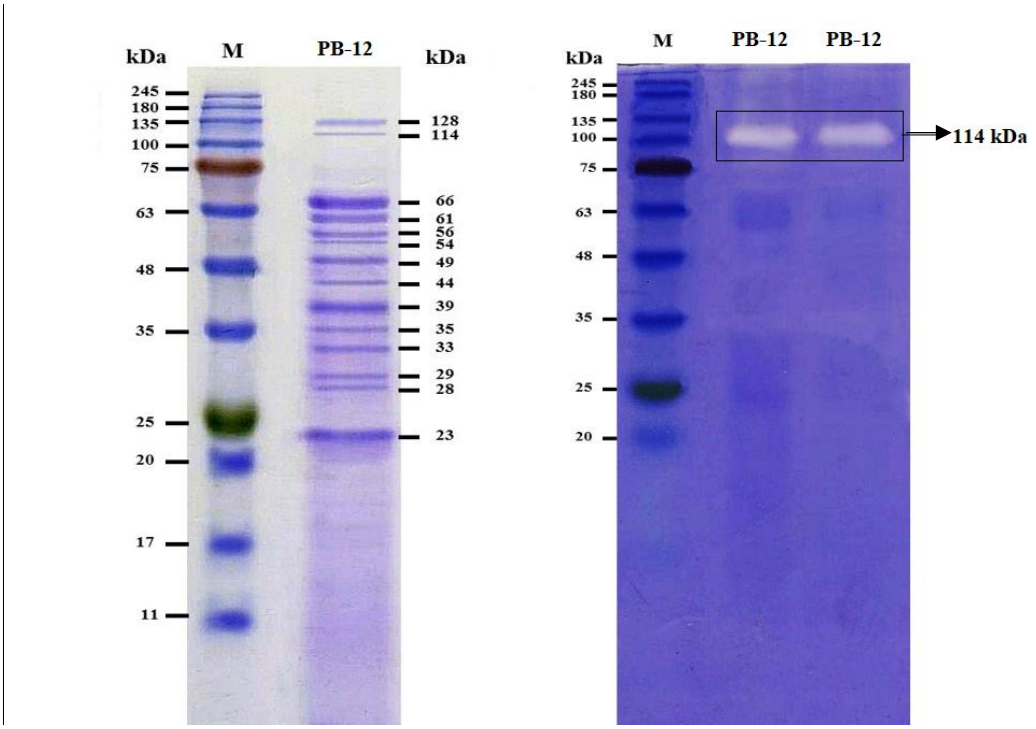
Based on fibrin zymography, we can also examine the molecular size of the fibrinolytic enzyme. The Coomassie Brilliant BlueR250 stained gels showed one white band on blue background with a molecular mass of ~114 kDa (Figure 1C, 1D). Fibrin hydrolytic activities indicate the presence of one fibrinolytic enzyme. This protein could degrade the fibrin substrate on the gel directly.



(A) Fibrinolytic activity of PP on a fibrin agar plate. (1) h-24, (2) h-36, (3) h-48



(B) Lytic diameter of CPE and PP



(C) SDS PAGE, (C) Zymogram of fibrinolytic activity of PP. (M) Protein Ladder, (arrow) molecular weight of the fibrinolytic enzyme was approximately 114 kDa

(C) **(D)**

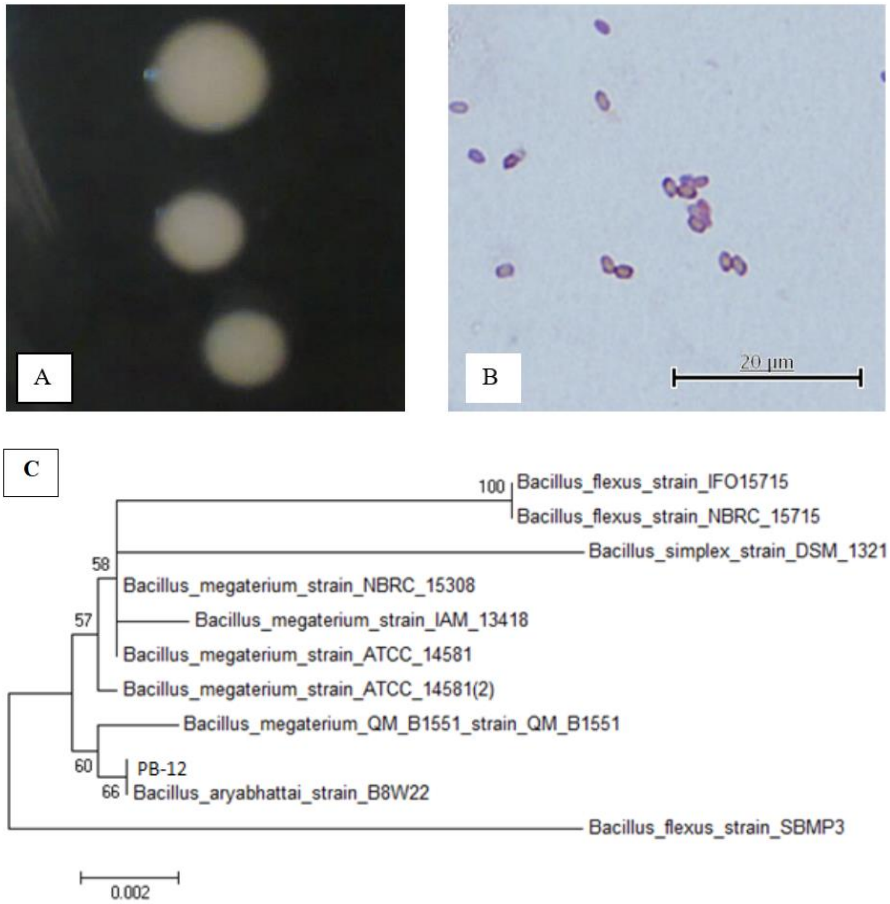
Figure 1: Fibrinolytic activity and Fibrin zymography

Identification of isolate PB-12

The bacterial of PB-12 is a gram-positive, short rod with smooth edges (Figure 2A). Biochemical analysis showed that isolate PB-12 was a positive catalase activity, capable of producing acid from sucrose, fructose, glucose, and lactose.

This bacterium did not reduce nitrate, produce indole, and cannot utilise citrate. Based on its cultural characteristic, isolate PB-12 was able to grow at pH 5 - 9 (Table I).

The 16 S rRNA gene PCR product using 27F - 907R and 533F - 1492R primers were approximately 880 bp and 959 bp, respectively. BLASTN results of PCR products sequence showed that PB-12 has a 99.0% similarity with *Bacillus aryabhattai.* The phylogenetic analysis using MEGA5 software supported the BLASTN result that PB-12 strongly correlates with *B. aryabhattai* (Figure 2C). Based on 16S rRNA gene sequence analysis and biochemical tests, PB-12 isolate was identified as *Bacillus aryabhattai* and named *Bacillus aryabhattai* PB-12.



(A). Morphology of colony on the agar plate, (B) Gram staining image (1000x), (C). Phylogeny tree showing relationships of isolate PB-12 and related bacterial strains from GenBank

Figure 2: Morphological and phylogenetic tree of isolate PB-12

Table I: Morphological and biochemical characteristics of PB-12 bacterial isolate

Characteristics	Results
Morphology	Round, convex, smooth edges,
Gram staining	Coccus, Gram-positive
Hydrolysing ability	
Starch	+
Casein	+
Catalase	+
Motility	+
Nitrate reduction	-
Indole production	-
H_2S production	-
Citrate utilisation	-
Acid Fermentation	
Sucrose	+
Fructose	+
Glucose	+
Lactose	-
pH	5-9

Discussion

Coastal lands harbour high microorganism diversity which produces a potential source of new bioactive compounds for industrial, pharmaceutical, and agricultural uses (Baharum, 2010). The unique habitats of the marine environment involving high salinity, low temperature, and nutrition concentration make enzymes generated from marine microorganisms differ from enzymes from terrestrial microorganisms (Cheng et al., 2020).

In this study, PB-12 isolate from Papuma Coastal had fibrinolytic activities. Some fibrinolytic enzymes were already found in marine microorganisms such as Marinobacter aquaeolei MS2-1, Bacillus velezensis BS2, Bacillus subtilis A26, Streptomyces lusitanus, Bacillus vallismortis, Bacillus pumilus BS15, Streptomyces violaceus VI-TYGM, Alteromonas piscicida, Pseudoalteromonas sp. Shewanella sp. IND20, Serratia rubidaea KUAS001 (Barzkar, Jahromi, & Vianel, 2022), Serratia Marcescens Subsp. Sakunensis (Krishnamurthy & Belur, 2018),

Bacillus licheniformis VITLMS (Kumar et al., 2021), and Aspergillus versicolor ZLH-1 (Zhao et al., 2022).

The enzyme production yields in fermentation are strongly dependent on the incubation period. The incubation period significantly influences the density of cells and fibrinolytic enzyme production. Generally, the optimal fibrinolytic enzyme production is in the early stationary phase (Khursade et al., 2019). In this result, the fibrinolytic enzyme production increased fibrinolytic activity from 12 hours to 24 hours, and remained steady at 36 hours and 48 hours. This condition could be due to the increased number of isolated PB-12 cells in the log phase until the early stationary phase.

SDS-fibrin zymography analysis showed that isolate PB-12 produced one distinct band. The molecular weight of the fibrinolytic enzyme was approximately 114 kDa (Figure 1D). This molecular weight is similar to the fibrinolytic enzyme produced by Weissella thailandensis (116 KDa), but almost twice the amount of fibrinolytic enzyme from Shewanella sp. IND20 (55.5 Kda) (Vijayaraghavan & Prakash, 2015), and Arthosira platensis (72Kda) (Barros et al., 2020). Barzkar, Jahromi, and Vianel (2022) reported the molecular mass of marine fibrinolytic enzymes ranging from 21 kDa to 72 kDa. In conclusion, the molecular weight of marine fibrinolytic enzymes varies depending on the species of microorganism.

Various studies have shown the diversity of marine microorganisms producing fibrinolytic enzymes. In the present research, isolate PB-12 obtained from Papuma Coastal was identified as Bacillus aryabhattai. Marine microorganisms belonging to the genus Bacillus are considered the most important source of fibrinolytic enzymes. Some species of Bacillus were reported had fibrinolytic activity were Bacillus flexus, B.pumilus BS2, B licheniformis KJ-31, B subtillis JS2, B subtillis HQS-3, B vallimortis, and B. velezensis BS2 (Barzkar, Jahromi, & Vianel, 2022). A finding of the marine fibrinolytic enzyme of B. aryabhattai is considered surprising because the fibrinolytic enzyme from B. aryabattai has not been reported previously.

Conclusion

The fibrinolytic enzyme produced by PB-12 originated from Papuma Coastal could effectively degrade fibrin clots directly. This isolates closely related to Bacillus aryabhattai with 99% identity using 16S rRNA sequence. The molecular weight fibrinolytic enzyme harboring isolate PB-12 was approximately 114 kDa. Bacillus aryabhattai PB-12 is considered a potential isolate to produce fibrinolytic enzymes.

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Conflict of interests

The authors declared that there is no conflict of interest regarding the publication of this paper.

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