A marine-derived fibrinolytic protease from *Bacillus paramycoides* AL6: Partial purification, preliminary characterization and *in vivo* antithrombotic efficacy

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Abstract. Thrombosis is a pathological condition characterized by blood clot formation that obstructs blood flow and contributes substantially to morbidity and mortality worldwide. Limitations of conventional thrombolytic therapies have prompted the development of enzyme-based alternatives, particularly microbial fibrinolytic enzymes. The present study aimed to identify the AL6 bacterial isolate, partially purify and preliminarily characterize its fibrinolytic protease and evaluate its antithrombotic efficacy *in vivo*. Molecular identification using the 16S rRNA gene revealed that the AL6 isolate shared 98.27% similarity with *Bacillus paramycoides*. The enzyme preparation was subjected to anion-exchange chromatography, yielding a partially purified fibrinolytic enzyme preparation with protein bands in the 30-50 kDa range on SDS-PAGE. *In vitro* fibrinolytic activity, assessed by clot lysis, increased with increasing enzyme volume, with clot lysis values of 47.02±3.00, 57.00±3.67 and 76.30±3.17% at enzyme volumes of 100, 200 and 300 μl, respectively. However, they remained lower than those of streptokinase (81.25-92.31%). *In vivo* antithrombotic efficacy was evaluated using a κ-carrageenan-induced mouse tail thrombus model, in which the partially purified enzyme markedly reduced thrombus length compared with the negative control and exhibited a dose-dependent effect. The highest thrombus reduction (52.54%) was achieved at 0.88 mg/kg body weight and was not markedly different from the positive control. Preliminary safety assessment showed that bleeding time remained within normal limits, suggesting no apparent alteration in hemostasis under the tested conditions. Overall, the partially purified fibrinolytic protease preparation from *B. paramycoides* AL6 demonstrated promising *in vitro* fibrinolytic activity and *in vivo* antithrombotic efficacy. However, further purification, biochemical characterization and comprehensive safety evaluation are required to determine its potential for future therapeutic development.

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Introduction

Thrombosis is a pathological condition characterized by the formation of a blood clot (thrombus) within a blood vessel, which can obstruct blood flow in arteries, veins and the microcirculation (1). Virchow's triad explains the mechanism of thrombosis through hypercoagulability, blood flow stasis and endothelial damage (2). These three factors reflect disruption of the hemostatic system, which normally balances coagulation and anticoagulation. In pathological conditions, this balance is disrupted, requiring therapeutic intervention to prevent further complications (3). Globally, thrombotic diseases remain a leading cause of morbidity and mortality, with ~19.8 million deaths from cardiovascular disease in 2022, or 32% of all deaths worldwide, primarily due to heart attacks and strokes (4).

Conventional thrombolytic therapies, including streptokinase, tissue plasminogen activator and urokinase, are effective but have several limitations, including high cost, short half-life, low fibrin specificity and risks of bleeding and immunological reactions (3,5). The economic burden of conventional thrombolytic therapy remains considerable, with recent reports estimating the cost of alteplase at US$2,200-2,400 per 100 mg and urokinase at ~US$140 per 250,000 IU (6). In recent years, enzyme-based therapies have gained increasing attention due to their high specificity, good catalytic efficiency and adequate stability (3). Fibrinolytic enzymes from microorganisms can degrade fibrin, the main component of thrombus and thus potentially dissolve blood clots effectively (7). Furthermore, these enzymes offer low production costs and ease of large-scale production (8).

Microorganisms, particularly bacteria, are a major source of enzymes because they can secrete various extracellular enzymes. The genus *Bacillus* is one of the most widely used in biotechnology because it can produce various enzymes, including proteases (9). Proteases break peptide bonds and support important biological functions such as cell growth and nutrient cycling (10). Compared with other sources, microbial proteases are superior because of their rapid growth, ease of genetic modification and economic feasibility for large-scale production (11). In particular, fibrinolytic enzymes from Bacillus have been widely reported to have promising antithrombotic activity (12-15) and have the potential to be developed as a safer, less invasive therapy (16).

Fibrinolysis is an important physiological mechanism in maintaining hemostasis through the degradation of fibrin in thrombi. Microbial fibrinolytic enzymes, including plasminogen activators, have been extensively studied for their ability to hydrolyze thrombi efficiently with minimal side effects (17). As well as thrombolytic therapy, these enzymes also have potential applications in antimicrobial activity and blood pressure regulation. However, challenges remain in identifying new enzymes with high stability, optimal activity and good specificity for fibrin, as well as in the often complex enzyme purification process (18).

Growing exploration of enzyme sources shows that microorganisms from specific environments can produce fibrinolytic enzymes with superior characteristics. Growing exploration of enzyme sources shows that microorganisms from specific environments can produce fibrinolytic enzymes with superior characteristics. A new fibrinolytic enzyme from *Bacillus sp.* with high specific activity (736.4 U/mg), broad pH stability, and high affinity for fibrin has been reported (19). Furthermore, bacterial isolates from certain environments have been shown to produce proteases with very high thrombolytic activity, with up to 99.7% clot lysis (20). These findings confirm that exploring microbial isolates still offers significant opportunities to discover candidate fibrinolytic enzymes with superior performance. Furthermore, recent advances in therapeutic discovery, including artificial intelligence-guided drug repurposing, highlight the growing importance of integrating innovative approaches with experimental validation and translational assessment to develop potential therapeutic agents (21).

One isolate demonstrating this potential is AL6, which was reported to have high fibrinolytic activity *in vitro*, with clot lysis reaching 79.48% (22). However, information regarding its molecular identity, enzyme characteristics and biological effectiveness *in vivo* remains limited. Furthermore, studies that comprehensively integrate enzyme purification, molecular characterization and *in vivo* thrombolytic activity evaluation remain rare.

Therefore, the present study aims to molecularly identify the AL6 bacterial isolate, partially purify and preliminarily characterize its fibrinolytic protease and evaluate its *in vivo* antithrombotic efficacy using a mouse (*Mus musculus*) tail thrombus model at doses of 0.22, 0.44 and 0.88 mg/kg body weight.

Materials and methods

Isolation and identification of fibrinolytic enzyme-producing marine microorganisms. Isolation and identification of fibrinolytic enzyme-producing marine microorganisms from the Tanjung Dewa coast of South Kalimantan, Indonesia, were carried out following the procedure described by Lutpiatina *et al* (22). The isolation stage was performed using serial dilution, followed by cultivation of the bacterial suspension on Nutrient Agar (NA) medium and incubation at 37˚C for 24-48 h to obtain distinct colonies. Pure colonies were obtained by repeated streaking on NA medium. Initial identification was performed based on colony morphology and Gram staining. For Gram staining, bacterial smears were heat-fixed, sequentially stained with crystal violet and iodine, decolorized with alcohol or acetone-alcohol, and counterstained with safranin before microscopic examination. Spore formation was confirmed using the Schaeffer-Fulton method by preparing a heat-fixed smear, staining with malachite green with gentle heating to facilitate penetration into spores, rinsing with water to remove excess stain, and counterstaining with safranin. The preparations were examined microscopically to determine cellular and spore morphology.

Proteolytic activity was tested by growing the isolates on Skim Milk Agar (SMA) medium containing 1% skimmed milk. After incubation at 37˚C for 48 h, a clear zone around the colonies was observed to assess casein degradation ability. Fibrinolytic activity was tested using fibrin agar medium prepared from fibrinogen and agar in phosphate buffer, followed by the addition of thrombin to form a fibrin matrix. A bacterial suspension or crude enzyme was added to the wells of the medium and incubated at 37˚C for 18-24 h. The formation of a clear zone due to fibrin degradation is an indicator of fibrinolytic activity. Isolate AL6 was selected due to its good proteolytic and fibrinolytic activity (22).

Molecular identification of isolate AL6. Molecular identification of isolate AL6 was performed using 16S rRNA gene analysis. Genomic DNA was extracted from a 24-h pure culture of isolate AL6 grown in Nutrient Broth using the Presto Mini gDNA Bacteria Kit (cat. no. GBB100/101; Geneaid Biotech Ltd.), according to the manufacturer's instructions. The concentration and purity of the extracted DNA were assessed using a spectrophotometer. The extracted DNA had a concentration of 67.34 ng/μl and an A260/A280 ratio of 1.84. Genomic DNA extracted from a 24-h pure culture of isolate AL6 grown in Nutrient Broth using the Presto Mini gDNA Bacteria Kit (GBB100/101; Geneaid Biotech Ltd.) was used as the template for PCR amplification of the bacterial 16S rRNA gene. PCR was performed using KAPA2G Robust DNA Polymerase (Roche Diagnostics GmbH) with the universal primers 27F (5'-AGAGTTTGATCMTGGCTC AG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The thermocycling conditions consisted of an initial denaturation at 98˚C for 30 sec, followed by 30 cycles of denaturation at 98˚C for 10 sec, annealing at 55˚C for 30 sec, and extension at 72˚C for 45 sec, with a final extension at 72˚C for 2 min. PCR products were separated by electrophoresis on a 1% agarose gel prepared in 1X TAE buffer and visualized using GelRed® under a UV transilluminator. Successful amplification was confirmed by the presence of an approximately 1,500-bp DNA band. The 16S rRNA analysis was performed for bacterial identification using conventional PCR followed by Sanger sequencing. No loading control was used because

the assay was performed for bacterial identification rather than quantitative gene-expression analysis.

PCR products showing the expected ~1,500-bp band on electrophoresis were subjected to Sanger sequencing using the 27F and 1492R primers. The resulting sequences were processed using BioEdit version 7.5.2 7.2.5 (https://www.bioz.com/product/bioedit+version+7+2+5/pm42291150-64-83-83?v=Bioedit+Company) to generate a consensus sequence and compared with 16S rRNA gene sequences in the GenBank database using BLASTn (NCBI, https://www.ncbi.nlm.nih.gov/) to determine sequence similarity. To further support the identification results, a phylogenetic tree was constructed using the neighbor-joining method based on multiple sequence alignment. This analysis was used to assess the phylogenetic relationship of isolate AL6 with other bacterial species.

Fibrinolytic protease preparation by anion-exchange chromatography and molecular weight estimation. Enzyme fractionation was performed using anion-exchange chromatography with the ÄKTApure system and a HiTrap Q FF column (1 ml). Crude enzyme was obtained from a culture in Nutrient Broth and then centrifuged at 10,000 x g for 10 min at 4˚C to separate the cells. The supernatant was filtered and concentrated before being subjected to chromatographic separation.

The column was equilibrated using a low-salt buffer (10 mM NaCl) at a flow rate of 1 ml/min. The sample was loaded onto the column and washed with 10-20 mM NaCl buffer to remove unbound proteins. Elution was performed using a 20-1,000 mM NaCl gradient over 20 column volumes. Protein absorbance was monitored at 280 nm and fractions were collected for further analysis. Fractions F8-F14 were pooled for subsequent fibrinolytic activity and SDS-PAGE analyses.

The molecular weight profile of the protein preparation was assessed using SDS-PAGE. Protein samples were denatured using SDS buffer and separated on a 12% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue at room temperature for 1 h to visualize protein bands, and apparent molecular weights were estimated by comparison with standard molecular weight markers. As total protein concentration and specific activity were not determined, the chromatographic separation could not be evaluated in terms of purification fold, enzyme enrichment, or purification efficiency.

Fibrinolytic activity test (clot lysis assay). The clot lysis assay was performed according to the method described previously (22). Peripheral venous blood samples were collected from two healthy adult female volunteers (median age, 22 years; age range, 22-22 years) following approval from the Research Ethics Committee and written informed consent from each participant. Participants were recruited at Poltekkes Kemenkes Banjarmasin in January 2026 (Ethics approval no. 881/KEPK-PKB/2025). Trained personnel collected 9 ml of peripheral venous blood from each participant using standard aseptic procedures. The blood was transferred into pre-weighed microtubes and allowed to clot. Following clot formation, the samples were centrifuged at 1.207 g for 5 min at room temperature to remove the serum, and the initial clot weight was determined from the difference in microtube mass before and after clot formation.

The clots were treated with distilled water (negative control), streptokinase (positive control), or a fibrinolytic enzyme at 100, 200 and 300 μl (1 mg/ml). All samples were incubated at 37˚C for 24 h. After incubation, the lysis fluid was discarded and the microtubes were reweighed. The percentage of lysis was calculated by comparing the clot weight before and after treatment using the formula: percentage of lysis=(initial clot weight - final clot weight)/initial clot weight x100%. This formula was used to determine the sample's effectiveness in lysing blood clots.

In vivo antithrombotic effect evaluation in experimental animals. The *in vivo* antithrombotic effect was evaluated using 55 male BALB/c mice (Mus musculus), aged 2-3 months and weighing 20-30 g. The mice were obtained from Balai Veteriner Banjarbaru, South Kalimantan, Indonesia, and were assigned to three enzyme-treatment groups and two control groups (negative and positive). Prior to and throughout the experiment, the mice were maintained under standard laboratory conditions at a mean temperature of 22±3˚C, relative humidity of 30-70%, and an automatic 12-h light/dark cycle. Each mouse was housed individually in a transparent plastic cage with adequate ventilation and rice-husk bedding, with standard laboratory feed and drinking water provided ad libitum. The animals were maintained and handled in accordance with the Guide for the Care and Use of Laboratory Animals (8th ed.) https://doi.org/10.17226/12910 (23) and applicable institutional animal welfare guidelines. At the end of the experiment, the mice were sacrificed by cervical dislocation performed by trained personnel at the Veterinary Center. No euthanasia drugs were used and death was confirmed by the absence of respiration and other signs of vital activity.

The mice were randomly divided into five groups (n=9 per group): a negative control group receiving 0.9% NaCl solution, a positive control group receiving streptokinase at a dose of 0.44 mg/kg body weight and three treatment groups receiving the partially purified fibrinolytic enzyme preparation at doses of 0.22, 0.44 and 0.88 mg/kg body weight, respectively. All treatments were administered intravenously at 0.1 ml per mouse.

The thrombosis model was induced by intravenous injection of κ-carrageenan at 40 mg/kg body weight into the tail vein. After 24 h, thrombus formation was assessed by measuring thrombus length using a caliper. This measurement was recorded as the pre-treatment thrombus length. The respective test preparations were then administered according to the assigned group.

After treatment, thrombus length was measured again using a caliper and recorded as the post-treatment thrombus length. Thrombus reduction was defined as the decrease in thrombus length after treatment relative to the pre-treatment length and was used as an indicator of *in vivo* antithrombotic activity. The percentage of thrombus reduction was calculated using the following formula: Thrombus reduction (%)=[(thrombus length before treatment - thrombus length after treatment)/thrombus length before treatment] x100%. A higher thrombus reduction

Table I. Results of isolate genomic DNA isolation.

No.	Sample code	Concentration (ng/μl)	A260	A230	A280	A260/A230	A260/A280
1	AL 6	67.34	1.34	0.69	0.74	2.0	1.84

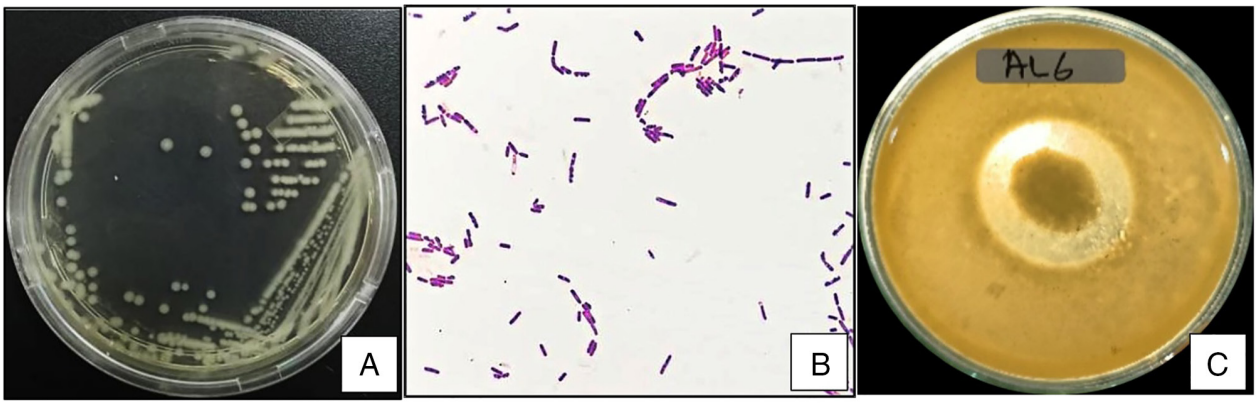

Figure 1. Morphological, microscopic and proteolytic characteristics of bacterial isolate AL6. (A) Colony morphology on nutrient agar, (B) microscopic morphology following Gram staining and (C) proteolytic activity on skim milk agar.

percentage indicated greater reduction of thrombus length under the tested conditions.

As an initial safety assessment, bleeding time was measured 24 h after treatment using a standardized wound at the distal end of the tail. The bleeding time was recorded as the time required for bleeding to cease. The animals were monitored throughout the experimental period for signs of distress or other adverse events in accordance with predefined humane endpoint criteria.

The Ethics Commission of the Banjarmasin Ministry of Health Polytechnic reviewed and approved the study protocol and it was declared ethically sound under Decree No. 881/KEPK-PKB/2025.

Statistical analysis. Clot lysis data were analyzed using parametric tests after meeting the assumptions of normality (Shapiro-Wilk) and homogeneity of variance (Levene's test). Comparisons between groups were performed using one-way ANOVA and Tukey's post hoc test. Data on thrombus length were analyzed using the Kruskal-Wallis test due to non-normality and non-homogeneity. Pairwise comparisons were performed using the Mann-Whitney U test with Holm adjustment for multiple comparisons. A Holm-adjusted $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of AL 6 bacterial isolate. AL 6 bacterial isolate was characterized by macroscopic and microscopic morphology after growth on Nutrient Agar and incubation for 24 h at 37˚C. Macroscopically, AL 6 isolate colonies appeared circular, had entire edges and were flat. The colonies were white, had a glistening surface, were translucent and had a dry consistency (Fig. 1A).

Microscopic observation using Gram staining showed that isolate AL6 had bacillus-shaped cells, was Gram-positive and could form spores (Fig. 1B). These characteristics indicate that isolate AL 6 belongs to the Gram-positive bacterial group and may belong to the genus Bacillus. The proteolytic index was calculated from a clear zone diameter of 21 mm and a colony diameter of 13 mm, yielding an index of 1.61 (Fig. 1C). Meanwhile, the fibrinolytic index was calculated from a clear zone 19 mm in diameter and a colony 8 mm in diameter, yielding a final index of 2.37.

Molecular identification. Molecular identification of isolate AL 6 was performed through 16S rRNA gene sequence analysis. Genomic DNA was successfully isolated at a concentration of 67.34 ng/μl with an A260/A280 purity ratio of 1.84 and an A260/A230 purity ratio of 2.0 (Table I), indicating adequate DNA quality for further analysis. PCR amplification of the 16S rRNA gene produced a single DNA band measuring ~1,500 bp. Agarose gel electrophoresis revealed a clear band corresponding to the marker, indicating successful amplification (Fig. 2).

The sequencing results were analyzed using BLASTn in the GenBank database. Based on the analysis results (Table II), isolate AL 6 showed the highest similarity to *Bacillus paramycoides* strain MCCC 1A04098, with 98.27% identity, an E-value of 0.0 and 100% query coverage. These values indicated a strong match between isolate AL 6 and the reference species.

To strengthen the identification, the present study performed phylogenetic analysis using 16S rRNA gene sequences. The results showed that isolate AL 6 clustered with several *Bacillus paramycoides* strains, including WS3, ASS-23 and MCCC 1A04098 (Fig. 3). This cluster is adjacent to the *Bacillus cereus* group, which includes *B. cereus*, *B. thuringiensis*, *B. wiedmannii*, *B. albus* and *B. anthracis*. Meanwhile, *B. subtilis* and *B. pumilus* are in different branches

Table II. BLASTn analysis results of 16S rRNA gene sequence bacterial isolate AL 6.

Isolate code	Sequence length	Bacterial name	Accession number	Percentage identity	E-value	Query cover
AL 6	1,389	*Bacillus paramycoides* strain MCCC 1A04098	NR_157734.1	98.27%	0.0	100%

Table III. Comparison of fibrinolytic activity of crude enzyme and partially purified fractions (F8-F14) based on clot lysis percentage.

Enzyme preparation	100 μl clot lysis (%)	200 μl clot lysis (%)	300 μl clot lysis (%)
Crude enzyme (Isolate AL 6)	40.47	55.00	69.48
Pooled fraction (F8-F14)	47.02±3.00	57.00±3.67	76.30±3.17

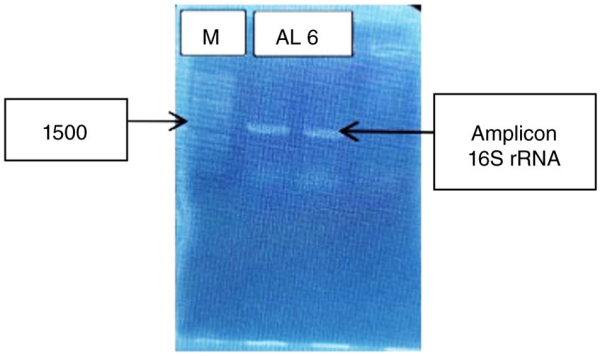

Figure 2. Agarose gel electrophoresis visualization of the amplified 16S rRNA gene of bacterial isolate AL6. DNA bands corresponding to the amplified 16S rRNA gene fragment used for molecular identification. M, DNA molecular weight marker.

but still within the same genus, while *Paenibacillus polymyxa* is a separate outgroup. These results support the tentative identification of isolate AL6 as *B. paramycoides*-related, although species-level identification cannot be confirmed based on 16S rRNA analysis alone.

Purification and characterization of fibrinolytic protease enzyme

Enzyme fractionation by anion-exchange chromatography. Fibrinolytic enzyme fractionation was performed using anion-exchange chromatography on a HiTrap Q FF column (1 ml) with the ÄKTA system. The chromatographic procedure consisted of equilibration, sample loading, washing and elution using a 20-1,000 mM NaCl gradient.

The chromatogram (Fig. 4) showed several peaks corresponding to protein-containing fractions separated during the chromatographic run. A main peak was observed at 30-50 ml. Fractions within this region were collected and subsequently evaluated for fibrinolytic activity. Fractions F8-F14 were pooled for further analysis.

Molecular weight analysis (SDS-PAGE). The partially purified enzyme preparation was analyzed using SDS-PAGE to characterize its protein band profile. The SDS-PAGE results (Fig. 5) showed several protein bands in the initial sample (S), flow-through (FT) and chromatographic fractions (F3, F6, F8, F10, F14, F18 and F20). Differences in band patterns and intensities were observed among the fractions, with prominent bands appearing in fractions F8, F10 and F14.

Compared with the Broad Multicolor Prestained Protein Standard (15-270 kDa) marker, the observed protein bands were estimated to be within the 30-50 kDa range. The presence of multiple protein bands indicates that the F8-F14 preparation was not electrophoretically homogeneous and therefore represents a partially purified enzyme preparation rather than a single purified protein.

In vitro fibrinolytic activity (clot lysis assay). The fibrinolytic activity test showed that the pooled F8-F14 preparation exhibited clot-lysis activity at all enzyme volumes tested (Table III). At a volume of 100 μl, clot lysis was 47.02±3.00%, compared with 40.47% for the crude enzyme. At 200 μl, clot lysis was 57.00±3.67% for the pooled F8-F14 preparation and 55.00% for the crude enzyme. At 300 μl, the corresponding values were 76.30±3.17 and 69.48%, respectively. These findings demonstrate measurable fibrinolytic activity in both the crude enzyme and pooled F8-F14 preparation. However, because total protein concentration and specific activity were not determined, the observed differences in clot-lysis percentage cannot be interpreted as evidence of enzyme enrichment or increased purification efficiency.

In vitro fibrinolytic activity testing using the clot lysis method showed that the fibrinolytic enzyme preparation was able to lyse blood clots in a volume-dependent manner. The percentage of lysis increased with increasing enzyme volume, reaching 44.00-50.00% at 100 μl, 52.94-60.00% at 200 μl and 73.91-80.00% at 300 μl. The positive control (streptokinase) produced the highest values (81.25-92.31%), whereas the negative control showed no lytic activity. These findings demonstrate measurable fibrinolytic activity of the enzyme preparation under the tested conditions, although the observed activity remained lower than that of the standard thrombolytic agent. The complete results of the clot lysis assay, including the values obtained for each enzyme volume and control group, are presented in Table IV.

Table IV. Clot lysis percentage results.

Group	Replicate	Initial blood clot weight (g)	Final blood clot weight (g)	Difference (g)	Clot lysis (%)
FPE 1 mg/ml 100 μl	1	0.28	0.14	0.14	50.00
	2	0.34	0.18	0.16	47.06
	3	0.25	0.14	0.11	44.00
FPE 1 mg/ml 200 μl	1	0.17	0.08	0.09	52.94
	2	0.20	0.08	0.12	60.00
	3	0.31	0.13	0.18	58.06
FPE 1 mg/ml 300 μl	1	0.23	0.06	0.17	73.91
	2	0.20	0.04	0.16	80.00
	3	0.16	0.04	0.12	75.00
Positive control	1	0.13	0.01	0.12	92.31
(streptokinase 3000 IU, 100 μl)	2	0.16	0.03	0.13	81.25
	3	0.13	0.02	0.11	84.62
Negative control (distilled	1	0.17	0.17	0.00	0.00
water 100 μl)	2	0.15	0.15	0.00	0.00
	3	0.20	0.20	0.00	0.00

FPE, fibrinolytic protease enzyme.

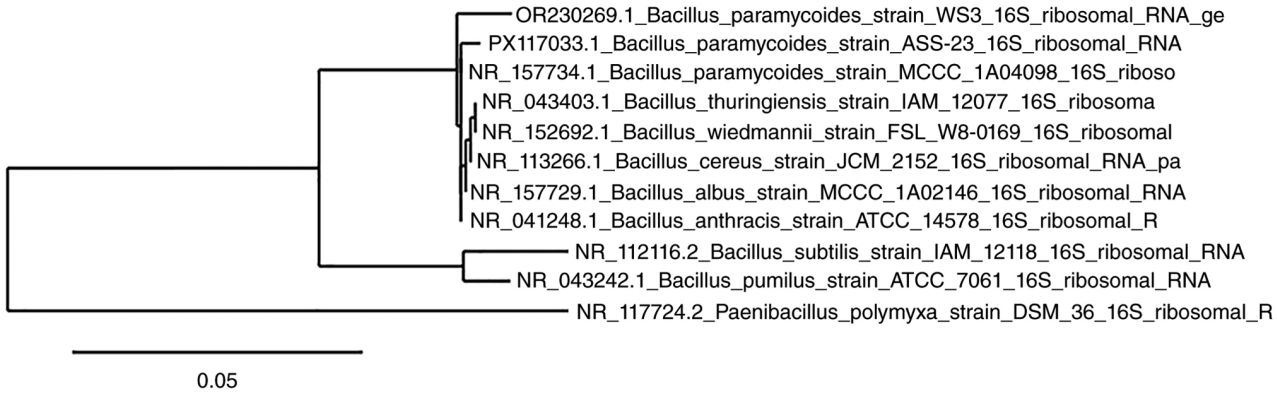

Figure 3. Phylogenetic analysis of bacterial isolate AL6 based on 16S rRNA gene sequences. Phylogenetic tree showing the relationship between isolate AL6 and closely related *Bacillus* species.

Statistical analysis of clot lysis activity. Statistical analysis showed significant differences among the tested enzyme volumes ($P<0.05$) and Tukey's post-hoc test identified significant differences between each pair of enzyme volumes. These findings support a volume-dependent difference in clot-lysis activity under the experimental conditions. However, the analysis does not establish whether chromatographic fractionation. The complete statistical analysis results are listed in Table V.

General condition of test animals and initial safety (bleeding time). During *in vivo* testing, all test animals remained physiologically stable with no mortalities or behavioral changes, indicating the treatment was well tolerated. Initial safety evaluation, based on bleeding time at the highest dose (0.88 mg/kg BW), showed a value of 150-180 sec. The observed bleeding time was consistent with the findings reported in our previous study evaluating the antithrombotic activity and hemolysis pattern of fibrinolytic protease-producing bacterial isolates (22). Thus, the fibrinolytic protease preparation showed no apparent prolongation of bleeding time under the tested conditions, although comprehensive safety and toxicity assessments are required.

In vivo antithrombotic activity in a tail thrombus model. An antithrombotic activity test using a mouse tail thrombus model showed that the fibrinolytic protease enzyme markedly reduced thrombus length compared with the negative control. The negative control group showed no change in thrombus length, while the positive control (streptokinase) reduced thrombus length by 68.20%. In the treatment group, thrombus reduction increased gradually with dose: 46.47% at 0.22 mg/kg BW, 47.72% at 0.44 mg/kg BW and 52.54%

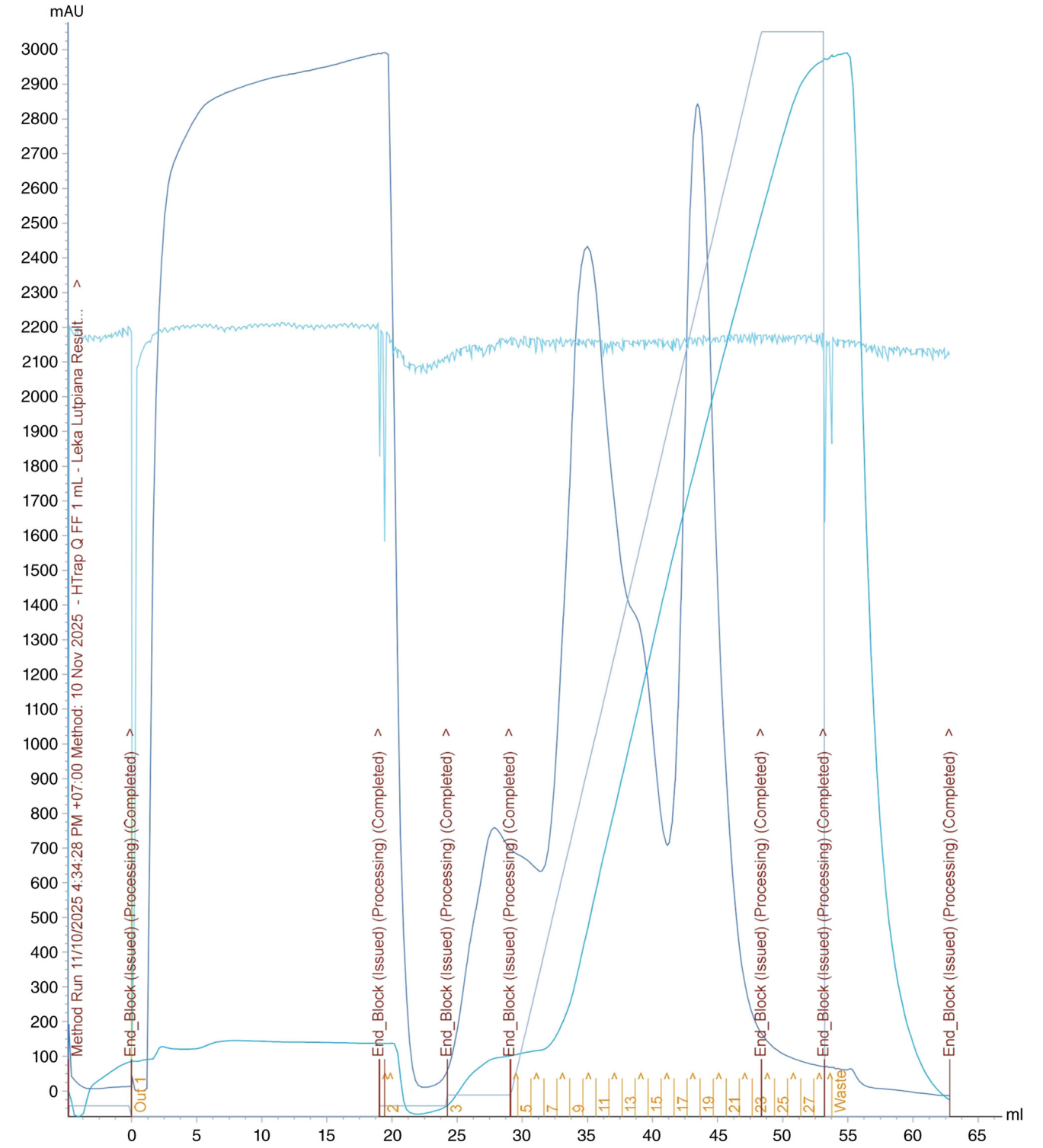

Figure 4. Elution chromatogram of fibrinolytic protease purification using an anion-exchange chromatography HiTrap Q FF (1 ml) column. Protein fractions were collected during purification to identify fractions with potential fibrinolytic activity.

at 0.88 mg/kg BW. These findings indicate a dose-dependent antithrombotic effect.

The present study calculated the percentage reduction as the difference in thrombus length before and after treatment, relative to the initial thrombus length. The higher the value, the greater the enzyme's ability to reduce or lyse thrombi. Complete data on thrombus length and reduction percentage can be seen in Table VI, while a visual representation of the thrombus before and after treatment is shown in Fig. 6.

Statistical analysis of thrombus length differences. Statistical analysis revealed that thrombus length was not normally distributed across all treatment groups, with normality test results ranging from 0.002 to 0.965. In addition, the homogeneity test indicated unequal variances among groups

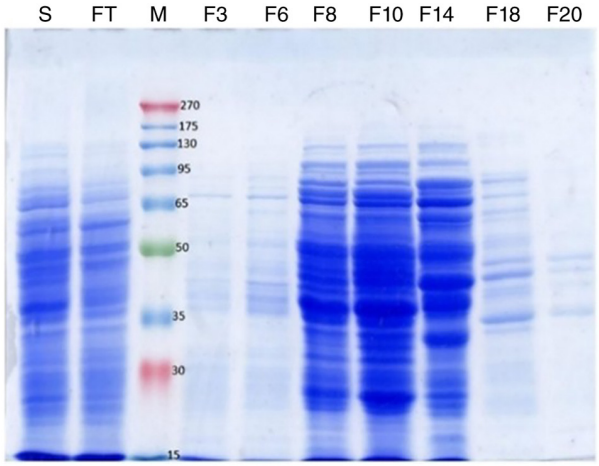

Figure 5. SDS-PAGE profile of the partially purified fibrinolytic protease from bacterial isolate AL6. Protein band patterns of the crude sample, flow-through fraction, protein marker and eluted fractions following purification by anion-exchange chromatography. Prominent protein bands were observed predominantly in fractions F8-F14, with estimated molecular weights ranging from 30-50 kDa based on comparison with the Broad Multicolor Prestained Protein Standard marker. S, crude sample; FT, flow-through fraction; M, protein marker; F3, F6, F8, F10, F14, F18 and F20, eluted fractions.

Table V. Statistical analysis of *in vitro* fibrinolytic activity based on clot lysis percentage.

Test/comparison	P-value (Sig.)
Normality test	0.322-0.978 (Normally distributed)
Homogeneity test	0.850 (Homogeneous variance)
One-way ANOVA	0.000[a]
Post hoc Tukey:	
100 μl vs. 200 μl	0.024[a]
100 μl vs. 300 μl	0.000[a]
200 μl vs. 300 μl	0.001[a]

[a]P<0.05.

(P=0.001). Therefore, the present study applied a non-parametric Kruskal-Wallis test, which showed a statistically significant difference in thrombus length among the treatment groups (P<0.001). The present study then performed pairwise comparisons using the Mann-Whitney U test with Holm adjustment for multiple comparisons.

The post-hoc analysis showed that all fibrinolytic protease enzyme (FPE) treatment groups had markedly different thrombus lengths compared with the negative control after Holm adjustment (P<0.001 for all comparisons). Compared with the positive control, the FPE 0.22 mg/kg BW group differed markedly (adjusted P=0.035). In contrast, the FPE 0.44 mg/kg BW group did not reach statistical significance after Holm adjustment (adjusted P=0.057). Notably, the FPE 0.88 mg/kg BW group also showed no statistically significant difference from the positive control (adjusted P=0.290), suggesting that its antithrombotic effect was statistically comparable to that of the positive control.

Among the FPE treatment groups, no significant difference was observed between the 0.22 and 0.44 mg/kg BW doses (adjusted P=0.566). However, significant differences were observed between the 0.22 and 0.88 mg/kg BW groups (adjusted P=0.024) and between the 0.44 and 0.88 mg/kg BW groups (adjusted P=0.048). These findings indicate a significant difference in thrombus length at the highest FPE dose compared with the lower-dose groups. Overall, the results demonstrated that FPE treatment markedly affected thrombus length, with the 0.88 mg/kg BW dose showing an antithrombotic effect statistically comparable to the positive control. Table VII presents the complete statistical analysis.

Discussion

The results indicate that bacterial isolate AL6 has potential as a producer of a fibrinolytic protease, supported by its molecular characteristics and the fibrinolytic activity of its partially purified enzyme preparation. Molecular analysis based on the 16S rRNA gene showed 98.27% similarity to *Bacillus paramycoides* (100% query coverage) and phylogenetic analysis placed the isolate in the same cluster as the *B. cereus* group, confirming its affiliation with the genus *Bacillus*. This finding is consistent with previous findings in marine *Bacillus* isolates showing lower sequence similarity and identification limited to the genus level (19). This difference suggests that the AL 6 isolate is genetically closer to certain species, although its specific identity cannot be confirmed.

Interpretation of these results requires consideration of the limitations of 16S rRNA identification. Liu *et al* (24) explained that conservation of the 16S rRNA gene in the B. cereus group can result in >97% sequence similarity. Johnson *et al* (25) added that intragenomic heterogeneity and multiple copies can affect identification accuracy. Therefore, the present study putatively categorizes isolate AL 6 as *B. paramycoides* and recommends confirmation through whole-genome sequencing.

During purification, ion-exchange chromatography produced several protein peaks, with the main fraction eluting at 30-50 ml. The multiple peaks indicate that the preparation contained Several protein components were observed, consistent with the enzyme's partially purified nature. This pattern aligns with previous reports showing that Bacillus fibrinolytic enzymes eluted during increasing salt concentrations, while the flow-through fraction showed no detectable fibrinolytic activity (13,19). The separation of multiple protein fractions is also consistent with previous findings, indicating that the purification process reduced protein complexity but did not yield a homogeneous enzyme preparation (15).

SDS-PAGE showed protein bands with molecular weights of 30-50 kDa, with dominant bands in certain fractions. This range is comparable to those reported for fibrinolytic enzymes from Bacillus, including 32.3 and 27.3 kDa, respectively (14,15). The presence of multiple protein bands indicates that the preparation was not electrophoretically homogeneous, supporting its classification as a partially purified enzyme preparation. Other studies support this finding, including fibrinolytic enzymes *from Bacillus cereus* S46 (26), *Bacillus subtilis* K42 (26,27), *Bacillus amyloliquefaciens* MCC2606 (28) and *Bacillus halodurans* IND18 and *Bacillus cereus* IND1 (29,30).

Table VI. Mean initial and final thrombus length, difference, and percentage of thrombus reduction.

Group	Mean initial thrombus length (mm)	Mean final thrombus length (mm)	Difference (mm)	Thrombus reduction (%)
Negative control (κ-carrageenan only)	50.52	50.52	0.00	0.00
Positive control (streptokinase)	44.22	16.07	28.16	68.20
FPE 0.22 mg/kg BW	49.01	27.04	21.97	46.47
FPE 0.44 mg/kg BW	49.56	26.41	23.14	47.72
FPE 0.88 mg/kg BW	57.70	23.98	33.72	52.54

FPE, fibrinolytic protease enzyme; BW, body weight.

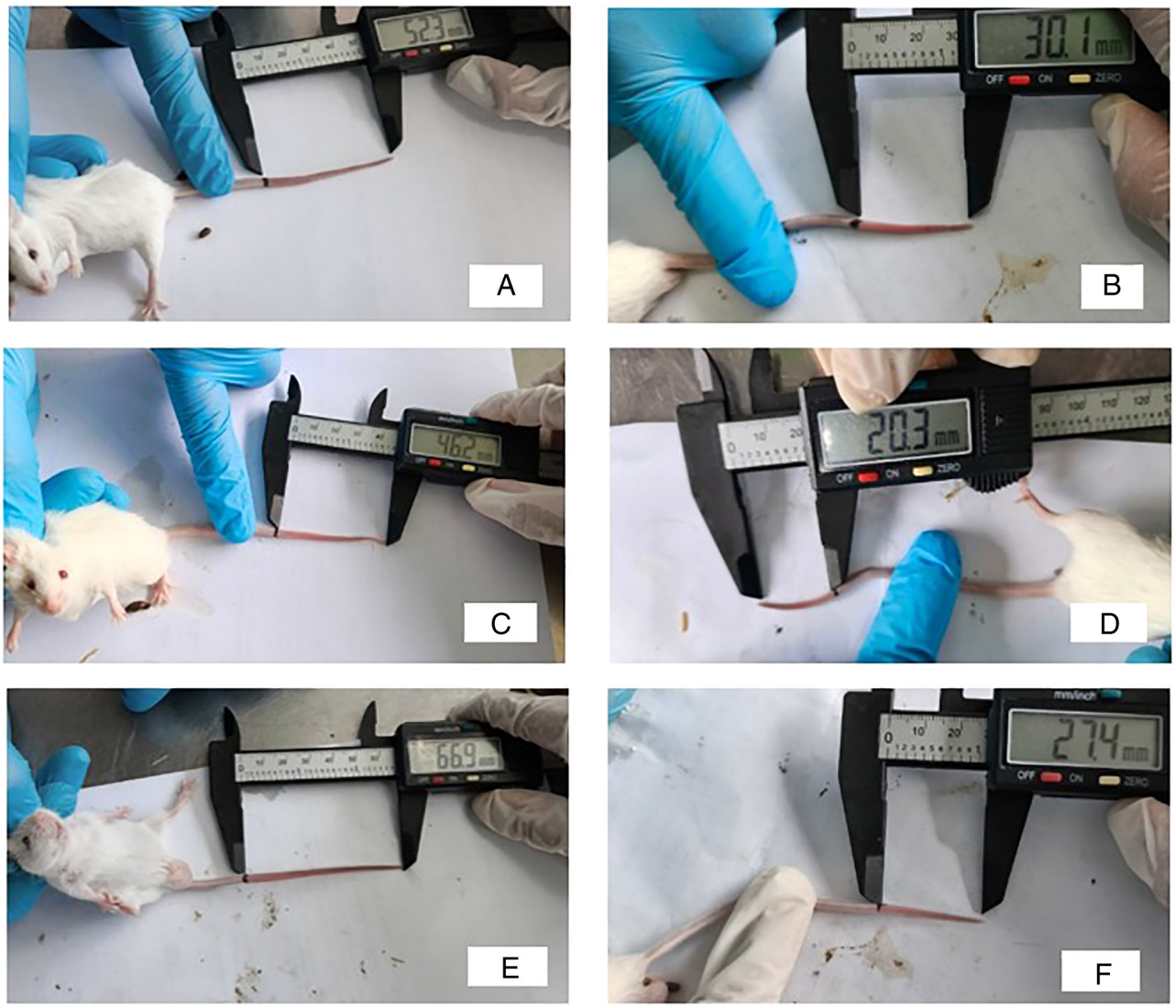

Figure 6. Representative thrombus formation in mouse tails before and after treatment with fibrinolytic protease enzyme. (A, C and E) Tail thrombus length 24 h after κ-carrageenan induction; (B, D and F) thrombus condition following administration of EPE at doses of 0.22, 0.44 and 0.88 mg/kg body weight.

In vitro clot lysis assays demonstrated that clot lysis activity increased with increasing volume of the partially purified fibrinolytic protease preparation (F8-F14). The pooled fraction showed clot lysis percentages of 47.02±3.00, 57.00±3.67 and 76.30±3.17% at volumes of 100, 200 and 300 μl, respectively. Although the activity remained slightly lower than that of the positive control streptokinase (81.25-92.31%), the partially purified fraction exhibited clear dose-dependent fibrinolytic activity. By contrast, the negative control showed no clot lysis. This pattern confirms the dose-dependent relationship reported by Lutpiatina *et al* (22) and Zhou *et al* (15). Differences in

Table VII. Statistical test results of antithrombotic activity based on differences in thrombus length.

Test/comparison	P-value (Sig.)	P-value adjusted (Holm)
Normality test	0.002-0.965 (partially normally distributed, partially not normally distributed)	-
Homogeneity test	0.001 (not homogeneous)	-
Kruskal-Wallis	0.000[a]	-
Post-Hoc Mann-Whitney:		
Negative control vs. Pos. Control	0.000	0.000[a]
Negative control vs. FPE 0.22 mg/kg BW	0.000	0.000[a]
Negative control vs. FPE 0.44 mg/kg BW	0.000	0.000[a]
Negative control vs. FPE 0.88 mg/kg BW	0.000	0.000[a]
Poaative control vs. FPE 0.22 mg/kg BW	0.007	0.035[a]
Poaative control vs. FPE 0.44 mg/kg BW	0.019	0.057
Poaative control vs. FPE 0.88 mg/kg BW	0.145	0.290
FPE 0.22 mg/kg BW vs. FPE 0.44 mg/kg BW	0.566	0.566
FPE 0.22 mg/kg BW vs. FPE 0.88 mg/kg BW	0.004	0.024[a]
FPE 0.44 mg/kg BW vs. FPE 0.88 mg/kg BW	0.012	0.048[a]

[a]P<0.05. FPE, fibrinolytic protease enzyme; BW, body weight.

activity compared with other studies may be explained by enzyme purity (31).

Mechanistically, the enzyme works by directly degrading fibrin or by activating plasminogen (31,32), the specific mechanism of action of the partially purified AL6 enzyme preparation remains to be determined. The difference in activity compared with streptokinase may be because streptokinase acts systemically with rapid plasminogen activation (32,33), while microbial enzymes tend to be more specific for fibrin (15).

Statistical analysis showed significant differences between groups (P<0.05) and further Tukey's test confirmed significant differences across all enzyme volume variations. These results align with enzyme kinetics and ANOVA theory (34).

In vivo testing showed that the partially purified fibrinolytic protease preparation markedly reduced thrombus length in mice. The highest reduction occurred at 0.88 mg/kg body weight (52.54%). These results are consistent with previous reports of dose-dependent antithrombotic effects of fibrinolytic enzymes (14,15). Although its effectiveness was slightly below that of the positive control, these findings support the potential of the partially purified enzyme preparation as a candidate for further antithrombotic investigation.

Physiologically, the κ-carrageenan thrombus model triggers inflammation, which activates coagulation and forms fibrin as a thrombus scaffold (35,36). The balance between coagulation and fibrinolysis is clinically important, as dysregulation of fibrinolytic regulators such as plasminogen activator inhibitor-1 has been associated with thrombotic and reproductive disorders (37). The reduction in thrombus length observed in the present study therefore reflects the enzyme's fibrinolytic activity (32). The Kruskal-Wallis test showed a significant difference in thrombus length among groups (P<0.001), followed by pairwise Mann-Whitney U tests with Holm adjustment, which identified significant differences in several treatment comparisons.

Overall, the present study demonstrated that bacterial isolate AL6 produces a fibrinolytic enzyme preparation that can be fractionated by anion-exchange chromatography and exhibits measurable *in vitro* fibrinolytic activity and *in vivo* antithrombotic effects. The F8-F14 preparation showed volume-dependent clot-lysis activity and was associated with a reduction in thrombus length in the mouse model. However, the absence of total protein concentration and specific activity measurements prevents assessment of purification fold, enzyme enrichment and purification efficiency. Therefore, the findings should be interpreted as preliminary evidence supporting further investigation rather than as confirmation of purification effectiveness.

The enzyme preparation was obtained through anion-exchange chromatographic fractionation and remained partially purified, as indicated by the presence of multiple protein bands on SDS-PAGE. However, total protein concentration and specific activity were not determined for the crude enzyme or pooled F8-F14 fractions. Consequently, purification fold, enzyme enrichment and purification efficiency could not be assessed. The fibrinolytic activity data therefore demonstrate activity of the enzyme preparation but cannot be used to establish an increase in enzyme-specific activity attributable to the chromatographic procedure.

Furthermore, enzyme production remained at the laboratory scale and its broader applicability and translational potential have not been evaluated. Recent advances in therapeutic discovery, including artificial intelligence-guided drug repurposing, emphasize the importance of integrating innovative approaches with experimental validation and translational assessment in the development of potential therapeutic agents (21). Prior to clinical implementation, further safety evaluation, risk assessment and translational planning are

essential (38). These limitations highlight the need for further studies to confirm species identification, improve enzyme purification and characterization and comprehensively evaluate its *in vivo* safety, efficacy and translational potential.

In conclusion, bacterial isolate AL6 was molecularly identified as a *Bacillus paramycoides* related strain and demonstrated the ability to produce a putative fibrinolytic protease with an estimated molecular weight of 30-50 kDa. The enzyme preparation was partially purified by anion-exchange chromatography and exhibited promising *in vitro* fibrinolytic activity, as demonstrated by its volume-dependent clot lysis. *In vivo* evaluation using a κ-carrageenan-induced mouse tail thrombus model demonstrated significant antithrombotic efficacy, with the highest dose of 0.88 mg/kg body weight producing a 52.54% reduction in thrombus length and showing no significant difference from the positive control. Preliminary safety assessment indicated that bleeding time remained within normal limits under the tested conditions, with no apparent adverse effect on hemostasis. Overall, the partially purified fibrinolytic protease preparation from isolate AL6 demonstrated promising *in vitro* fibrinolytic activity and *in vivo* antithrombotic effects, supporting its potential for further investigation as a candidate antithrombotic agent. However, further purification, biochemical and stability characterization, comprehensive safety evaluation and subsequent translational studies are required to establish its therapeutic potential.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LL was responsible for conceptualization, methodology, supervision, funding acquisition, data analysis, review, editing and writing the original draft. RDD and AI were responsible for sample administration, experimental work, data organization and verification and data analysis. LS was responsible for conceptualization, methodology, reviewing and editing. NFN was responsible for experimental work, data organization and verification and manuscript preparation. LL, RDD and AI confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of the Ministry of Health Polytechnic of Banjarmasin (Poltekkes Kemenkes Banjarmasin), Indonesia, approved the present study (approval no. 881/KEPK-PKB/2025; approved on December 15, 2025). All animal experiments were conducted in accordance with institutional ethical guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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