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Novel Fibrinolytic Enzyme by *Scopulariopsis brevicaulis* OS 3456: Production, Characterization, *In vitro,* and *In vivo* Activity

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FIBRINOLYTIC enzyme production from *Scopulariopsis brevicaulis* OS 3456 isolated from a local soil sample was studied. The enzyme was purified by ammonium sulfate precipitation and gel filtration chromatography using Sephadex G-100, increasing its specific activity to 370 U/mg with a yield of 1.5% and a purification fold of 3.4. The molecular weight of the purified enzyme was 61.5 kDa determined by SDS-PAGE analysis. The optimum temperature of the enzyme was 37°C, and it was stable over a pH range of 5.0–9.0 with maximum stability at pH 7.0. The activity was increased in the presence of ß-mercaptoethanol, Mn^{2+}, Ba^{2+}, triton X-100, and xylene by 137.1, 51.6, 41.4, 37.5, and 23%, respectively. Furthermore, the enzyme activity was inhibited by Cd^{2+}, Al^{3+}, EDTA, PMSF, and acetone. The *in vitro* thrombolytic activity of the undiluted purified enzyme (370 U/mg) was found to be 100%. Meanwhile, in the cases of 185, 92.5, 46.25, 23.125, and 11.562 U/mg, the clot lysis percentage was 76.8, 67.4, 57.8, 39.5, and 28%, respectively. A carrageenan-induced tail thrombosis model was applied to test the *in vivo* thrombolytic activity of the enzyme. The result indicated no obvious thrombus in the tails of mice treated with the tested enzyme (370 U/mg). However, when the enzyme was diluted, its thrombolytic activity decreased gradually. All these results explore the promising thrombolytic activity of the extracted fibrinolytic enzyme. Hence, more purification steps and more experimental animal studies are required in the future for its use as a commercial drug.

Keywords: Fibrin, Fibrinolytic enzyme, *In vitro* activity, *In vivo* activity, *Scopulariopsis* sp, Thrombosis.

Introduction

Blood clots (thrombosis) are one of the most widely occurring diseases worldwide. Thrombosis is associated with the higher content of fibrinogen in plasma. Fibrinogen is converted into insoluble fibrin (the main protein component of the blood clot) by thrombin (EC 3.4.21.5) action after trauma or injury (Kotb et al., 2015). Fibrin accumulation in blood vessels usually limits the blood flow through veins and arteries, resulting in cardiovascular diseases (CVDs), such as acute myocardial infarction, peripheral vascular disease, heart diseases, high blood pressure, and stroke, and it is a major cause of death worldwide (Barzkar et al., 2022). Public health data on CVDs is well documented by the World Health Organization (WHO) in its recently published reports. According to the WHO, 17.9 million people die each year from CVDs, accounting for an estimated 31% of all deaths worldwide with the number expected to rise to 23.6 million by 2030 (Roth et al., 2020).

Fibrin clots are naturally hydrolyzed by the plasmin enzyme (EC 3.4.21.7), activated from plasminogen-by-plasminogen activators (PAs). There is also a biological balance in the tissues between blood coagulation and fibrinolysis. However, when this balance is disturbed as a result of several disorders including tissue injury or high pressure or genetic variation or disease-like diabetes mellitus, the need for intravenous administration of a fibrinolytic agent arises to restore the uniform blood flow and thus aid in the healing process (Krishnamurthy et al., 2018).

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Current fibrinolytic drugs available for clinical treatment of thrombosis are mostly plasminogen activators (PAs), including tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), and bacterial plasminogen activator streptokinase (Liu et al., 2015). All of these agents have some limitations, such as high costs, preventing them from being utilized on a large scale, and undesirable side effects, including allergic reactions, gastrointestinal bleeding, and thrombus recurrence (Wu et al., 2019). Therefore, significant studies have been conducted to produce other novel, potent, and safe fibrinolytic enzymes from various sources, enhancing their efficacy and fibrin specificity for clinical use. It is well known that microbial fibrinolytic enzymes are highly recommended due to their powerful tools for large-scale production, modification, and improvement, such as fibrinolytic proteases from *Bacillus cereus* RSA1 (Sharma et al., 2020) and *Bacillus subtilis* K42 (Hassanein et al., 2011). Fungal fibrinolytic enzymes may be better alternatives to those of bacterial origin due to their high stability, specificity, and ability to withstand extreme conditions. Thus, they are applied in the pharmaceutical industry as thrombolytic agents such as fibrinolytic proteases from *Aspergillus brasiliensis* AUMC 9735 **(**Kotb et al., 2015), *Aspergillus carbonarius* S-CSR-0007 (Afini et al., 2016), *Paecilomyces tenuipes* (Kim et al., 2011), and *Oidiodendron flavum* **(**Tharwat, 2006).

The batch submerged fermentation process involves producing enzymes by microorganisms in a liquid nutrient medium in which the substrate is broken down by the microbial isolate and the desired enzyme is extracellularly released into solution. The reactor is filled with the medium passing the fermentation, and the product is collected at the end of the process. The produced enzyme is in the crude form and must be purified for further use. Several purification techniques are well known, including salting out by adding ammonium sulphate, acetone, or ethanol, ion exchange chromatography, adsorption chromatography, affinity chromatography, gel filtration chromatography, and ultrafiltration (dialysis). The optimal levels of temperature, pH, substrate concentration, incubation time, and kinetic properties have to be detected to enhance the enzyme activity (Sharma et al., 2020).

In vitro studies are considered a key step in evaluating the potential of a microbial enzyme to be developed into a drug (Krishnamurthy et al., 2018). Regarding the assessment of thrombolytic activity, the *in vitro* clot lytic effect was utilized for determining the ability of the enzyme to dissolve the blood clots for thrombosis treatment (Sharma et al., 2020). Regarding the assessment of anti-thrombus activity, the *in vitro* anticoagulant effect was utilized for detecting the preventive effect of the enzyme in thrombosis (Ma et al., 2015). In order to confirm the enzyme activity and understand the entire process of initiation, propagation, and resolution of thrombosis, the application of *in vivo* approaches becomes pivotal (Krishnamurthy et al., 2018). For this purpose, a carrageenan-induced tail thrombosis model was investigated (Yan et al., 2009), as well as the effect of a purified fibrinolytic enzyme on bleeding and clotting times (Yuan et al., 2012).

The aim of the current study was to produce a fibrinolytic enzyme from *Scopulariopsis brevicaulis* OS 3456, isolated from a soil sample collected from a location near a slaughterhouse on the 10th of Ramadan in Sharkia Governorate, Egypt, and to study the *in vitro* and *in vivo* activities of the purified enzyme.

Materials and Methods

Collection of samples and isolation of proteolytic fungi

Twelve soil samples were collected from various locations near slaughterhouses and near garbage dumps in different regions on the 10th of Ramadan in Sharkia Governorate, Egypt. Each sample was screened on a skimmed-milk agar plate. The fungal isolates showing a clear zone were considered proteolytic isolates (Kotb et al., 2015) and were preserved at -20°C in glycerol solution until further use.

Screening of fungal isolates producing fibrinolytic protease

The proteolytic fungal isolates were spot inoculated on fibrin salt basal agar medium optimized by Tharwat (2006) and composed of (g/L): fibrin, 2.0; agar, 20.0; $KHPO_4$, 1.0; NH_4NO_3, 0.05; $(NH_4)_2SO_4$, 0.5; at pH 6.5. The plates were incubated at 28°C for 3–5 days. The fibrinolytic activity of fungal isolates was confirmed by the occurrence of a clear hallow zone around the colony. The most potent isolate was then subjected to identification and enzyme production.

Identification of the most potent fungal isolate

The selected fungus was identified at the species level based on the morphology of fungal culture and microscopic characteristics according to Humber (1997). Then, identification was confirmed through the use of molecular tools.

Molecular identification of the most active fungal isolate was performed by Sigma Company Scientific Services, Egypt (www.sigma-co-eg.com). The DNA was extracted from the fungal culture using Zymo-Spin™ Technology and then subjected to PCR using a universal primer pair, forward primer ITS1 (5'-TCCG TAGGTGAACCTGCGG-3'), and reverse primer ITS4 (5'-TCCTCCGCTTATT GATATGC-3'). PCR amplicons were sequenced at GATC Biotech AG (Konstanz, Germany) by using an ABI 3730x1 DNA sequencer by Sanger technology and analyzing the amplified ITS1-5.8S-ITS2 rRNA-gene. The Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) was utilized for aligning sequence data with closely related sequences (Altschul et al., 1997). The fungal isolate was submitted at GenBank and was also deposited at Culture Collection Ain Shams University (CCASU), located at the Faculty of Pharmacy, Ain Shams University. The phylogenetic analysis of isolate was conducted through the use of MEGA version 11.0 using the neighbor-joining method (Tamura et al., 2021).

Fibrinolytic Protease Production

A basal production medium containing (g/L): sucrose, 5; KH_2PO_4, 30; $MgSO_4.7H_2O$, 1.0; $FeSO_4.7H_2O$, 0.5; KCl, 0.5; and fibrin (0.25%) at pH 7.0 was utilized. Each flask containing 50 ml of media was inoculated with 1mL of 10^5 conidia/ml spore suspension from a 7-day-old culture of the fibrinolytic isolates. The flasks were incubated at 28°C under shaking conditions at 100rpm for 7 days. After the incubation period, the contents of the flasks were filtered through Whatman No.1 filter paper, and the filtrates were centrifuged at 5000rpm for 10min to obtain cell-free filtrates. The supernatant was used as the crude enzyme (Afini et al., 2016).

Optimization of fibrinolytic enzyme production process

Different nutritional and environmental conditions such as different incubation periods (5, 7, and 9 days), different inoculum sizes (0.5, 1, 1.5, and 2mL) of spore suspension, different incubation temperatures (20, 30, 35, 40, 45, 50, and 60°C), different pH values (3, 4, 5, 6, 7, 8, and 9), and various carbon sources (glucose, fructose, sucrose, maltose, and starch) were tested for optimizing the culture conditions of the production medium in order to enhance the fibrinolytic enzyme production through the use of shake flask studies by submerged fermentation (Afini et al., 2016; Khursade et al., 2019).

Enzyme assay

The reaction mixture containing 1mL of 1.2% of bovine fibrin solution in 0.1M phosphate buffer (pH 7.0) and 1mL of crude enzyme was incubated for 2h at 37°C. The reaction was stopped with adding 2mL of 10% (w/v) trichloroacetic acid (TCA) and centrifuged at 4000rpm for 10min at 4°C. Then, 5mL of 0.44M Na_2CO_3 was added to the supernatant, followed by adding 1mL of diluted Folin-Ciocalteu's phenol reagent. The mixture was incubated for 30min at 37°C in the dark **(**Kotb et al., 2015). The optical density was measured at 660nm using a UV-Spectrophotometer (UNICO™ UV-2000) against a blank reagent prepared without adding enzyme. One unit of enzyme activity was defined as the amount of enzyme required to release 1µg of L-tyrosine per minute under assay conditions. Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Purification of fibrinolytic enzyme from Scopulariopsis brevicaulis OS 3456

According to Dixon & Webb (1962), the crude enzyme was fractionally precipitated by adding solid ammonium sulphate gradually to obtain 20, 40, 60, and 80% saturations in ice salt bath conditions with constant stirring on magnetic stirrer until all added ammonium sulphate was completely dissolved and then allowed to stand overnight at 4°C followed by centrifugation at 10,000 rpm for 30 min in a cooling centrifuge. The precipitate was collected, re-suspended in a minimum volume of 0.2 M phosphate buffer (pH 7.0), and dialyzed using a dialysis bag with a 10-kDa cut-off against the same buffer at an ice-salt bath (Schuett et al., 2020). Finally, a 5mL sample was applied to gel filtration chromatography using Sephadex G-100 (Fluka, Switzerland) using a 1.5 x 100cm column pre-

equilibrated and eluted with the same buffer at a flow rate of 2mL/min. Next, eluates (5mL fractions) were collected for measuring enzyme activity and protein content (Distasio et al., 1982).

Determination of the molecular weight of the produced enzyme by SDS-PAGE

The purified fibrinolytic enzyme obtained from Sephadex G-100 column chromatography was applied to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in order to estimate the molecular weight of the fibrinolytic enzyme using the standard pre-stained protein ladder 245kDa (Sigma-Aldrich Co., St Louis, MO, USA) (Laemmli, 1970). Fifteen µL of the sample was mixed with an equal volume of sample buffer and de-natured by heating in a boiling water bath for 3 minutes. Thirty µL of the sample mixture was loaded in the stacking gel wells. Five µL of broad range protein molecular weight marker was loaded in the first well in the stacking gel. This analysis was carried out in the Central Lab of the Faculty of Science at Ain Shams University. The profile pattern generated by SDS-PAGE was analyzed using SYNGENE software.

Characterization of the purified fibrinolytic enzyme

The reaction mixture was incubated at various temperatures ranging from 20°C to 60°C in order to detect the temperature effect on the enzyme activity. The thermal stability was investigated by pre-incubating the purified enzyme for 1h without the fibrin at the same temperatures. The optimal pH of the enzyme was evaluated by measuring fibrinolytic activity at a pH range of 2 to 12 using different buffers: 0.1 M phosphate-citrate buffer (pH 2, 3, 4, 5, and 6), 0.1M sodium phosphate buffer (pH 7), 0.1M Tris-HCL buffer (pH 8 and 9), and 0.1M glycine-NaOH buffer (pH 10,11, and 12). Moreover, in order to observe the pH stability, the purified enzyme was pre-incubated for 1 hour at room temperature in different buffers (Hassanein et al., 2011). The effect of various metal ions on the fibrinolytic enzyme was assessed using 5 mM concentrations of Na^{+}, K^{+}, Mn^{2+}, Co^{2+}, Hg^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Mo^{5+}, Ca^{2+}, Cd^{2+}, Ba^{2+}, and Al^{3+} in chloride or sulphate salts in 0.1 M phosphate buffer at pH 7.0 (Sharma et al., 2020). Furthermore, the effects of some inhibitors/activators such as ethylene diamine tetra acetic acid (EDTA), urea, sodium azide (NaN_3), and SDS in 5 mM concentration, Triton-X100, phenyl methane sulfonyl fluoride (PMSF), Tween-80, Tween-20, and ß-mercaptoethanol in 5% concentration were tested (Taneja et al., 2017). The purified fibrinolytic enzyme was incubated with different organic solvents (isopropanol, butanol, ethanol, acetone, di-ethyl ether, toluene, methanol, xylene, hexane, ethyl acetate, dimethyl sulfoxide (DMSO), and chloroform) at 20% (v/v) in the same buffer in order to determine the best solvent that has stabilizing effect on the enzyme for assessing the enzyme storage for extended time periods (Hassanein et al., 2011). The buffer without any additives was utilized as a control. The reaction mixture was incubated for different incubation periods (10, 20, 30, 60, 120, and 140min) in order to detect the optimum incubation time with maximum enzyme activity (El-Naggar et al., 2017). The residual activity was measured at the defined standard assay conditions as shown previously.

In vitro assessment of thrombolytic activity

a) Clot lytic effect using pasteur pipette

One ml of buffalo citrated blood and 100µL of 0.25M $CaCl_2$ were filled into the tip of a Pasteur pipette. Blood clots were formed inside the Pasteur pipette. A piece of Pasteur pipette was placed in a glass watch, and enzyme extract was added to it for 2h in order to observe the clot lysis inside the Pasteur pipette by the fibrinolytic activity of enzyme extract, using PBS as a negative control (Afini et al., 2016).

b) Clot lytic effect using blood clot as a substrate

In order to prepare a blood clot, 100µL of 0.25M $CaCl_2$ was added to 1.0mL of buffalo blood containing 3.8% sodium citrate in pre-weighed sterile Eppendorf tubes. The weight of the clot formed was determined as follows: weight= weight of tube with clot – weight of empty tube (Sharma et al., 2020). Aliquots of 500µL with various concentrations of the purified enzyme (370, 185, 92.5, 46.25, 23.125, and 11.562U/mg) were incubated with blood clots at 37°C for 10h (Zhou et al., 2022). For the negative control, phosphate buffered saline (PBS) was added to a clot, and for the positive control, Heparin sodium inj. USP 26 (5000 I.U/mL) from (Allmed medical) with expiry date from 3/2021 until 4/2023) (a commercial thrombolytic drug) was added to a clot under identical conditions. The following equations were utilized for calculating the clot lysis percentage (Zubair et al., 2015):

Clot lysis (%)= (weight of the lysed clot**)/ (weight of clot before lysis)×100 (1)

** Weight of the lysed clot= Weight of clot before treatment- weight of clot after treatment (2)

In vitro assessment of anticoagulant activity

One ml of buffalo citrated blood and different concentrations of purified enzyme (370, 185, 92.5, 46.25, 23.125, and 11.562U/mg) were mixed gently. The mixture was incubated at 37°C for 30min. Then, 100µL of 0.25M $CaCl_2$ was added to the mixture to induce blood coagulation. Then, the anticoagulant effects of the enzyme were observed (Yuan et al., 2012). Under identical conditions, PBS and Heparin (a commercial anticoagulant drug) were added to a clot for negative and positive control, respectively.

In vitro cytotoxic activity of the purified enzyme

The cytotoxic activity of the purified fibrinolytic enzyme from *S. brevicaulis* OS 3456 was evaluated against the WI-38 cell line (normal human lung fibroblast cells obtained from the American Type Culture Collection (ATCC, Rockville, MD)). The optical density was measured using the microplate reader (SunRise, TECAN Inc., USA) at 590nm in order to identify the number of viable cells and estimate the cytotoxic concentration (CC_{50}, the enzyme concentration required to cause toxic effects in 50% of the tested WI-38 cellular population) (Mosmann, 1983). This analysis was conducted at the Regional Center for Mycology and Biotechnology at Al-Azhar University.

In vivo assay of the purified fibrinolytic enzyme

Male Swiss albino mice (*Mus musculus*) of the CD1 strain, which were 8 weeks old and weighed 25–30g, were utilized in the present study. They were obtained from the breeding unit of the Theodor Bilharz Research Institute (Giza, Egypt).

a) κ-Carrageenan-induced mouse tail thrombosis model

In order to detect the best dose of carrageenan and optimize the tail thrombosis model, male Swiss albino mice were intravenously injected with different doses of sterile carrageenan (5–25 mg/kg) dissolved in physiological saline at a concentration of 0.2% (w/v). The length of a wine-colored thrombus in the tail of mice was observed after 8h (Yan et al., 2009).

A total of 25 male Swiss albino mice were randomly divided into 5 groups (n = 5). Group 1 served as a negative control with PBS. Group 2 was given Heparin as a positive control. Groups 3, 4, and 5 were treated with 3 different concentrations of the purified fibrinolytic enzyme (92.5, 185, and 370U/mg, respectively). Each mouse received an intravenous injection of 20mg/kg carrageenan dissolved in physiological saline half an hour after the last intraperitoneal drug treatment. Thrombus lengths were measured and photographed at 24h (Ma et al., 2015). The percentage of *in vivo* thrombus dissolution after 24 hours of the enzyme administration was calculated using the following equations (Majumdar et al., 2016):

% of thrombosis (X)= (length of tail thrombus in treated mice (cm))/ (length of tail thrombus in control mice (cm))×100 (3)

% Actual thrombus dissolution (Y)= 100 – (X) (4)

b) The effect of the purified fibrinolytic enzyme on bleeding and clotting time

A total of 25 male Swiss albino mice were divided into 5 groups (n= 5): PBS as a negative control, Heparin as a positive control, and the three enzyme concentrations (370, 185, and 92.5U/mg). The mice were injected intraperitoneally each day for a week. After an hour of the last treatment, the bleeding time was measured by fixing the mice and cutting 3mm tail tips. The bleeding time was measured until the bleeding stopped naturally. A drop of blood was put on a clean slide in order to determine the clotting time that was measured until blood clotted naturally (Yuan et al., 2012).

Statistical Analysis

The statistical analyses were carried out using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA). Statistical differences between the treatments and the control were calculated using a one-way ANOVA, followed by Tukey's multiple comparison tests. The experiment's data was expressed as a mean standard error of mean (SEM). $P \leq 0.05$ were considered statistically significant.

Results

Isolation and screening of the fibrinolytic fungal isolates

A total of 116 fungal isolates were isolated from various soil samples collected from different locations near slaughterhouses and

near garbage dumps on the 10th of Ramadan in Sharkia Governorate, Egypt. Only 24 isolates produced a clear zone on skimmed milk agar plates. Further screening of these isolates for qualitative production of fibrinolytic enzyme on fibrin plates indicated that only three fungal isolates Nos. (3), (4), and (7) were able to produce fibrinolytic enzyme as pointed out by a clear zone formed around their growth (Fig. 1). The quantitative assay using Folin-Ciocalteu's phenol reagent demonstrated that isolates Nos. (3) and (4) provided the highest fibrinolytic enzyme activity (5.38 and 7.25U/mL, respectively), while isolate No. (7) gave the lowest enzyme activity (3.11U/mL). Thus, isolate No. (4) was selected as the most potent fibrinolytic enzyme manufacturer. Based on the optimization of the production process, the maximum fibrinolytic enzyme activity was obtained with a 7-day incubation period, 1mL inoculum size, glucose as a carbon source, and fibrin of 0.25% associated with the same concentrations of the other components (30g/L KH_2PO_4, 1g/L $MgSO_4.7H_2O$, 0.5g/L $FeSO_4.7H_2O$, and 0.5g/L KCl) at pH 7.0 and incubation temperature of 30°C. These conditions increased the activity from 7.2U/ml to 8.51U/mL by 1.2-fold in comparison with the non-optimized medium.

Identification of the most potent fungal isolate

The morphological appearance of a 7-day old culture of the producer isolate No. (4) was observed on Potato Dextrose Agar media (PDA) and exhibited tan to beige powdery colonies, and the reverse side of the colony was tan with a brown center (Fig. 2A). The microscopic examination of the isolate indicated the production of the annellides (conidiogenous cells) from unbranched or branched penicillate-like conidiophores. Conidia are in chains with the youngest conidium at the chain base next to the annellide tip. The conidia are thick-walled, round to lemon-shaped (Fig. 2B). Both morphological characteristics and microscopic examination of the isolate provided its preliminary identification as *Scopulariopsis brevicaulis.*

The results of molecular identification revealed that the amplified sequence alignment of the isolate with reference sequences at the NCBI database confirmed it to be *S. brevicaulis*. The sequence data for *Scopulariopsis brevicaulis* OS 3456 had been deposited in "GeneBank" under accession number [ON329197] and at CCASU under strain number CCASU-2022-F5. A maximum likelihood phylogenetic tree was constructed, presenting its closely related strains (Fig. 2C).

Purification of the Fibrinolytic Enzyme

Fibrinolytic enzyme from *S. brevicaulis* OS 3456 was purified by fractional precipitation using different ammonium sulphate saturations (20, 40, 60, and 80%). Compared to the specific activity of the crude enzyme (106.37U/mg) with the four fractions, it was obvious that 80% saturation was the best fraction that indicated the maximum specific activity (183.33 U/mg) with a yield of 3.23% and a purification fold of 1.7 (Table 1). This fraction was applied to gel filtration chromatography through the use of Sephadex G-100. Then, 25 fractions were collected using phosphate buffer eluting solution. As illustrated in elution profile in Fig. 3, the enzyme was successfully eluted at fraction (No. 11) with maximum enzyme activity (4.8U/mL). The specific activity increased to 369.23 U/mg with a yield of 1.5% and a purification fold of 3.4 (Table 2).

Determination of the molecular weight of the produced enzyme by SDS-PAGE

A single band of the enzyme was observed, as displayed in lane 3 (Fig. 4A). The molecular weight of the enzyme was found to be 61.5kDa as calculated using SYNGENE software from gel documentation (Fig. 4B), presenting the only peak of the purified enzyme with a molecular weight of 61.5kDa.

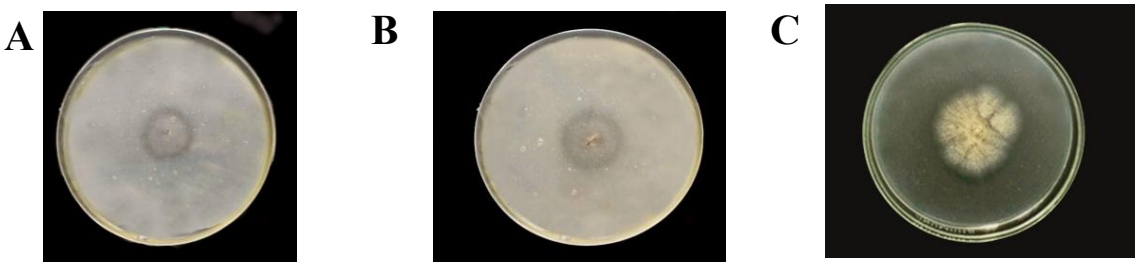

Fig. 1 Fibrinolytic activity of the most potent isolates on minimal fibrin basal medium; (A) Isolate No. 3, (B) Isolate No. 4, (C) Isolate No. 7

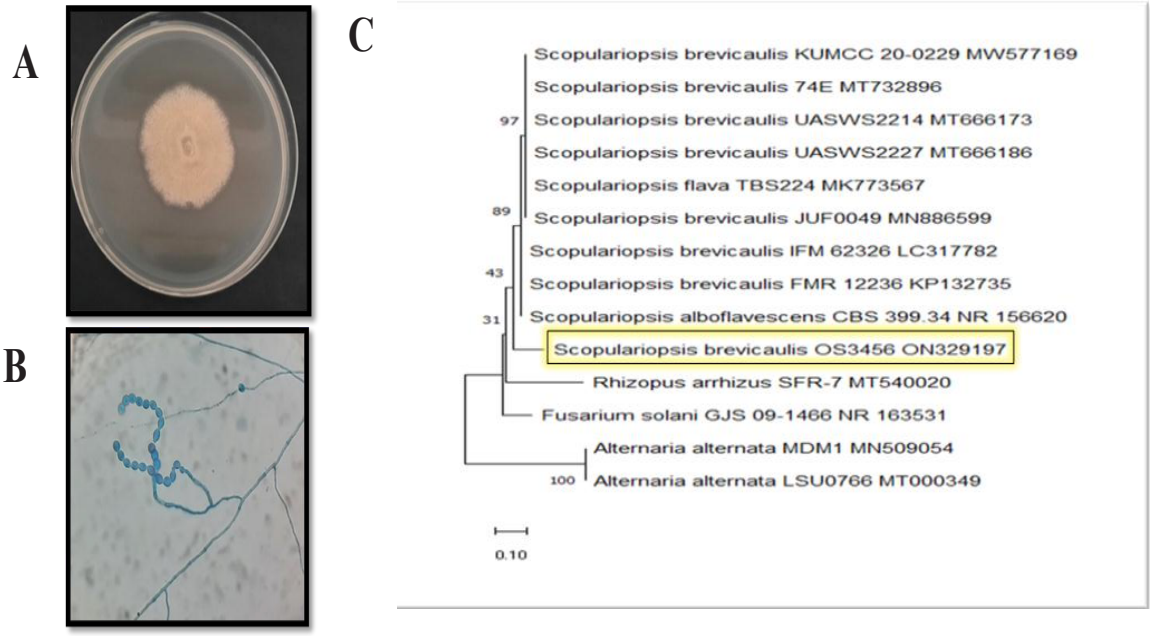

Fig. 2. Identification of the most potent fibrinolytic isolate (*S. brevicaulis*); (A) Macroscopic morphology of 7 days old culture as observed on PDA, (B) Microscopic morphology showing conidia and conidiophores, (C) Phylogenetic tree of *Scopulariopsis brevicaulis* OS 3456 showing closely related strains based on 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence

TABLE 1. Fibrinolytic activities of ammonium sulphate fractions of the fibrinolytic enzyme from *Scopulariopsis brevicaulis* OS 3456

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U\mg)	Relative activity or yield (%)	Purification fold
Crude	51060	480	106.37	100	1.00
20%	0.00	0.18	0.00	0.00	0.00
40%	243	2.16	112.5	0.47	1.06
60%	688.5	4.6	150	1.35	1.41
80%	1650	9	183.33	3.23	1.7

TABLE 2. Summary of the purification steps, purification folds, and percentages of yield recovery of the fibrinolytic enzyme from *Scopulariopsis brevicaulis* OS 3456

Step of purification	Total activity (U)	Total protein (mg)	Specific activity (U\mg)	Relative activity or yield (%)	Purification fold
Crude	51060	480	106.37	100	1.00
Amm. Sulphate precipitation (80%)	1650	9	183.33	3.23	1.7
Sephadex G-100 gel filtration column	768	2.08	369.23	1.5	3.4

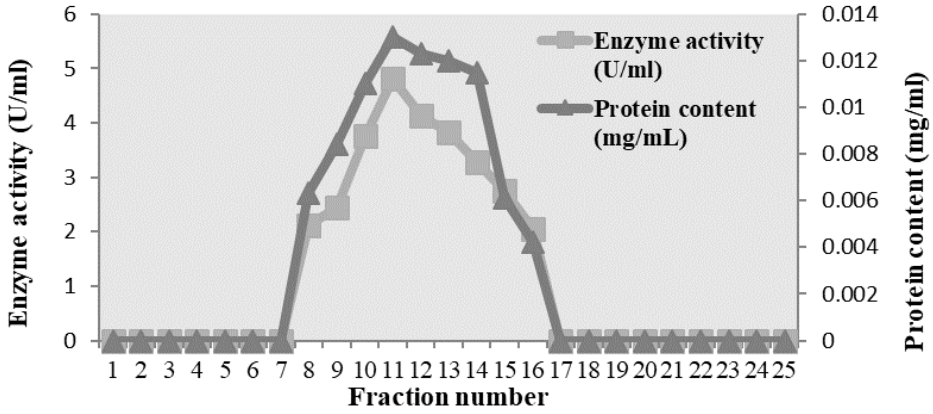

Fig. 3. Elution profile of the purified fibrinolytic enzyme from *S. brevicaulis* OS 3456 eluted from Sephadex G-100 column

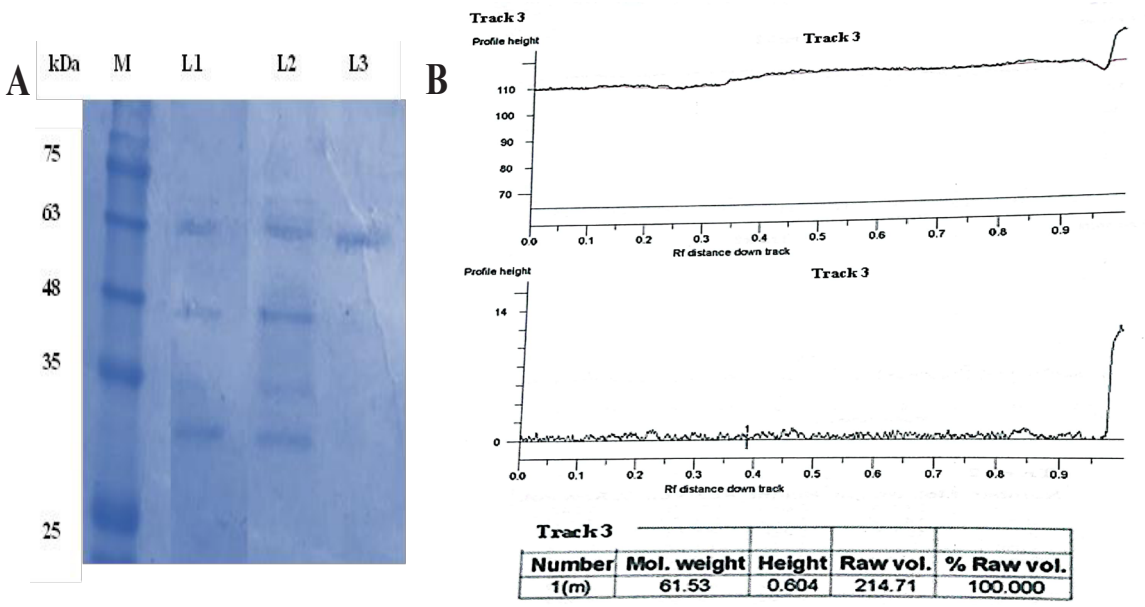

Fig. 4. (A) SDS-PAGE of the purified fibrinolytic enzyme from *S. brevicaulis* OS 3456 [(M): Protein marker. Lane (1): Crude enzyme. Lane (2): Ammonium sulphate fraction 80%. Lane (3): Purified enzyme after Sephadex G-100 gel filtration chromatography], (B) Gel document of lane 3 that represents the purified enzyme by SYNEGENE software

Characterization of the purified fibrinolytic enzyme

Results in Fig. 5A demonstrated that the enzyme activity increased gradually till it reached 37°C which was the optimum temperature. At higher reaction temperatures, a notable reduction in enzyme activity was evident. From the thermal stability profile, the enzyme appeared to be catalytically stable from 30°C to 45°C. Results in Fig. 5B indicated that as the pH increased, the enzyme activity increased till it reached its maximum activity at pH 7.0. Above that, the enzyme activity decreased gradually. The pH stability profile revealed that the enzyme was stable over a broad pH range of 5.0–9.0 with a maximum stability at pH 7.0.

It was obvious in Fig. 5C that the addition of Mn^{2+}, Ba^{2+}, Co^{2+}, Ca^{2+}, K^{+}, Mg^{2+}, Na^{+}, and Mo^{5+} increased the enzyme activity by 51.6, 41.4, 40.1, 14.4, 12.8, 10.3, 10, and 6.2%, respectively. Meanwhile, the presence of Cd^{2+}, Al^{3+}, Hg^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} reduced the enzyme activity. On the other hand, Fe^{2+} exerted no effect on enzyme activity, and it was found that EDTA and PMSF inhibited the enzyme by 59.2 and 38.4%,

respectively. On the contrary, ß-mercaptoethanol, triton X-100, urea, SDS, sodium azide, and tween-80 improved the fibrinolytic activity by 137.1, 37.5, 35.1, 13.2, 6.4, and 5.5%, respectively. Meanwhile, tween-20 nearly had no effect on the activity (Fig. 5D). The results in Fig. 5E revealed that the enzyme activity was enhanced by xylene and hexane by 23 and 4%, respectively. However, it was inhibited by the rest of the solvents (methanol, ethanol, butanol, toluene, acetone, chloroform, isopropanol, ethyl acetate, DMSO, and diethyl ether). It was found that the enzyme activity increased gradually as the incubation time increased to 120 min, and after that, it slightly decreased (Fig. 5F).

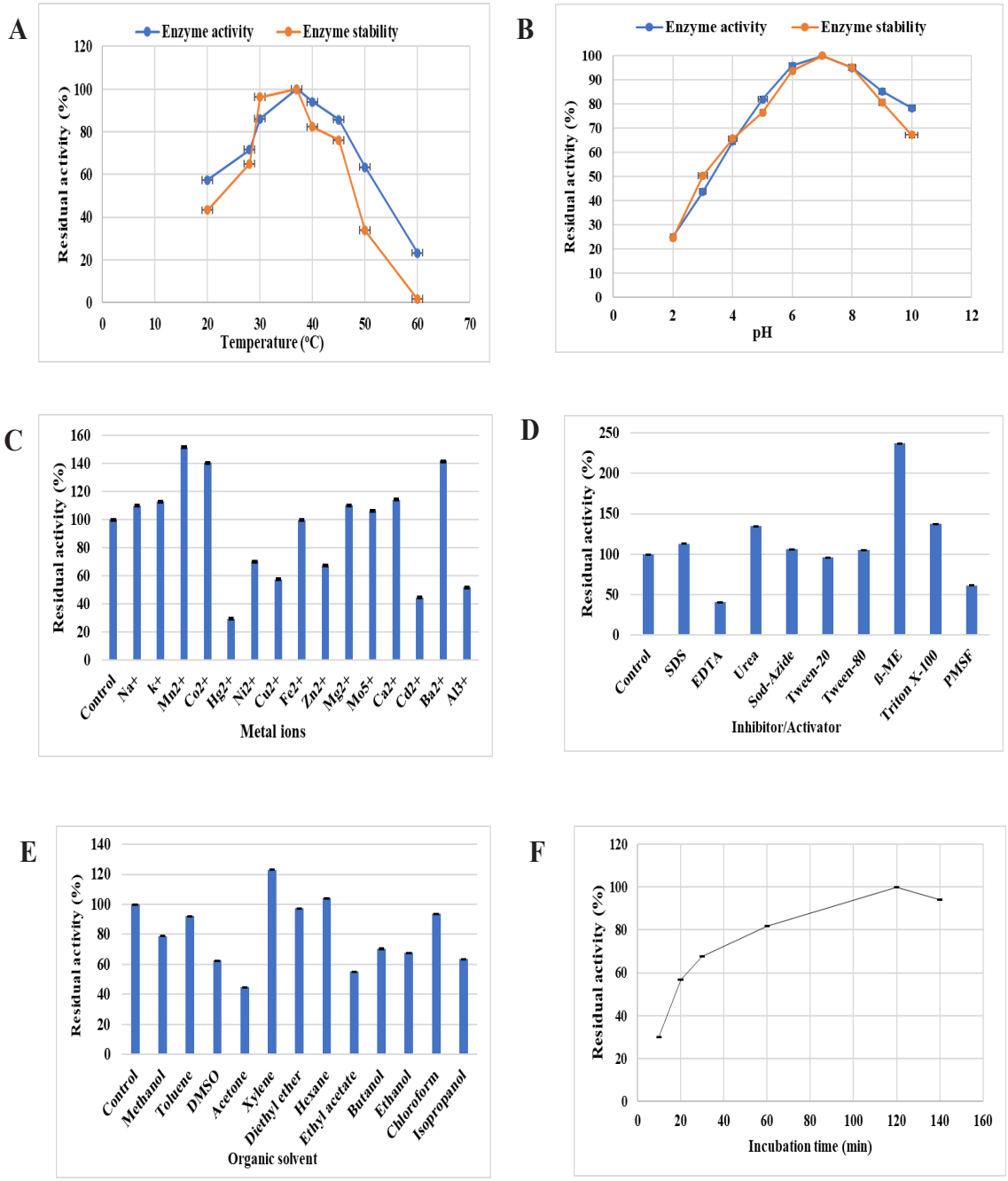

Fig. 5. Effect of (A) temperature, (B) pH, (C) Metal ion, (D) Inhibitor/activator, (E) Organic solvents, (F) Incubation time on the activity of the purified fibrinolytic enzyme from *S. brevicaulis* OS 3456. Error bars represent one standard deviation from the mean (n=3)

In vitro assessment thrombolytic activity

a) Clot lytic effect using pasteur pipette

The blood clot inside the Pasteur pipette was lysed by the fibrinolytic activity of the purified enzyme within 2h (Fig. 6A), and no lysis was observed in the negative control (PBS) (Fig. 6B).

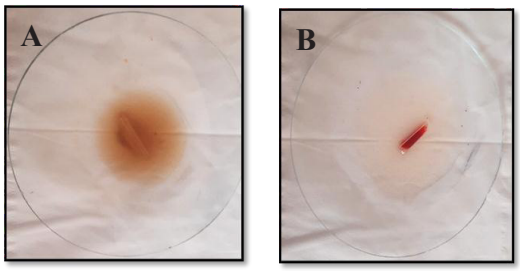

Fig. 6. In vitro thrombolytic activity using blood clot inside Pasteur pipette that incubated with (A) Purified enzyme, (B) PBS

b) Clot lytic effect using blood clot as a substrate

The results in Table 3 revealed that the thrombolytic activity of the undiluted purified enzyme (370U/mg) was 100% with no significant difference in comparison with the reference drug (Heparin) and a very high significant difference with PBS, a negative control exhibiting a negligible percentage of clot lysis of 21% ($P \leq 0.0001$) as presented in Fig. 7 (A, G, and H). As for the different concentrations of the enzyme 185, 92.5, 46.25, 23.125, and 11.562U/mg, the clot lysis percentages were 76.8, 67.4, 57.8, 39.5, and 28%, respectively, as shown in Fig. 7 (B-F).

In vitro assessment of anticoagulant activity

The anticoagulant effects of different concentrations of the purified fibrinolytic enzyme, PBS, and Heparin on animal blood were tested. The results indicated that the lower enzyme concentrations (11.562 and 23.125U/mg) had no notable effect on the blood clot, as did PBS as a negative control (Fig. 8F, G, and H). However, the anti-coagulant effect was gradually enhanced as the enzyme concentrations increased. At an enzyme concentration of 46.25 U/mg, the blood was partly coagulated (Fig. 8E), and the anticoagulant effect was obvious when the enzyme activity reached 92.5, 185, and 370U/mg (Fig. 8B–D), at which the blood was fluid and not clotted, the same as the effect of Heparin as a positive control (Fig. 8A).

In vitro cytotoxic activity of the purified enzyme against the WI-38 cell line

The cytotoxic activity of the enzyme was evaluated against the WI-38 cell line at various concentrations ranging from 0.35 to 45µg/mL. It was found that the cytotoxic concentration CC_{50} value was equivalent to 8.11±0.97µg/mL after treatment for 24h (Fig. 9).

In vivo assay of the purified fibrinolytic enzyme

a) κ-Carrageenan-induced mouse tail thrombosis model

As displayed in Fig. 10B, carrageenan dose was positively correlated with the acquired thrombus length of the mouse's tail until it reached 20mg/kg. Above this concentration, there was no change in thrombus tail length. Thus, 20mg/kg carrageenan was chosen for testing the thrombolytic effect of the produced enzyme.

TABLE 3. Clot lysis percentages of different concentrations of the purified enzyme compared with positive and negative controls

Treatment	Weight of clot before treatment (g)	Weight of clot after treatment (g)	Weight of lysed clot (g)	Clot lysis (%)
PBS	1.06±0.01	0.83±0.06	0.224±0.065	21±6.15
Heparin		0	1.06±0.01	100 [a]
11.562U\mg		0.76±0.014	0.3±0.014	28±1.3 [b]
23.125U\mg		0.64±0.017	0.42±0.017	39.5±1.65 [aa, b]
46.25U\mg		0.44±0.018	0.61±0.018	57.8±1.67 [a, b]
92.5U\mg		0.34±0.014	0.71±0.014	67.4±1.35 [a, b]
185U\mg		0.24±0.027	0.78±0.057	76.8±2.6 [a, bb]
370U\mg		0	1.06±0.01	100 [a]

Values are presented as mean ± SEM (n=3), [a] $P \leq 0.0001$ significant difference from PBS group, [aa] $P \leq 0.01$ significant difference from PBS group, [b] $P \leq 0.0001$ significant difference from Heparin group, [bb] $P \leq 0.001$ significant difference from Heparin group.

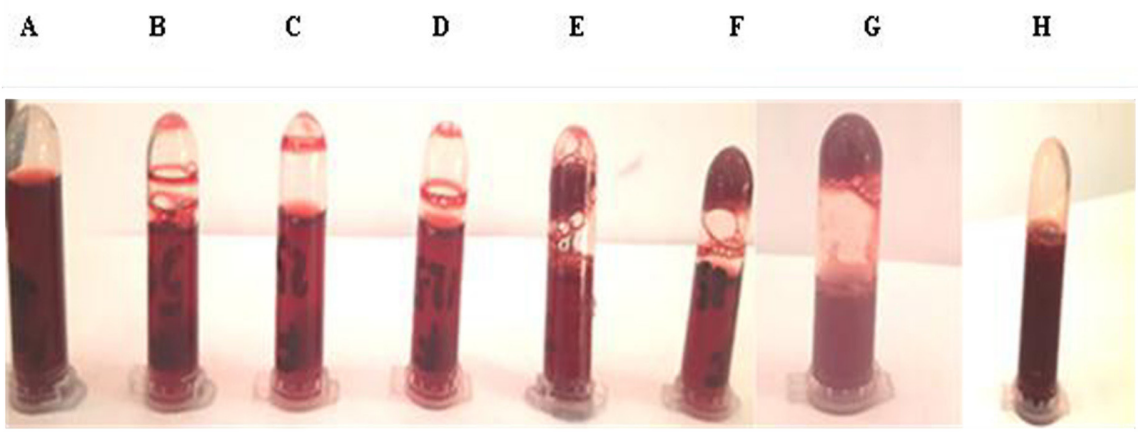

Fig. 7. *In vitro* thrombolytic activity of the fibrinolytic protease using blood clot as substrate (A-F). The different concentrations of the enzyme (G) (PBS), a negative control (H) (Heparin) a positive control

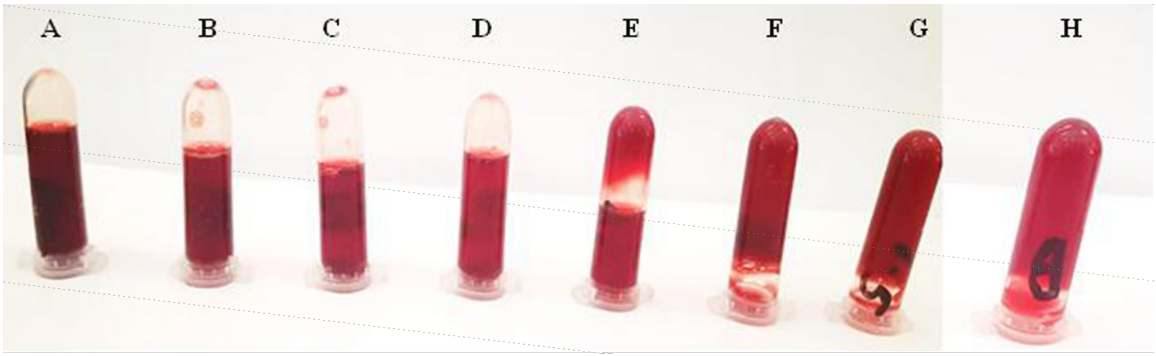

Fig. 8. *In vitro* anticoagulant activity of different concentrations of the purified enzyme on animal blood compared with positive and negative control, (B-D) 370, 185, and 92.5U/mg, respectively with strong anti-coagulant activity, (E) 46.25U/mg of enzyme, the blood was partly coagulated, (F, G) 11.562 and 23.125U/mg have no anti-coagulant activity, (A) Heparin, anti-coagulant reference drug, (H) PBS, a negative control

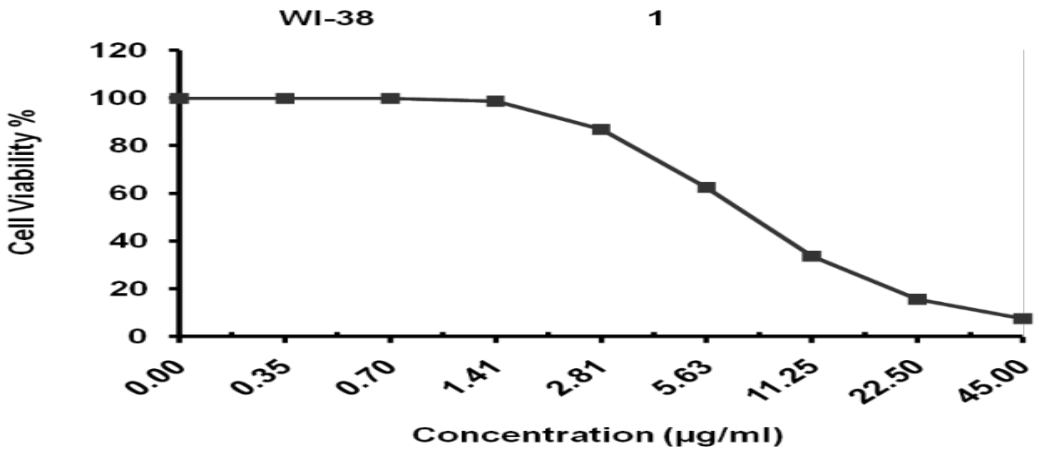

Fig. 9 . *In vitro* cytotoxic activity of the purified fibrinolytic enzyme from *S. brevicaulis* OS 3456 against the WI-38 cell line

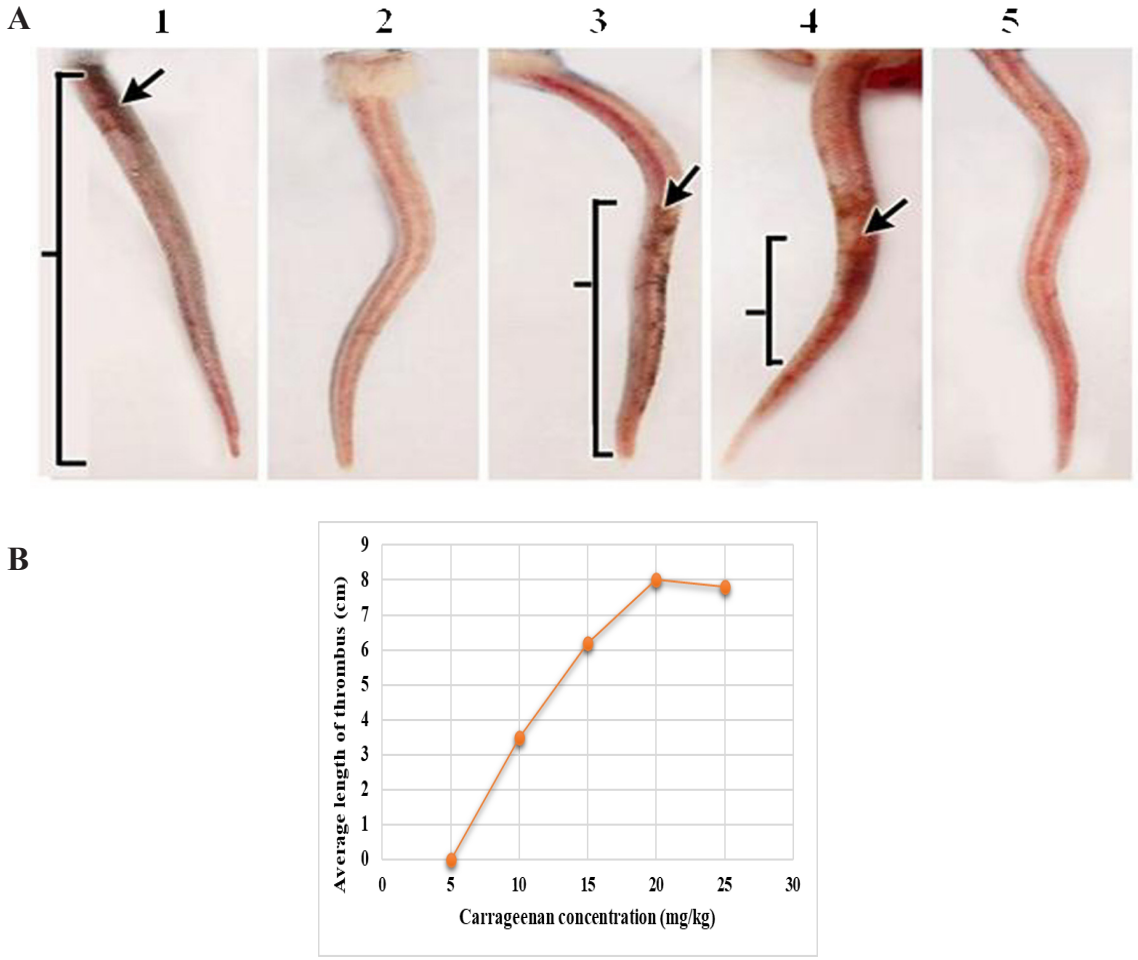

Fig. 10. (A) The dose-dependent thrombolytic effect of the produced enzyme was compared to Heparin and PBS [The arrows indicate the thrombus formation area (wine color) in the tail]; (A1) group 1: κ-carrageenan (20mg/kg) and 200µL of PBS (A2) group 2: κ-carrageenan (20mg/kg) and 100µL of Heparin (A3) group 3: κ-carrageenan (20mg/kg) and 200µL of 92.5U/mg of enzyme (A4) group 4: κ-carrageenan (20mg/kg) and 200µL of 185U/mg of enzyme (A5) group 5: κ-carrageenan (20mg/kg) and 200µL of 370U/mg of enzyme (B) The relation between carrageenan dose and mouse tail thrombus length

Results in Table 4 and Fig. 10A revealed that treatment with the produced enzyme at 370U/mg (Group 5) dissolved most mice tail thrombi by carrageenan with a dissolution percentage of 98.2±0.63% with no significant difference from Heparin, the +ve control (Group 2) exerting complete clot lysis with a dissolution percentage of 99±0.46%, and with a highly significant difference from PBS, the -ve control (Group 1) ($P \leq 0.0001$) having no effect on the blood clot. As the enzyme concentration decreased (Groups 3 and 4), the thrombolytic activity also decreased gradually.

b) The effect of the purified fibrinolytic enzyme on bleeding and clotting time

The results indicated a positive correlation between the bleeding and clotting times and the concentration of the enzyme. The high concentration of the enzyme (370U/mg) postponed bleeding and clotting times to 12.84±0.19 and 4.76±0.27min, respectively, with no significant difference from the Heparin group and a very high significant difference from the PBS group ($P \leq 0.0001$). Moreover, in the case of an enzyme with 185 U/mg activity, the bleeding and clotting times were less postponed to 9.42±0.22 and 3.28±0.14min, respectively. Meanwhile, with an enzyme concentration of 92.5U/mg, the bleeding and clotting times were similar to those of the negative control group (Fig. 11).

TABLE 4. *In vivo* Thrombolytic activity of different doses of the enzyme compared with PBS and Heparin

Thrombolytic agent	Length of tail thrombus (cm)	Thrombosis (%)	Actual thrombus dissolution (%)
PBS	7.8 ± 0.09	100	0
Heparin	0.08 ± 0.03 [a]	1 ± 0.46 [a]	99 ± 0.46 [a]
92.5U\mg	5.66 ± 0.2 [ab]	72.3 ± 2 [ab]	27.7 ± 2 [ab]
185U\mg	3.66± 0.19 [ab]	47 ± 3 [ab]	50.8 ± 3.35 [ab]
370U\mg	0.14 ± 0.05 [a]	1.7 ± 0.6 [a]	98.2 ± 0.63 [a]

Values are presented as mean ± SEM (n=5). [a] $P \leq 0.0001$ significant difference from PBS group. [b] $P \leq 0.0001$ significant difference from Heparin group.

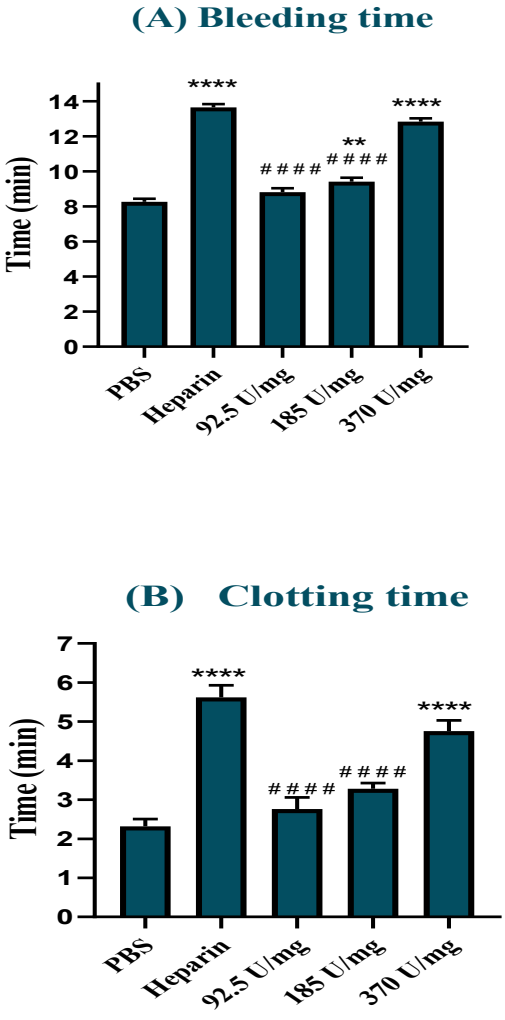

Fig. 11. (A) Bleeding time, (B) Clotting time of mice treated with different concentrations of the enzyme compared with PBS and Heparin. [** $P \leq 0.0001$ statistically significant difference from the PBS group. ** $P \leq 0.01$ significant difference when compared to the PBS group. #### $P \leq 0.0001$ significant difference when compared to the heparin group]**

Discussion

Currently, enzymes have a major role in various applications in the fields of food, detergent, medicine, and so forth, since enzymes can solve problems of conventional methods in the breakdown of complex matters.

The fibrinolytic enzyme has medical applications and is regarded as a life-saving drug. In the current study, production, purification, and biochemical characterization of fibrinolytic protease from *Scopulariopsis brevicaulis* OS 3456 isolated from soil near a slaughterhouse were reported. It was the first time to extract fibrinolytic enzyme from *Scopulariopsis* sp. Afini et al. (2016) isolated fibrinolytic protease from *Aspergillus carbonarius* S-CSR-0007 from the same soil source near the slaughterhouse.

The enzyme was partially purified by fractional precipitation through the use of ammonium sulphate having protein molecule exclusionary power, which helps in effective protein precipitation. The maximum amount of protein was precipitated at 80% saturation, and specific activity was 183.33 U/mg with purification fold of 1.7. The enzyme was then subjected to gel filtration chromatography using Sephadex G-100 in order to minimize the amount of precipitated protein with specific activity of 370 U/mg and purification fold of 3.4. The purification method was very close to that of Al Farraj et al. (2020). Other researchers utilized different enzyme purification techniques, such as Zhou et al. (2022), who used ethanol precipitation and DEAE-Sephadex chromatography to purify the Velefibrinase from *Bacillus velezensis* Z01, and Hu et al. (2019), who used UNOsphere Q column chromatography, Sephadex G-75 gel filtration, and high-performance liquid chromatography to purify the fibrinolytic enzyme from *Bacillus subtilis* DC27.

The enzyme purity was confirmed by SDS-PAGE, showing one protein band with a molecular weight of about 61.5kDa. The molecular weight was comparable with that of serrapeptase (60kDa) purified from *Serratia marcescens* Strain E 15 (Ethiraj & Gopinath, 2017). However, its molecular weight was found to be higher than fibrinolytic protease from *Aspergillus ustus* 1 (33kDa) (Popova et al., 2021) and Velefibrinase from *Bacillus velezensis* Z01 (32.3kDa) (Zhou et al., 2022). However, it was lower than fibrinolytic enzymes from *Arthrospira platensis* (72kDa) (de Barros et al., 2020) and *Lasiodiplodia pseudotheobromae* (80kDa) (Meshram & Saxena, 2016).

The purified enzyme maintained fibrinolytic activity across a broad pH range of 5.0–9.0 with a maximum stability at pH 7.0, and the optimum temperature was 37°C, which is in line with the physiological environment of the human body. The lower temperature and lower pH reduced the enzyme activity by slowing down the reaction rate because they changed the shape of the enzyme active site. However, extreme temperatures can cause denaturation of the enzyme, causing it to lose its shape and stop working. Furthermore, the enzyme became unstable at temperatures above 50°C, verifying that the enzyme has somewhat good thermal stability. These findings are the same as those of fibrinolytic enzymes from *Streptomyces radiopugnans* VITSD8 (Dhamodharan et al., 2019) and *Bacillus amyloliquefaciens* strain KJ10 (Rajaselvam et al., 2021). Meanwhile, the fibrinolytic enzyme produced by Biji et al. (2016) was thermophilic with an optimum temperature of 50°C, and the enzyme obtained by Kornienko et al. (2021) was alkaline with a pH of 10.0. On the other hand, Kim et al. (2011) produced an acidic fibrinolytic enzyme with an optimum pH of 5.0. It was found that after 120 minutes, the activity slightly decreased because an enzyme began to lose its catalytic activity due to denaturation. Therefore, the enzyme would lose a significant amount of activity after a specific period of incubation (120min) (El-Naggar et al., 2017).

Generally, metal ions and inhibitors play a crucial role in the regulation of enzyme activity by their effect on the active site of the enzyme. It was found that the addition of ß-mercaptoethanol, Mn^{2+}, Ca^{2+}, and Mg^{2+} at 5mM concentration significantly increased the enzyme activity by 137.1, 51.6, 14.4, and 10.3%, respectively, acting as activators. The activity increased in the presence of β-mercaptoethanol as a reducing agent, indicating the involvement of sulfhydryl groups (Sami et al., 2007). On the contrary, EDTA, PMSF, Cu^{2+}, and Zn^{2+} reduced the enzyme activity, revealing that these cations bind to carboxyl groups that may be an essential component of the active site of the enzyme. This suggests that the enzyme is a serine and metalloprotease enzyme. These results are in good agreement with Yao et al. (2019). On the other hand, Kumar et al. (2020) reported that the enzyme activity decreased in the presence of Mn^{2+}. Moreover, Krishnamurthy & Belur (2018) found that Zn^{2+} activated the fibrinolytic enzymes.

In the current study, the *in vitro* thrombolytic and anticoagulant activities of the enzyme were evaluated. The clot lysis activity reached its maximum (100%) at 370U/mg. When the enzyme activity decreased to 185, 92.5, 46.25, 23.125, and 11.562U/mg, the clot lysis percentage decreased gradually to 76.8, 67.4, 57.8, 39.5, and 28%, respectively. The anticoagulant activity was obvious at the enzyme activities of 370, 185, and 92.5 U/mg. Similarly, the results of Yuan et al. (2012) demonstrated that the thrombolytic and anti-thrombus effects of the enzyme improved when the enzyme activity increased to 2070U. Meanwhile, Sharma et al. (2020) reported that a significant reduction in clot weight was observed with 25, 50, and 75U/mL concentrations of fibrinolytic protease with 70, 57, and 22% residual clot weights, respectively.

The cytotoxic activity of the purified enzyme was evaluated against the WI-38 cell line. The cytotoxic concentration $\mathbf{CC_{50}}$ value of the enzyme was equivalent to 8.11±0.97 µg/ml which is lower than that reported by Mukherjee et al. (2012), who found that a dose up to 15 µg/ml did not demonstrate cytotoxicity towards HT29 mammalian cells and it induced only 3.0% hemolysis of the washed mammalian erythrocytes.

Moreover, the *in vivo* thrombolytic activity of the enzyme was determined in κ-carrageenan-induced tail thrombosis model, which has the major advantages of visibility of the thrombus infraction region, simplicity, and accuracy without sacrificing the animal. Carrageenan is a straight-chained sulfur-containing macromolecular polysaccharide inducing tissue inflammation and tail thrombosis in experimental animals, especially the Kappa type. The results demonstrated that the enzyme significantly prevented the tail thrombosis

at a dose of 370 U/mg, confirming the anti-thrombotic power of the enzyme. Similarly, Yan et al. (2009) reported that when the amount of subtilisin QK reached 12000 IU, the thrombus nearly disappeared in the mouse tail. Moreover, Ma et al. (2015) found that a medium dose of aspirin eugenol ester (36 mg/kg) was a strong anti-thrombotic agent. The same results obtained by Zhou et al. (2022) confirmed that the high dosage of Velefibrinase reduced the tail thrombus region by 36.82%.

Conclusion

The present study revealed that *Scopulariopsis brevicaulis* OS 3456 has effective fibrinolytic activity. The purified fibrinolytic enzyme with a molecular weight of 61.5 kDa exhibits significant *in vitro* thrombolytic and anti-coagulant activities. Furthermore, the thrombolytic effect of the produced fibrinolytic enzyme was confirmed *in vivo* for use as a commercial thrombolytic drug. Therefore, the current study indicated that this enzyme from this fungal isolate can be utilized efficiently as a new medication for thrombus lysis.

Competing interests The authors report no conflicts of interest regarding this work.

Authors' contributions: Omnia Shaaban Mousa: conceptualization, methodology, validation, formal analysis, software, investigation, data curation, writing - original draft, visualization. Noha Mohamed Abd Elhameed: methodology, review & editing, supervision, project administration. Adel Ahmed El Mehalawy: conceptualization, investigation, review & editing, visualization, supervision, project administration. Samar Samir Mohamed: conceptualization, methodology, investigation, data curation, writing - review & editing, visualization, supervision, project administration.

Ethics approval: The scientific research ethics committee has approved the current research study with a code ASU-SCI/MICR/2023/1/2.

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انتاج و توصيف واختبار النشاط المعملي و الحي لانزيم جديد محلل للفيبرين بواسطة سكوبيولاريوبسيس بريفيكالس

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تمت دراسة إنتاج إنزيم محلل للفيبرين من نوع فطري محلي معزول من عينة من التربة وهو سكوبيولاريوبسيس بريفيكالس و قد تمت تنقية الإنزيم بطريقة الترسيب باستخدام كبريتات الأمونيوم وكروماتوجرافيا باستخدام مادة (Sephadex G-100) .

قد وجد ان النشاط النوعي للانزيم بعد عملية التنقية اصبح 370 وحدة\ مجم بعائد 1.5 % و معدل تنقية 3.4 . وكان الوزن الجزيئي للإنزيم المنقى 61.5 كيلو دالتون. وقد وجد ان درجة الحرارة المثلى للإنزيم هي 37 درجة مئوية وكانت مستقرة على نطاق واسع من الأس الهيدروجيني من 5.0 إلى 9.0 مع أقصى ثبات عند 7.0.

تم العثور على نشاط التخثر في المختبر للإنزيم المنقى غير المخفف (370 وحدة / مجم) بنسبة ٪100 وعند تخفيفه الي 185 ، 92.5 ، 46.25 ، 23.125 ، 11.562 وحدة / مجم ، كانت نسبة تحلل الجلطة 76.8 ، 67.4 ، 57.8 و 39.5 و ٪28 على التوالي. ثم تم تطبيق نموذج تجلط الذيل الناجم عن الكاراجينان لاختبار نشاط التخثر في الجسم الحي للإنزيم. أظهرت النتيجة عدم وجود جلطة واضحة في ذيل الفئران التي عولجت بالإنزيم المختبَر 370 وحدة / مجم ولكن عندما تم تخفيف الإنزيم، انخفض نشاط التخثر تدريجيًا.