JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

FIBRINOLYTIC PROTEASES OF ENTOMOPATHOGENIC MICROMYCETES: PRODUCTION AND CHARACTERISTICS

Daria L. Basalaeva [1], Anna A. Bogomolova [2], Alexander A. Osmolovskiy[1]*

Address(es):
[1] Department of Microbiology, Lomonosov Moscow State University, 1-12 Leninskie Gory, 119991, Moscow, Russia.
[2] Utrecht University, Utrecht, 80125, 3508 TC, Netherlands.

*Corresponding author: dbasalaewa@yandex.ru

https://doi.org/10.55251/jmbfs.11899

ARTICLE INFO

Received 4. 10. 2024
Revised 21. 5. 2025
Accepted 22. 5. 2025
Published 1. 8. 2025

Regular article

OPEN ACCESS

ABSTRACT

The search for new fibrinolytic enzymes is of great interest, as they are widely used in the treatment of thromboembolic conditions. However, existing treatments are limited and may cause side effects. The aim of the study is to find out whether entomopathogenic micromycetes can produce fibrinolytic enzymes and to study their properties. Primary screening of proteolytic enzyme production was performed among 15 strains of entomopathogenic micromycetes. After cultivation of the producer strain and precipitation with ammonium sulfate, followed by purification by dialysis, enzyme extracts were obtained. Fractionation was then performed using isoelectric focusing to isolate individual samples for the analysis of the proteolytic activity of fungal strains.

Three most promising strains from the Cordycipitaceae and Ophiocordycipitaceae families were selected. The most suitable cultivation conditions were determined, and the producing strain was selected. Micromycetes have demonstrated a significant ability to break down protein molecules. Consequently, a comprehensive analysis of proteolytic activity was conducted in 15 entomopathogenic strains. The dynamics of protease accumulation in the nutrient medium was investigated in three strains after submerged cultivation. A preparation of a proteolytic enzyme from the micromycete *Akanthomyces* cf. *aculeatus*, exhibiting both overall proteolytic and fibrinolytic activities, was obtained. The results indicate that the proteolytic agent under consideration is a protein with a molecular weight estimated at approximately 35 kDa and an isoelectric point of 2.15. Moreover, it has been confirmed that this enzyme can cleave both fibrin and fibrinogen according to SDS-PAGE analysis.

Keywords: fibrinolytic enzymes, entomopathogenic fungi, micromycetes, proteases, fibrinolysis, submerged cultivation

INTRODUCTION

Micromycetes are a diverse group of fungi that can be divided into various ecological categories, one of which is entomopathogenic fungi that cause insect diseases. These fungi produce a wide range of enzymes, in particular, proteolytic enzymes play a significant role in their metabolism.

Proteolytic enzymes from the class of hydrolases are used in medicine to dissolve blood clots that form inside blood vessels, in case of violations of the natural process of blood clotting. Blood clots consist of fibrin, platelets and red blood cells. Fibrinolytic enzymes break down fibrin and other components of blood clots by direct action on fibrin or by activating the plasminogen protein, which then turns into plasmin and also cleaves fibrin. In addition, antithrombotic drugs also include anticoagulants and antiplatelet drug (**Leger *et al*, 1994; Wilberts *et al*, 2022**).

Blood clot-related diseases such as atherosclerosis, heart attack, high blood pressure and other heart-related problems are among the leading causes of death worldwide (**Marin *et al*, 1961; Sharkova *et al*, 2016**).

Each type of antithrombotic medicines has its own limitations and should be prescribed based on the individual characteristics of the disease and the patient's body. Although anticoagulant and antiplatelet drugs are effective in preventing the formation of new blood clots, they are not as effective as fibrinolytic enzymes in destroying existing blood clots.

In this regard, there is an urgent need to develop more effective drugs that affect various components of blood clotting and completely prevent the formation of blood clots, affecting the processes of coagulation and fibrinolysis.

Fungi have been found to produce fibrinolytic enzymes and could potentially be used in medicine to treat patients with blood clots. Examples of promising fungal drugs include the plasminogen activator proteolytic complex Longolitin, produced by a micromycete of the species *Arthrobotrys longa*. It has been shown that Longolitin is effective against hematoma formation in rats, which indicates its ability to lyse blood clots (**Gautam *et al*, 2022**). Thrombolytics, fungal agents that directly lyse blood clots and have been widely used in human therapy in the past, include aspergillin O, which is extracted from *Aspergillus oryzae* (**Dijksterhuis, 2019**).

Fibrinolytic drugs derived from bacteria have numerous side effects, which forces scientists to look for other sources (**Moore *et al*, 2020**). Entomopathogenic micromycetes, which require numerous proteases from host organisms to obtain nutrients, have not been widely studied for their fibrinolytic properties.

Entomopathogenic fungi produce proteolytic enzymes, in particular fibrinolytic ones, which they secrete to spread throughout the insect body. These enzymes suppress the immune system of insects and change the pH level in their body (**Sánchez-Pérez *et al*, 2014**). Fibrinolytic enzymes are an integral part of modern thrombolytic therapy and are of increasing interest to scientists. However, a huge number of producers of proteolytic enzymes useful for humans remain unknown.

One of these groups of fungi that have not been fully studied in terms of their production of fibrinolytic enzymes are entomopathogenic micromycetes (**Sharma *et al*, 2021**). These fungi are a promising source of unique enzymes that can find significant medical applications.

Thus, the study of these fungi not only has the scientific potential to obtain new information about the species, but also contributes to the development of thrombolytic therapy and the medical use of mushrooms.

MATERIALS AND METHODS

Objects of study

Fifteen strains of micromycetes from the collection of the Department of Mycology and Algology, Lomonosov Moscow State University, Russia were used (Tabl. 1). The strains used in this study are entomopathogenic microorganisms, which act as parasites to insects from the order Ascomycota.

Table 1 Strains of micromycetes

No. from collection	Name of the strain	Family
4599	*Akanthomyces* cf. *aculeatus* Lebert, 1858	Cordycipitaceae
4606	*Akanthomyces muscarius* (Petch) Spatafora, Kepler & B. Shrestha, 2017, strain 1	
4607	*Akanthomyces muscarius* strain 2	
4614	*Akanthomyces muscarius* strain 3	
4616	*Akanthomyces muscarius* strain 4	
4204	*Beauveria bassiana* (Bals.-Criv.) Vuill., 1912	
4200	*Conoideocrella luteorostrata* (Zimm.) D. Johnson, G.H. Sung, Hywel-Jones & Spatafora, 2009	
4206	*Cordyceps fumosorosea* (Wize) Kepler, B. Shrestha & Spatafora, 2017	
5380	*Cordyceps militaris* (L.) Fr., 1818	
4205	*Cordyceps tenuipes* (Peck) Kepler, B. Shrestha & Spatafora, 2017	
4611	*Simplicillium wallacei* H.C. Evans, 2001, strain 1	
4612	*Simplicillium wallacei*, strain 2	
4207	*Purpureocillium lilacinum* (Thom) Luangsaard, Houbraken, Hywel-Jones & Samson, 2011, stain 1	Ophiocordycipitaceae
5376	*Purpureocillium lilacinum* , strain 2	
4241	*Purpureocillium takamizusanense* (Kobayasi) S. Ban, Azuma & Hiroki Sato, 2015	

Overall Proteolytic Potential

Evaluation of proteolytic potential was conducted using a solid medium with casein, collagen, elastin, keratin, hemoglobin, and fibrin as protein substrates. The media composition was: KH_2PO_4 - 0.5 g/l (Reachim, Russia), $MgSO_4$ - 0.25 g/l (Reachim, Russia), peptone – 5.0 g/l (Obolensk, Russia), agar – 15.0 g/l (Obolensk, Russia), and protein substrate casein/collagen/elastin/keratin – 10.0 g/l (Sigma-Aldrich, Germany) or hemoglobin/fibrin– 5.0 g/l) (Sigma-Aldrich, Germany). Each microorganism was cultivated for 4 days at 28°C. The zones of hydrolysis were visualized using 0.08% Coomassie G-250 (Sigma-Aldrich, Germany) in 3.5% perchloric acid (Sigma-Aldrich, Germany). The selection of active strains was based on the measured enzymatic index (EI), which is the ratio of the diameter of the hydrolysis zone to the diameter of the colony. The strains with the highest rank among the studied fungi were considered active and were further examined.

Cultivation

Cultures of the strains were grown in tubes with slant agar (wort – 40.0 g/l, agar (Obolensk, Russia) – 18.0 g/l) for 7 days at a temperature of 28° C.
Then the cultures were transferred to a liquid nutrient medium (wort – 87.0 g/l, glucose (Sisco Research Laboratories Pvt. Ltd., India) – 20.0 g/l and peptone (Obolensk, Russia) – 1.0 g/l; pH 5.5–6.0) and grown for 2 days at 200 rpm, 28° C. Then 3% of the biomass was sterically transferred to two fermentation media to continue cultivation.
To study the dynamics of enzyme accumulation, active strains were cultured for two weeks in FM1 and FM2, measuring proteolytic activity daily. The culture liquid was separated from the biomass of the micromycete by filtration through filter paper (FS, Russia) using a Bunsen flask.
Composition (g/l) FM1: glycerin («Reachim», Russia) - 70.0 g/l, glucose («Sisco Research Laboratories Pvt. Ltd.», India) - 30.0 g/l, fish meal hydrolysate («Obolensk», Russia) - 5.0 g/l, $NaNO_3$ («Reachim», Russia) - 2.0 g/l, $MgSO_4$ («Reachim», Russia) - 0.5 g/l, KH_2PO_4 («Reachim», Russia) - 0.5 g/l, pH 5.5-6.0; and FM2: glucose («Sisco Research Laboratories Pvt. Ltd.», India) - 35.0 g/l, fishmeal hydrolysate («Obolensk», Russia) - 5.0 g/l, NaCl («Reachim», Russia) - 2.0 g/l, starch («Sigma-Aldrich», Germany) - 12.0 g/l, peptone («Obolensk», Russia) - 5.0 g/l, KH_2PO_4 («Reachim», Russia) - 0.5 g/l, $MgSO_4$ («Reachim», Russia) - 0.5 g/l, pH 5.5-6.0.
Thus, the fermentation media differed in nitrogen sources - FM2 contained only a source of amine nitrogen (fishmeal hydrolysate and peptone**),** while FM1 contained sources of both amine and mineral nitrogen (fishmeal hydrolysate and sodium nitrate).

Measurement of proteolytic activity

After preliminary separation of biomass by filtration, the protease activity of fungi was determined. The proteolytic activity of fungal proteases was observed using azocasein (Sigma-Aldrich, Germany) for overall enzymatic activity, bovine fibrinogen (Sigma-Aldrich, Germany) for fibrinogenolytic activity, azocoll (EMD Millipore Corp., USA) for collagenolytic activity, chromogenic peptide substrates (CPS)
S-2251 (H-D-Val-Leu-Lys-pNA) (Sigma-Aldrich, Germany) for plasmin activity (direct fibrinolytic), S-2444 (pyro-Glu-Gly-Arg-pNA) (Sigma-Aldrich, Germany) for urokinase activity (plasminogen activator) and S-4760 (N-Succinyl-Ala-Ala-Ala-pNA)
(Sigma-Aldrich, Germany) for elastase activity.

To start the reaction, 100 µl of culture liquid was added to 200 µl of the substrate. The reactions were carried out for 10 (fibrinogen), 20 (azocoll) and 30 (azocasein) minutes and stopped by adding 300 µl of 10% trichloroacetic acid («Sigma-Aldrich», Germany). The samples were then measured after centrifugation of the sample (12.400 × g, for 5-10 min). Optical density measurements were performed using a BioSpectrometer® kinetic (Eppendorf, Germany) at 275 nm for fibrinogen, 340 nm for azocasein and 519 nm for azocoll.
To carry out reactions with CPS, 200 µl of the fungi culture liquid sample was mixed with 50 µl of 0.05 M Tris-HCl buffer with a pH of 8.2 and 100 µl of a chromogenic substrate. The reaction was stopped by 200 µl of 50% acetic acid. The optical density of CPS was measured at a wavelength of 405 nm.
The elastase activity of these strains has been measured to assess their safety in terms of their pathogenic potential. Elastase is an enzyme responsible for the hydrolysis of elastin, a key component of human connective tissue. Further research will involve evaluating the cytotoxicity of any products resulting from this process.

Obtaining of protease preparations.

Extracellular proteases are precipitated from culture filtrates with ammonium sulfate («Lenreactive», Russia). After the proteins are salted out, they are centrifuged at 15.000 × g for 20 minutes at 4 ° C. The resulting precipitate is dissolved in 0.0005 M Tris-HCl buffer (pH 8.2) and purified in dialysis tubes with the same buffer at a temperature of 4 °C for 18 hours. This is followed by freezing of the dialyzed protein preparation with liquid nitrogen and lyophilization.
The separation of the obtained samples is carried out using preparative isoelectric focusing, which is carried out according to the Westerberg method using ampholines with a pH range of 3-10 and a sucrose density gradient of 0-40% in a 110 ml column (LKB, Bromma, Sweden), at a voltage of 800 V for 36 hours. At the end, fractions are collected using a fraction collector and the pH, protein content and proteolytic activity in relation to the target substrates are determined.

Determination of protein content

The protein concentration is determined spectrophotometrically by measuring the optical density at 280 nm.

Denaturing gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE)

To assess the homogeneity of the isolated enzymes, electrophoresis was performed under denaturing conditions in 12% polyacrylamide gel (PAAG) in the presence of sodium dodecyl sulfate (SDS). A buffer for samples with dithiotriethol (DTT) was used to prepare the samples. For denaturation, the samples were heated for 3 minutes at 100 °C. Electrophoresis was performed at a voltage of 120 V and a current strength of 35 mA. The gel was then incubated in a fixing solution (10% acetic acid and 30% isopropanol) for 30 minutes.
To stain the gel, a 0.25% solution of Coomassie R-250 in 50% isopropanol and 10% acetic acid was used, in which the gel was kept for 30 minutes. The gel was then washed with a 7% solution of acetic acid for 10-12 hours.

Fibrin zymography

Polyacrylamide gel for fibrin zymography contains 100 µl of thrombin and 0.12% fibrinogen without the addition of SDS. Preparations from fractions with high

proteolytic activity are passed through the gel under native (non-denaturing) conditions at 12 mA and room temperature. After electrophoresis, the gel is incubated for 30 minutes in 50 mm Tris-HCl, pH 8.0, containing 2.5% Triton X-100, at room temperature. It is then washed for 30 minutes in distilled water and incubated for 18 hours at 37 ° C in a zymogram reaction buffer consisting of 0.02% NaN3 and 30 mm Tris-HCl, pH 8.0. To visualize the areas of hydrolyzed fibrin, the gel is stained with a standard solution of Coomassie R-250 for 2 hours and washed 3 times with 7% acetic acid.

Fibrinolysis and fibrinogenolysis

To determine the activity of fibrinolytic enzymes against fibrin, an experiment was carried out consisting of conducting a hydrolysis reaction between fibrin and fibrinogen using the resulting preparation. Visualization of the hydrolysis products was then performed using SDS-PAGE.
The reaction mixture consisted of 25 µl of a mixture of active fractions obtained after isoelectric focusing (IEF), 50 µl of 0.1% fibrinogen or 0.5% fibrin. In the case of fibrin, the mixture was incubated at different time points of 10 sec, 30 sec, 1 min, 2.5 min, 5 min and 10 min. For fibrinogen, the incubation time was 1, 2.5, 5, 10, 20 and 30 min. The reaction was terminated using a sample buffer for gel electrophoresis containing mercaptoethanol (25 µl). After termination of the reaction, the samples were heated to 100°C for 3 minutes.
As a control, two additional samples were prepared and used to determine the background level of activity. These included 25 µl of distilled water and 50 µl each of fibrin or fibrinogen mixed with 25 µl of sample buffer. After the reaction, SDS-PAGE was conducted to visualize the products of fibrin and fibrinogen hydrolysis.

Statistical analysis

The statistical analysis of the findings was conducted using the software suites Statistica 10.0 for Windows (developed by StatSoft Inc., USA) and Microsoft Excel 2016 for Windows 11. Statistical significance was determined based on a p-value threshold of less than 0.05. Each experimental procedure was replicated at least three times in biological terms and three times in analytical terms.

RESULTS

Results of overall proteolytic potential

The analysis of proteolytic potential resulted in selection of the following active strains: *Akanthomyces* cf. *aculeatus*, *A. muscarius* strain 4, *Conoideocrella luteorostrata*, *Purpureocillium takamizusanense* and *Simplicillium. wallacei* strain 1 (Fig. 1).
Table 2 summarizes the data on proteolytic potential of strains cultivated on agar media with protein substrates. The result demonstrates that entomopathogenic fungi degrade different substrates, including both fibrous and globular proteins.

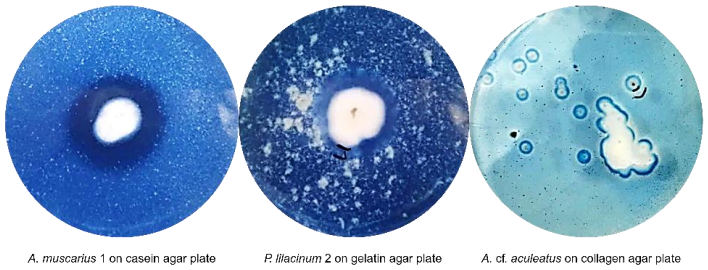


Figure 1 Growth of colonies of micromycetes on agar plates

Table 2 EI of strains cultivated on different substrates (M±m, p≤0.05)

Micromycete strains	EI_{casein}	$EI_{collagen}$	$EI_{gelatin}$	$EI_{keratin}$	$EI_{elastin}$	$EI_{hemoglobin}$	EI_{fibrin}
Akanthomyces cf. *aculeatus*	1.16±0.01	2.27±0.04	1.00±0.01	1.00±0.02	1.09±0.02	1.00±0.02	1.17±0.01
A. muscarius strain 1	1.75±0.02	1.26±0.03	1.00±0.01	1.17±0.03	1.32±0.03	1.00±0.01	1.14±0.02
A. muscarius strain 2	1.12±0.01	1.29±0.01	1.00±0.02	1.06±0.05	1.00±0.01	1.09±0.04	1.11±0.01
A. muscarius strain 3	1.11±0.03	1.29±0.02	1.00±0.01	1.13±0.04	1.00±0.01	1.28±0.02	1.10±0.01
A. muscarius strain 4	1.26±0.02	1.15±0.02	1.05±0.02	1.07±0.01	1.00±0.02	1.37±0.03	1.20±0.03
Beauveria bassiana	1.00±0.01	1.27±0.01	1.00±0.01	1.07±0.01	1.00±0.02	1.17±0.01	1.00±0.01
Conoideocrella luteorostrata	1.23±0.01	1.00±0.02	1.00±0.02	1.36±0.02	1.00±0.01	1.00±0.01	1.00±0.01
Cordyceps fumosorosea	1.21±0.02	1.16±0.03	1.40±0.03	1.05±0.01	1.00±0.02	1.21±0.02	1.08±0.02
C. militaris	1.96±0.03	1.00±0.01	1.31±0.02	1.00±0.02	1.00±0.02	1.00±0.01	1.00±0.01
C. tenuipes	1.00±0.01	1.27±0.02	1.00±0.01	1.00±0.01	1.00±0.03	1.26±0.03	1.00±0.01
Purpureocillium lilacinum strain 1	1.17±0.01	1.23±0.04	1.49±0.04	1.04±0.01	1.00±0.01	1.23±0.01	1.11±0.03
P. lilacinum strain 2	1.63±0.02	1.00±0.01	1.46±0.01	1.00±0.03	1.11±0.01	1.21±0.05	1.06±0.02
P. takamizusanense	1.03±0.01	1.26±0.03	1.21±0.02	1.20±0.02	1.00±0.02	1.00±0.01	1.20±0.01
Simplicillium wallacei strain 1	1.13±0.03	1.19±0.02	1.00±0.01	1.06±0.01	1.38±0.04	1.17±0.03	1.29±0.02
S. wallacei strain 2	1.57±0.02	1.15±0.01	1.43±0.02	1.05±0.01	1.33±0.02	1.24±0.01	1.08±0.01

Screening results of isolated strains.

Regarding submerged cultivation, all 5 strains, except *Conoideocrella luteorostrata* in FM2, possessed overall enzymatic activity (azocasein hydrolysis) when cultivated in FM1 and FM 2. Strains *Akanthomyces* cf. *aculeatus*, *Purpureocillium takamizusanense* and *Simplicillium wallacei* 1 showed relatively high activity against the majority of substrates. Therefore, they were chosen for further investigation and daily measurement of proteases' accumulation. *C. luteorostrata* was the least active strain. It only hydrolysed azocasein in FM1. Moreover, it was the lowest figure among all strains. Table 2 represents overall enzymatic, collagenolytic, plasmin-like, activator of plasminogen (urokinase) and elastase activity of the strains cultivated in submerged cultures in 2 media of different composition (Tabl. 3).

Table 3 Enzymatic activity of micromycetes in fermentation media

Micromycete strains		Activity, U/ml		Activity, µmol pNA×10-3/ml/min		
		Overall proteolytic	Collagenolytic	Elastase (S-4760)	Plasmin-like (S-2251)	Urokinase (S-2444)
Akanthomyces cf. *aculeatus*	FM1	0,223±0,005	0,023±0,001	0,522±0,006	64,322±0,050	0,116±0,010
	FM2	0,085±0,003	0,015±0,001	6,380±0,001	31,204±0,042	6,728±0,012
A. muscarius strain 4	FM1	0,084±0,001	0,000±0,001	0,000±0,001	0,696±0,034	4,640±0,051
	FM2	0,031±0,002	0,000±0,001	2,668±0,002	0,000±0,010	3,132±0,023
Conoideocrella luteorostrata	FM1	0,017±0,002	0,001±0,002	0,870±0,004	0,406±0,023	0,058±0,011
	FM2	0,009±0,001	0,000±0,001	0,000±0,001	0,464±0,024	0,000±0,010
Purpureocillium takamizusanense	FM1	0,295±0,004	0,028±0,001	6,670±0,003	0,000±0,011	6,032±0,024
	FM2	0,178±0,004	0,048±0,002	0,000±0,001	0,870±0,031	0,870±0,036
Simplicillium wallacei strain 1	FM1	1,110±0,001	0,122±0,003	2,610±0,005	75,400±0,064	72,094±0,031
	FM2	0,213±0,003	0,036±0,003	2,668±0,004	74,704±0,032	94,946±0,067

The results of studying the dynamics of proteolytic enzymes

To study the dynamics of enzyme production. strains of *Akanthomyces* cf. *aculeatus*, *Purpureocillium takamizusanense* and *Simplicillium wallacei* strain 1 were cultured in two fermentation media under submerged conditions and samples were taken every day for 9 days. An increase in urokinase, plasmin-like, elastase and fibrinogenolytic activity was observed on day 4-5 in all strains. *P. takamizusanense* and *S. wallacei* strain 1 actively hydrolyzed azocasein in fermentation medium 2 (Fig. 2a). In contrast, *A.* cf. *aculeatus* did not cleave azocasein, but had the highest fibrinogenolytic and urokinase activity on day 5 (Fig. 2c and Fig. 2f). In addition, the strain effectively hydrolyzed CPS with respect to plasmin-like and CPS elastase activity and was able to hydrolyze azocoll. It is worth noting that *S. wallacei* strain 1 also proved to be a promising producer of proteolytic enzymes, particularly with regard to urokinase. However, this culture was less convenient to work with and required more frequent inoculation. This can be a disadvantage for the biotechnology process, as it entails time and energy costs. Therefore, it was decided to use *A.* cf. *aculeatus* for the preparation of proteolytic enzymes. The peak of enzyme secretion was detected on the fifth day of cultivation with fermentation medium 1.

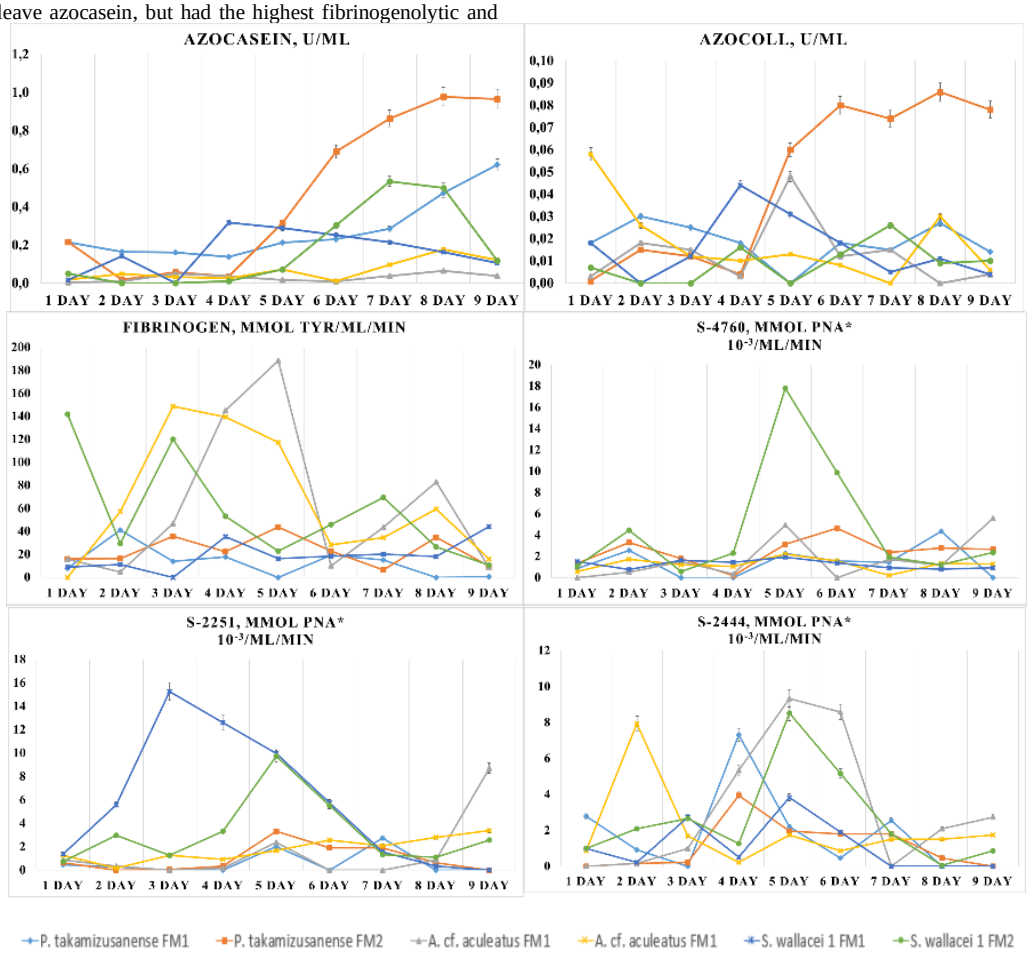


Figure 2 Dinamycs of proteolytic activity of active strains of entomopathogens

Results of obtaining of protease preparations and isoelectrofocusing

As a result of preparation of the *Akanthomyces* cf. *aculeatus* strain and separation of the protein preparation using isoelectrofocusing, two peaks of protein content were determined at acidic pH values of 2,15 and 5,05 (Fig. 3). In addition, the peak of total proteolytic activity was found in the neutral pH region of 6,75. In addition, reactions with target substrates were carried out for active fractions. The analysis of proteolytic activity allowed us to identify the five most active fractions: No. 7, 11 13, and 17.

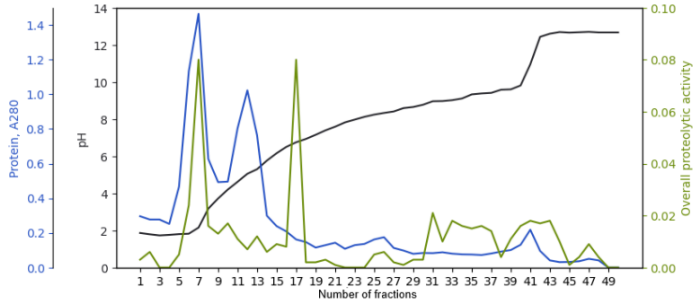


Figure 3 Isoelectrofocusing of proteases of *Akanthomyces* cf. *aculeatus* strain on FM1

The enzymes in fraction No. 7 showed an exceptionally high ability to hydrolyze fibrinogen (61,887 ± 0,198 µmol Tyr/ml/min), as well as noticeable plasmin and urokinase like activity. At the same time, the peak activity of elastase and urokinase was observed in fraction No. 12 (6,438 ± 0,105 µmol pNA×10-3/ml/min S-4760, 9,976 ± 0,099 µmol pNA×10-3/ml/min S-2444), while plasmin like activity was observed in fraction No. 13 (6,728 ± 0,090 µmol pNA×10-3/ml/min S-2251), None of the fractions hydrolyzed azocoll to any significant extent. The study of the substrate specificity of drug fractions has shown that they are capable of hydrolyzing various previously used substrates.

A comparison of the protein content, proteolytic activity and substrate specificity in fractions 1-8 shows that fibrinolytic enzymes are present in fraction 7. This is indicated by the peaks of these indicators.

The low pH level of fraction 7 (2,15) suggests that the micromycete *A.* cf. *aculeatus* secretes proteolytic enzymes that are active at acidic pH values. Fractions No. 7, 11 13, and 17 were also used to study enzymatic uniformity and the ability to hydrolyze fibrin.

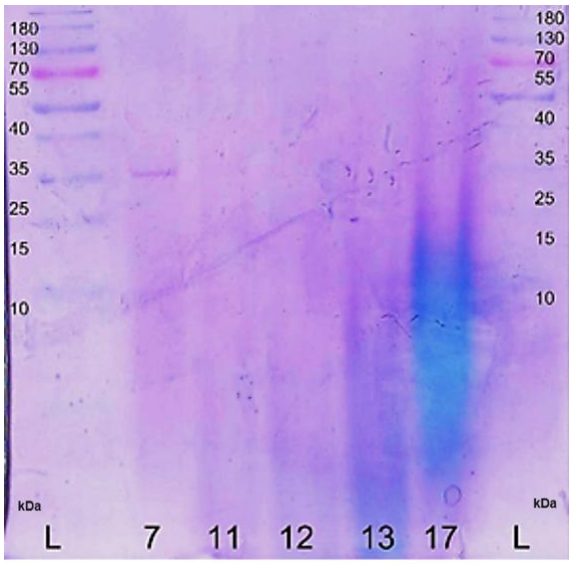
SDS-PAGE, zymography, fibrinolysis and fibrinogenolysis

Electrophoresis was used to evaluate the enzymatic uniformity of these fractions of the purified protein preparation. Denaturing electrophoresis by the Laemmli method showed that the entomopathogen *Akanthomyces* cf. *aculeatus* secretes a single protein with a molecular weight of approximately 35 kDa (Fig. 4). The zymography of fibrin fractions allowed us to establish that the drug can directly hydrolyze fibrin formed by mixing fibrinogen and thrombin in a gel (Fig. 5). The hydrolysis zone on the nomogram corresponds to a more transparent band in the gel on the track with the fraction number 7. This band is also located at the level of a marker with a mass of approximately 35 kDa.

Comparison of the results of Laemmli denaturing electrophoresis and fibrin zymogram showed that the micromycete *A.* cf. *aculeatus* does secrete a protease with a molecular weight of approximately 35 kDa and fibrinolytic activity.

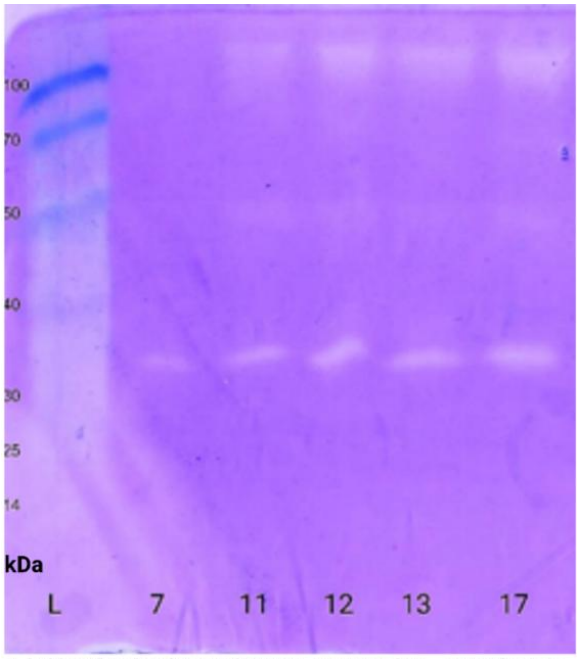
The results of an electrophoretic analysis of the products of the hydrolysis of fibrin and fibrinogen by a proteolytic enzyme isolated from the microorganism A. cf. aculeatus in culture medium indicate that this enzyme is capable of effectively

cleaving fibrinogen within 180 seconds after incubation, and fibrin within 30 minutes of the start of the reaction. For both fibrin and fibrinogen, the hydrolysis of alpha subunits is observed (Fig. 6). These findings allow us to draw the conclusion that the results of fibrin zymography correspond to the activity against natural fibrin and fibrinogen proteins.



L- ladder of molecular weight; 7, 11, 12, 13 and 17 - active fraction of preparation after IEF

Figure 4 SDS-PAGE of fractions after IEF of enzyme preparation of *Akanthomyces* cf. *aculeatus*



L- ladder of molecular weight; 7, 11, 12, 13 and 17 - active fraction of preparation after IEF

Figure 5 Fibrin zymography of fractions after IEF of enzyme preparation of *Akanthomyces* cf. *aculeatus*

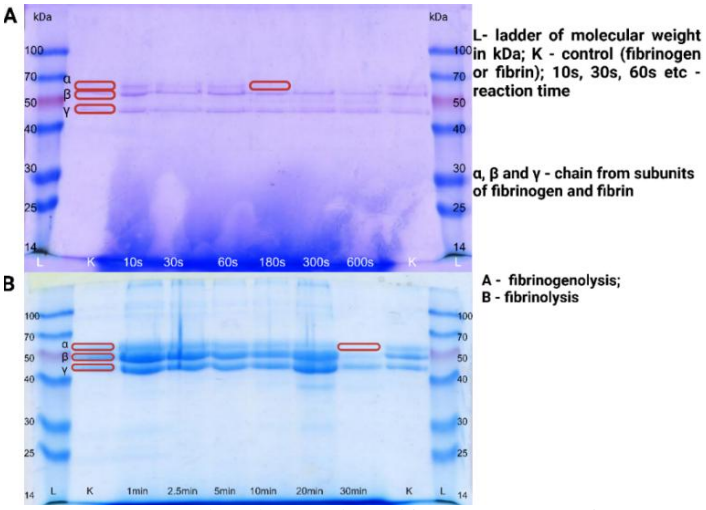


Figure 6 SDS-PAGE of reaction's products of fibrinogenolysis and fibrinolysis

The results of the analysis of the properties of the isolated protein preparation from the entomopathogenic micromycete *A.* cf. *aculeatus* showed that the fungus produces fibrinolytic protease with a molecular weight of approximately 35 kDa and a pI of 2,15. It also has plasmin and urokinase like activity. The presence of these fibrinolytic properties was confirmed by the results of zymography, which demonstrated the hydrolysis of fibrin.

DISCUSSION

The kingdom of fungi plays a significant role in nature due to its symbiotic interactions with other organisms and their influence on biological processes.
Akantomyces cf. *aculeatus*, *Purpureocillium takamizusanense* and *Simplicillium wallacei* strain 1 caused the appearance and development of maximum enzyme release on the fifth day of deep cultivation. Purification of extracellular proteins of the fungus *A.* cf. *aculeatus* with subsequent isoelectrofocusing revealed one target protease fraction at pi 2,15 in fraction No. 7. Electrophoresis in Laemmli denaturing gel and fibrin zymography allowed us to determine that the fibrin cleaving protease has a molecular weight of approximately 35 kDa. These results demonstrate that entomopathogenic micromycetes can be a promising source of both fibrinolytic proteases and other proteolytic enzymes that can be used in medicine and many other fields of biotechnology.
Further studies of entomopathogenic fungi as fibrinolytic agents may lead to the discovery of promising new drugs for the treatment of thrombosis. Future research could focus on choosing the optimal composition of the medium, cultivation conditions and pH to increase the production of the desired product, as well as on studying other types of entomopathogenic microorganisms to expand the range of potential producers. It would also help identify more enzymes that are has fibrinolytic activity.
In addition, it would be important to thoroughly investigate the properties and toxicology of the enzymes isolated from these microorganisms in order to determine their suitability for medical use.

CONCLUSIONS

Consequently, a comprehensive analysis of proteolytic activity was conducted in 15 entomopathogenic strains, both at the primary and secondary levels. The dynamics of protease accumulation in the nutrient medium was investigated in three strains after submerged cultivation.
A preparation of a proteolytic enzyme from the micromycete *Akanthomyces* cf. *aculeatus*, exhibiting both general proteolytic and fibrinolytic activities, was obtained. This preparation was then purified, fractionated, and characterized. The molecular weight of the enzyme and its fibrin/fibrinogen cleavage activity were determined.
The results indicate that the proteolytic agent under consideration is a protein with a molecular weight estimated at approximately 35 kDa and an isoelectric point of 2.15. Moreover, it has been confirmed that this enzyme can cleave both fibrin and fibrinogen according to polyacrylamide gel electrophoresis analysis.

Funding: The authors received no specific funding for this work.

Author contributions: D.L.B. designed the experiments; D.L.B. A.A.B. and A.A.O. performed the experiments; A.A.O. provided technical assistance; D.L.B. A.A.B. and A.A.O. analyzed the data; D.L.B. A.A.B. and A.A.O. wrote the paper. All authors have read and agreed to the published version of the manuscript.

Conflict of interest: The authors declare that they have no conflicts of interest.

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