Microbiol. Biotechnol. Lett. (2023), 51(2), 167–173
http://dx.doi.org/10.48022/mbl.2205.05002
pISSN 1598-642X eISSN 2234-7305

MBL
Microbiology and Biotechnology Letters

Action of Extracellular Protease of *Aspergillus terreus* on Human Plasma Hemostasis Proteins

Alexander A. Osmolovskiy[1,2]*, Elena S. Zvonareva[2], Nina A. Baranova[1], and Valeriana G. Kreyer[1]

[1]*Department of Microbiology, Faculty of Biology, Lomonosov Moscow State University, Moscow 119234, Russian Federation*
[2]*Higher School of Economics - National Research University, Moscow 109028, Russian Federation*

Received: May 6, 2022 / Revised: June 26, 2022 / Accepted: June 30, 2022

Proteolytic enzymes secreted by *Aspergillus*, as pathogenicity factors, affect blood coagulation and fibrinolysis, and therefore the target proteins of their action in the bloodstream are of significant interest. In the present study, the action of the isolated protease of *A. terreus* 2 on different human plasma proteins was shown. The protease of *A. terreus* 2 exhibited the highest proteolytic activity against hemoglobin, which was 2.5 times higher than the albuminolytic activity shown in both of the protein substrates used. In addition, the protease has significant ability to hydrolyze both fibrin and fibrinogen. However, the inability of the *A. terreus* 2 protease to coagulate rabbit blood plasma and coagulate human and bovine fibrinogen indicates the severity of the enzyme's action on human blood coagulation factors. It should be considered as a potential indicator of this isolated protease's participation in fungal pathogenesis. The protease shows no hemolytic activity. Furthermore, its activity is insignificantly inhibited by thrombin inhibitors, and is not inhibited by plasmin inhibitors.

Keywords: ***Aspergillus terreus*, fibrinolytic enzymes, thrombolytic activity, hemostatically-active proteases**

Introduction

Extracellular proteases of microscopic fungi are currently among the most intensively studied enzymes. Of particular interest is a possible application of these enzymes as components of agents for treatment, prevention and diagnosis of thromboembolic complications in biomedicine [1–4]. Despite the variety of their properties, such as substrate specificity, optimal pH and temperature parameters, some proteases of *Aspergillus* are able to substantially hydrolyze fibrin or coagulate blood plasma, exhibiting a hemostatic-active effect [5–10]. Proteolytic enzymes of *Aspergillus* are known for their definite fibrinolytic activity, as well as for the activating effect of such important enzymes of the human hemostasis system as protein C and factor X [5, 11, 12].

***Corresponding author**
Phone: +89 168168739
E-mail: aosmol@mail.ru

Alkaline exoproteases of *Aspergillus terreus* have found their practical application in many areas of industry and economy [13–15].

Micromycete *A. terreus* 2 is a producer of extracellular proteolytic enzymes capable of activating prekallikrein via its limited proteolysis and possessing plasmin-like activity. Previous studies of *A. terreus* 2 were the first to show that extracellular proteases produced by micromycetes are able to activate prekallikrein in human blood plasma, and also established that proteases of micromycetes are also capable to hydrolyze both fibrin and fibrinogen [5, 9, 16, 17]. Activation of prekallikrein makes it possible to consider the protease of this micromycete as a potential diagnostic component for quantity determining of the specified protein in the blood plasma of patients, and the fibrinolytic effect may indicate a potential use as a treatment for superficial microthrombosis and bruising. However, it has not been studied if *A. terreus* 2 proteases can participate in blood coagulation

and thrombolysis.

The hypothesis of the study was to determine the ability of the protease of *A. terreus* 2 to cause plasma coagulation or degradation of its constituent proteins (both globular and fibrillar).

In this work, we carried out studies aimed at the coagulation of fibrinogen and plasma in humans and some animals by extracellular protease *A. terreus* 2, its ability to directly degrade protein substrates in blood plasma, and sensitivity to some inhibitors of the hemostasis system.

Materials and Methods

Strain and growth conditions

Strain of *A. terreus* 2 from the collection of filamentous fungi-producers of proteasesactive toward hemostasis system proteins (Microbiology Department, M.V. Lomonosov Moscow State University, Russia) was used in this work.

For proteolytic potential determination the micromycete was cultivated on Petri dishes with Skim milk agar (SMA), Blood agar (BA) and Plasma agar (PA). As basal components of media BA and PA contained following substances (in %): tryptone - 0.7; peptone - 1.0; yeast autolysate - 0.5; NaCl - 0.5; Na_2CO_3 - 0.03; agar - 2.0, pH 7.0–7.4, and additionally 8% sheep blood or 5% lyophilized sheep plasma (Sigma-Aldrich, USA), respectively. The composition of SMA was as follows (in %): skim milk powder (Sigma-Aldrich) - 5.0; agar - 3.0, pH 6.0–6.5. Inoculation was performed by injecting into the center of each medium in a Petri dish. After 5 days at 24, 28 and 37℃ of incubation, hydrolysis of substrates around the colonies was visualized.

For protease production the micromycete was cultivated under submerged conditions at 28℃ and 200 ×g in orbital shaker (ES-20/60, Biosan, Latvia). It was two-stage cultivation on seeding (composition in %: wort - 6.7; glucose - 1.0; peptone - 0.1, pH 5.5–6.0) and fermentation media (composition in %: glucose - 3.0; glycerol - 7.0; fish flour hydrolysate - 0.5; $NaNO_3$ - 0.2; KH_2PO_4 - 0.05; $MgSO_4$ - 0.05, pH 5.5–6.0) in 750-ml shake flasks containing 100 ml of culture medium. The inoculum material was obtained via spore flushing with seeding medium and then transferred into the seeding-culture medium for cultivation (2 days), after a part of the biomass was transfused into the fermentation medium and further cultured for 7 days [17].

Isolation and separation of extracellular protease

The preparation of extracellular protease of *A. terreus* 2 was obtained with $(NH_4)_2SO_4$ precipitation of proteins from the culture fluid after biomass removing at the rate of 608 g per 1 liter of sample. Salted out proteins were collected by centrifugation at 15000 ×*g* for 20 min at 4℃. Then the precipitate was dissolved in 0.001 M Tris-HCl buffer (pH 8.2) and dialyzed in dialysis tubes against the same buffer at 4℃ for 18 h. The dialyzed preparation was frozen with liquid nitrogen in a thin layer and lyophilized. Separation of the preparation (25 mg/ml) was carried out using preparative column isoelectric focusing by Vesterberg method [18] in a pH gradient of ampholytes 2.5–5.0 and a sucrose density gradient of 0–40% in a 110 ml column (LKB, Sweden) for 36 h at a voltage of 800 V as described earlier [16]. Fractions with pI 4.4–4.7 were collected for further investigations. The purity of the isolated protease was tested with native (non-reducing) electrophoresis [19].

Protein content determination

Proteins were determined by the Bradford protein assay [20] by mixing 950 µl of the Coomassie Brilliant Blue G-250 reagent and 50 µl of the sample. After that A_{595} was recorded.

Proteolytic assays

1% (w/v) suspensions of Hammerstein's casein (Sigma-Aldrich), human serum albumin (Sigma-Aldrich), bovine serum albumin (Sigma-Aldrich) and horse hemoglobin (Reanal, Hungary) were used for proteolytic assays. The activities of isolated protease were determined by Anson-Hagihara's modified method [21]. 200 µl of the sample and 400 µl of suspension of the corresponding protein substrate (in 0.1 M Tris-HCl buffer (pH 8.2)) were used for the reaction. The mixtures were incubated 10 min at 37℃ with permanent shaking (600 rpm). The reaction was stopped using 600 µl of 10% trichloroacetic acid. Then samples were centrifugated (12000 ×*g*, 10 min) and A_{275} was measured in supernatant. The activity was expressed in µmoles of tyrosine formed in 1 min in 1 ml of culture liquid (U_{Tyr}).

http://dx.doi.org/10.48022/mbl.2205.05002

Zymogram analysis

Zymography was carrying out preparing fresh fibrin suspension in 12% polyacrilamide gel by mixing 0,12% (w/v) fibrinogen and 100 µl of thrombin (10 IU) [22, 23]. Electrophoresis was going on in non-reducing (without mercaptoethanol) conditions at 25 mA on ice bath at 4℃. After that, the gel was gently stirred in 2.5% (v/v) Triton X-100, preparing on 50 mM Tris-HCl, pH 8.0, for 30 min at 25℃, washed for 30 min in distilled water and incubated for 18 h at 37℃ in zymogram reaction buffer (0.02% (w/v) NaN_3 based on 30 mM Tris-HCl, pH 8.0). The gel was stained with Coomassie blue R-250 standard solution (2 h) and washed 3 times with 7% (v/v) acetic acid. Clear bands were detected as fibrin hydrolysis areas.

Clotting assay

For study coagulation of blood plasma by isolated protease 0.4% (w/v) solutions of human and bovine fibrinogen (Sigma-Aldrich) and non-diluted and diluted in 2 times human and rabbit plasma (Renam, Russia) were used. For 100 µl of the protease of *A. terreus* 2 (10 U) was added to 200 µl of substrate solution and incubated under static conditions for 10 min at 37℃. Thrombin (10 U, Sigma-Aldrich) was used as positive control. After the incubation, clotting of substrates was visualized.

Effect of plasma proteins inhibitors on protease of *A. terreus* 2 activity

On the isolated protease of the micromycete (0.25 mg/ml), the effect of natural inhibitors of proteins of the hemostasis system was studied. Hirudin (1.5 mg/ml, Sigma-Aldrich, USA) was used as a thrombin inhibitor, because it slows down or completely stops the activation of thrombin by coagulation factors V, VIII, XIII; heparin (5.3 mg/ml, Renam) - as an inhibitor of thrombin and coagulation factors IXa, Xa, XIa, XIIa; ε-aminocaproic acid (1.3 mg/ml, Roth, Germany), as an inhibitor of plasmin and tissue plasminogen activator; sodium ascorbate (1.75 mg/ml, Roth) - as an inhibitor of blood coagulation.

The residual activity was determined with the chromogenic peptide substrate Chromozyme TH (Chromogenix, Italy) after 1.5 h of preincubation of the enzyme and inhibitor at room temperature and was expressed as a percentage of control one (reaction without inhibitor). 200 µl of a sample were mixed with 50 µl of corresponding inhibitor solution for the reaction. After exposition 100 µl of 0.05% solution of the Chromozyme TH in 0.05 M Tris-HCl buffer, pH 8.2, was added and incubated with shaking (600 rpm) for 5 min at 37℃. After that, the reaction was stopped by adding 200 µl of 50% acetic acid [5].

TS-100 thermoshaker ("BioSan", Latvia) was used for all types of reactions. The optical densities of the solutions were measured with Eppendorf kinetics spectrophotometer (Eppendorf, Germany).

The experiments were carried out in triplicate, with the error not exceeding 5–7%. The data were statistically processed using MS Excel 2019 and Statistica 7.0. The Mann-Whitney U test was used to compare the data. Differences were considered statistically significant at $p < 0.05$.

Results and Discussion

Nutrient agar plates assessment

The optimal temperatures for the secretion of proteases by micromycete *A. terreus* 2 (28℃) and the growth of colonies (37℃), determined earlier [16], made it possible to study their development on media contains different protein substrates(milk casein, plasma and blood).

Among the three temperatures at which the study was carried out (24, 28, and 37℃), the minimum of the growth was registered at 24℃, but it was approximately the same for every media. Fig. 1 shows the colonies of the micromycete on the indicated media. It can be observed, that the culture produces extracellular proteases (SMA) and does not exhibit hemolytic activity (BA). Visual hydrolysis of PA was also not detected.

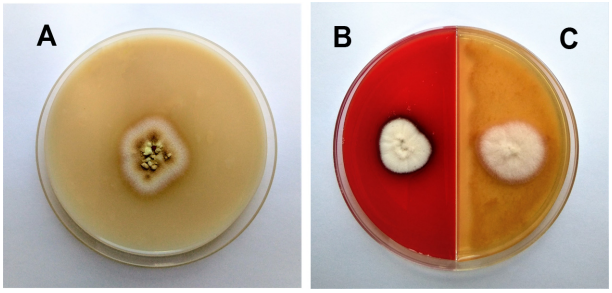

Fig. 1. Growth of *Aspergillus terreus* 2 on skim milk agar (A), plasma agar (B) and blood agar (C).

Fibrinolysis, albuminolysis and hemoglobinolysis by protease of *A. terreus* 2

The consumption of media components during the growth and development of micromycetes, secreting proteases effective against proteins of the hemostasis system, is an important indicator of presence of such activity. In addition to the already established, of particular interest is the ability to coagulate proteins of the hemostasis system, and proteolysis of albumin, fibrin and hemoglobin. Therefore, the next stage of the study was to isolate the protease of *A. terreus* 2 from the culture liquid and to determine the related reactions.

The producer's protease was isolated by isoelectric focusing, and its purity was proven electrophoretically (data not shown). Fibrin zymography (Fig. 2) also confirmed the presence of one protease of *A. terreus* 2 in the collected fractions, and it pronounced fibrinolytic activity, along with inability to perform hemolysis.

An investigation of the hemoglobinolytic and albuminolytic activities of the protease showed that it is capable of hydrolyzing both substrates to varying degrees. The obtained results are shown in Table 1. The protease of *A. terreus* 2 exhibited the highest proteolytic activity against hemoglobin, which was 2.5 times higher than the albuminolytic activity with both used protein substrates. Proteolytic activities against human and bovine albumin were comparable.

Fig. 2. Fibrin zymogram of *A. terreus 2* protease.

Table 1. Albuminolytic and hemoglobinolytic activity of extracellular protease of *A. terreus* 2.

Protease	Albuminolysis, U_{Tyr}/mg		Hemoglobinolysis, U_{Tyr}/mg
	Human albumin	Bovine albumin	
A. terreus 2	344.3	526.7	1139.4

Fibrinogen and plasma coagulation

Study of the protease activities concerning coagulation components are the clotting tests of blood plasma and fibrinogen. The protease activity was tested using both animal and human proteins (Table 2). As presented in the table, the protease of *A. terreus* 2 could not coagulate either human fibrinogen or bovine fibrinogen. Negative results were demonstrated when studying the coagulation of rabbit plasma by the protease. Regarding human plasma, isolated protease coagulated it both undiluted and diluted in 2 times. These results confirm our earlier data on the activity of *A. terreus* 2 protease toward prekallikrein in human blood plasma [17].

Hemostatic inhibitors effect

At the next stage we also analyzed the inhibition of *A. terreus* 2 protease by natural inhibitors of hemostatic factors (Fig. 3). It was shown that such inhibitors as ε-aminocaproic acid and sodium ascorbate did not decrease the protease activity under experimental conditions. The most significant effect on the enzymatic activity of the protease was exerted by hirudin (reduces the activity by 38.2%). Heparin also inhibited protease activity (decreases by 17.8%).

The data obtained in this study for *A. terreus* 2, a producer of hemostatically active proteases, showed an effect of both types of hemostasis activities such as coagulation and fibrinolysis. It is well known that filamentous fungi, especially *Aspergillus,* can secrete proteases active toward different factors of the hemostatic system

Table 2. Plasma and fibrinogen coagulation by protease of *A. terreus* 2.

Sample	Human plasma		Rabbit plasma		Human fibrinogen	Bovine fibrinogen
	Normal	Diluted in 2 times	Normal	Diluted in 2 times		
A. terreus 2 protease	+	+	-	-	-	-
Thrombin (control)	+	+	+	+	+	+

http://dx.doi.org/10.48022/mbl.2205.05002

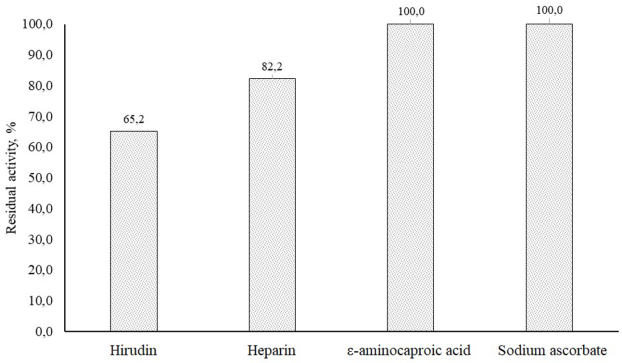

Fig. 3. Inhibitory analysis of *A. terreus 2* protease with natural inhibitors of the hemostasis system.

[5, 6]. This property is beneficial, and the study of a more significant number of producers can give data of the activity among micromycetes. Earlier, we showed that the addition of *A. terreus* 2 proteases to human plasma can stimulate coagulation at the contact phase via additional activation of prekallikrein. This property of extracellular proteases of micromycetes to activate that factor in human blood plasma has been shown for the first time [17].

This ability distinguishes *A. terreus* 2 protease from *A. ochraceus* L-1 protease, which can activate protein C and factor X and finds the application in the creation of diagnostic kits for determining the content of these proteins in patients plasma [7, 11, 12].

Along with activating properties for the proteins of the hemostasis system via limited proteolysis, fungal proteases also exhibit proteolytic activity against blood proteins. In several cases, when it is necessary to identify targeting proteases, concomitant proteolytic activity can interfere; therefore, it is necessary to assess its level with suitable protein substrates - key globular and fibrillar proteins of the hemostasis system.

The study investigated that protease of *A. terreus* 2 are highly active in reactions of cleaving both globular proteins (albumin) and fibrillar (fibrin), as well as, to an extent, can coagulate human plasma and cannot coagulate fibrinogen. The data obtained suggest that the inability of the *A. terreus* 2 protease to coagulate rabbit blood plasma and coagulate human and bovine fibrinogen indicates the severity of the enzyme's action on human blood coagulation factors. Perhaps this fact should be considered as a potential indicator of participation of the isolated protease in fungal pathogenesis. However, this also determines the possibility of its use as a hemostatically active enzyme for various purposes. Unfortunately, the protease exhibited high activity towards albumin and hemoglobin, despite the absence of hemolytic activity.

It is noteworthy that the activity of protease is insignificantly inhibited by thrombin inhibitors and is not inhibited by plasmin inhibitors. That confirms its activity against blood coagulation factors.

Similar properties of proteases of other aspergilli, studied earlier - *A. flavus* 1 (with a high degree of pathogenicity) and *A. ochraceus* L-1 (a conditional pathogen), showed similar effects on various components of the hemostasis system (*in vitro*). They were highly active in cleaving both globular and fibrillar proteins, and, to varying degrees, they could coagulate the plasma of humans and animals but were not able to coagulate fibrinogen. These micromycetes did not show hemolytic activity also but were able to break down hemoglobin. The comparison shows that the albuminolytic activity of *A. terreus* 2 protease was 1.40 and 1.03 times less than that of *A. flavus* 1 and *A. ochraceus* L-1, respectively. The hemoglobinolytic activity of the isolated protease was comparable to that of *A. flavus* 1 protease and 1.17 times higher than that of *A. ochraceus* L-1 protease [24].

The data obtained serve as the basis for further studies of the biochemical and physicochemical properties of the isolated *A. terreus* 2 protease in order to clarify the possibilities of its practical application in biomedicine.

The use of proteolytic enzymes of microscopic fungi in medical practice is possible in two directions: as part of thrombolytic drugs and as components of diagnostic kits as activators of blood plasma proenzymes. The activity of proteases in relation to different substrates and the severity of this activity to certain proteins of the hemostasis system determines the scope of their application. High fibrinolytic and fibrinogenolytic activity and accompanying albuminolysis and hemoglobinolysis, the ability to cause hemolysis, and sensitivity to plasma inhibitors limit the therapeutic effect of such proteases. At the same time, this does not mean that such enzymes are unpromising; they may well find a worthy application for *in vitro* studies of blood coagulation factors [11, 22, 23].

It was shown, that the *A. terreus* 2 protease exhibits a complex effect on the proteins of the hemostasis system,

affecting the proteolysis of a significant number of its components. Isolated protease is highly hydrolyze both globular (human albumin, bovine albumin and hemoglobin) and fibrillar (fibrin) proteins. The protease can coagulate human but not rabbit plasma, human and bovine fibrinogen. The protease shows no hemolytic activity. The activity is insignificantly inhibited by thrombin inhibitors, and is not inhibited by plasmin inhibitors. Considering our previous studies, one could argue that using this protease is better for diagnostic purposes than in therapy and the treatment of thromboembolic diseases.

Acknowledgments

This work was supported by the Financial Council for Grants of the President of the Russian Federation No. SP-3906.2021.4.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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