Fibrinogenolytic activity of protease from the culture fluid of *Pleurotus ostreatus*

Yevhenii M. Stohnii,[1] Valeriia V. Sakovich,[2] Volodymyr O. Chernyshenko,[1] Volodymyr I. Chernishov,[1] Tamara M. Chernyshenko[1], Iryna Kolesnikova,[1] Yevhenii P. Kucheriavyi[1], Dmitrii D. Zhernossekov[2]

[1]Palladin Institute of biochemistry NAS of Ukraine, Ukraine; [2]Polesskij State University, Republic of Belarus

Abstract

The use of proteases makes it possible to obtain partially hydrolyzed forms of macromolecules with unique properties. The importance of proteases for studying the structure and functions of fibrinogen forces scientists to search for new sources of highly specific proteases. Thus, the aim of this work was to study the content of the *Pleurotus ostreatus* culture fluid in search of fibrinogen-specific proteases. *P. ostreatus* was cultured for 14 days at 27°C. The culture fluid was collected and the protein fraction was salted out with NaCl and then dialyzed. Fibrinogen hydrolysis products by *P. ostreatus* protease were characterized using SDS PAGE under reducing conditions followed by immunoprobing using murine monoclonal antibodies I-5A (anti-Aα505-610) and 2d2a (anti-Bβ26-42). The study of turbidity and platelet aggregation was performed using a Multiskan FC spectrophotometric microplate reader and a SOLAR-2110 aggregometer, respectively. Electron microscopy of fibrils formed by truncated compared with native fibrins was performed using a transmission electron microscope N-600.

Analysis of the products of fibrinogen hydrolysis with a fungal protease using SDS-PAGE demonstrated the cleavage of the alpha chain of fibrinogen exclusively with the formation of a truncated form of fibrinogen in which there are no C-terminal portions of αC regions with a molecular weight of 25 kDa. A study of turbidity showed that the polymerization of truncated fibrin is significantly impaired. The rate of lateral association of protofibrils significantly decreased from 1.5 to 2.2 times in the case of truncated fibrinogen compared to the native one depending on the initial concentration of fibrinogen. It was shown that platelet aggregation in the presence of fibrinogen without 25 kDa fragments of αC regions was less effective than in the presence of native fibrinogen. Application of the preparation of the fungal protease allows us to obtain high molecular forms of the fibrinogen molecule with cleaved 25 kDa peptides, which provide new information on the role of these peptides in the fibrinogen functioning.

Correspondence: Volodymyr Chernyshenko, Palladin Institute of biochemistry NAS of Ukraine 9, Leontovych Str., Kyiv, 01054, Ukraine
Tel.: +380675906710; +380442355172.
E-mail: bio.cherv@gmail.com

Key words: Fibrinogen; limited proteolysis; Pleurotus osreatus; protease; platelets aggregration; fibrin polymerization.

Conflict of interest: The authors declare no potential conflict of interest.

Ethics approval: This study was preliminarily reported at the 44th FEBS Congress, From Molecules to Living Systems, Krakow, Poland, July 6−11, 2019.

Received for publication: 3 April 2020.
Accepted for publication: 19 July 2020.

Journal of Biological Research 2020; 93:9006
doi:10.4081/jbr.2020.9006

Introduction

Limited proteolysis is one of the most popular methods for studying the structure and function of a protein. This approach allows us to obtain fragments of a molecule, to study their features, and finally, we can conclude about the role of the certain fragment in the functioning of the whole molecule.[1-2] In addition, the limited proteolysis of macromolecules makes it possible to obtain functionally active fragments that can be used in medicine.[3]

Over the past decade, powerful fibrinolytic enzymes from various sources have been discovered, such as earthworms,[4] snake poisons,[5] insects,[6] microorganisms[7] and marine species.[8] An attractive source of various biologically active compounds are fungi.[9] They are commonly used as food and food flavoring substances, as well as in traditional oriental medicines. Mushroom extracts have antiviral, antitumor, hypotensive and hepatoprotective effects.[10] In particular, they represent an important source of thrombolytic agents. Many fibrinolytic enzymes have been identified in the fruiting bodies of fungi, such as *Armillaria mellea*,[11] *Grifola frondosa*,[12] *Pleurotus ostreatus*[13] and *Fomitella fraxinea*.[14] We have previously characterized an enzyme from the culture fluid of *Pleurotus ostreatus*.[15] It has been shown to be capable of splitting fibrinogen. The purpose of this work was to study the specificity of the action of this protease on fibrinogen molecule and to determine how fibrinogen hydrolysis by this enzyme affects the functional properties of fibrinogen.

Materials and Methods

Materials

Chemicals

Thrombin, Lysine-Sepharose and goat anti-mouse-HRP were purchased from "Sigma-Aldrich" (USA). ADP was purchased from Tekhnologia-standard (Russia). Acrylamide was from "Fluka"(Switzerland), N,N-methylenebisacrylamide from "Acros organics" (Belgium), Coomassi Brilliant Blue R-250 from "Sigma" (USA), Molecular Weight Calibration Kits - "Fermentas" (Lithuania).

Protease from the culture fluid of Pleurotus ostreatus

Pleurotus ostreatus was cultivated during 14 days at 27°C using liquid nutritious potato–sucrose medium to harvest mycelia in submerged culture as it was described previosly.[15] Enzyme-containing fraction was purified from the culture fluid of *Pleurotus ostreatus* by saturated solution of NaCl and dialyzed against water alkalized with ammonium to pH 7.5-8.0. Resulting solution was lyophilized (-83°C, 0.5 mBar) using Telstar (LyoQuest, Spain) Powder was resolved in 0.05 M Tris HCl buffer pH 7.4 with 0.13 M NaCl prior the experiments. Additional purification of the enzyme was performed as described in.[16] Protein concentration in the sample of enzyme was assayed by Bradford method, using bovine serum albumin as a standard protein and measuring the absorbance of the samples at 595 nm.[17]

Fibrinogen

Fibrinogen used in this study was purified from human citrated blood plasma according to the method described by Varetskaya TV[18] and was further plasminogen depleted on a Lysine-Sepharose affinity column. The plasminogen depletion was confirmed using non-enzymatical activator of plasminogen - streptokinase (Beacon Pharmaceuticals, UK) as follows. Sample of fibrinogen-depleted fibrinogen (2 mg/mL) or the equivalent volume of primary fibrinogen was admixed with 0.5 IU/mL of streptokinase in 0.05 M Tris HCl buffer pH 7.4 with 0.13 M NaCl and incubated at 37°C. 0.040 mM of plasmin-specific chromogenic substrate S2251 (Pharmacia, Sweden) was added to the mixture for the detection of the generation of plasmin. Sample was assumed as plasminogen-depleted when optical density of the mixture after 60 minutes of incubation was less than 0.020 (against 0.3 in samples with initial fibrinogen).

Monoclonal antibodies

Monoclonal mouse IgG1 antibodies I-3C to Bβ26-36 fragment of fibrinogen molecule and II-5C to Aα20-78 fragments of fibrinogen molecule were obtained in Palladin Institute of Biochemistry of NAS of Ukraine using hybridoma technique. Thrombin-treated N-terminal disulfide knot of fibrin was used for the immunization of mice.[19]

Preparation of platelet poor plasma and washed platelets

Blood plasma of healthy volunteers of both sexes age from 25 to 35 years who had not taken any medication for 7 days was collected into sterile plastic 10 mL tubes where was mixed immediately with 38 g/L sodium citrate (9 parts of blood to 1 part of sodium citrate). Blood was centrifuged at 160 g for 30 min at 25˚C. Platelet rich plasma was collected and centrifuged again at 300 g for 15 min. Platelet poor plasma was collected. Pellet of platelets was re-suspended in 0.004 M HEPES, 0.137 M NaCl, 0.0027 M KCl, 0.001 M $MgCl_2$, 0.0056 mM glucose, 0.003 M NaH_2PO_4, 0.35 mg/ml BSA, pH 7.4. Procedure of washing was repeated two times to obtain the homogenous suspension of washed platelets as recommended in Chernyshenko *et al.*[20]

Methods

Fibrinogenolytic activity of the protease

The mixture of fibrinogen (1 mg/mL) and enzyme (0.25 mg/mL) in 0.05 M Tris-HCl buffer pH 7.4 with 0.13 M NaCl was incubated during 6 hours at 37°C for SDS-PAGE. The hydrolysis was terminated by the addition of electrophoresis sample buffer containing 2% SDS, 5% glycerine and 2% β-mercaptoethanol. Samples were heated at 95ºC for 2 min before the experiment. Solubilised samples were separated by SDS-PAGE and immunoprobed in western-blotting.

SDS-PAGE/Western blotting

The composition of fibrinogen hydrolyzates obtained using protease from the culture fluid of *Pleurotus ostreatus* was characterized by SDS-PAGE using 10 % gel accordingly to Laemmli[21] in the presence of 0.2 % of β-mercaptoethanol. The separated proteins were further transferred to a nitrocellulose membrane in order to specifying the bands by immunoprobing. The membrane was blocked with 5% milk in PBS for an hour, incubated with 1-3C or II5-C antibody for another hour and then developed with a secondary HRP-labelled goat anti-mouse antibody. The bands were visualized using 0,001 M 4-chloro-1-naphtol solution in 0.5 M Tris pH 7.5 and 0.03% H_2O_2.

Preparation of digested fibrinogen

Solution of protease and fibrinogen were mixed 1:60 (w/w) in TBS and incubated during 6 hours at 37°C. Composition of polypeptide chains of truncated fibrinogen was confirmed by SDS-PAGE under reducing condition.

Effect of fibrinogen digestion by protease on polymerization properties of fibrin

Polymerisation of truncated fibrinogen *vs* native fibrinogen were monitored by measuring turbidity of the samples at 405 nm on the microplate reader Multiskan FC (ThermoFisher, USA) at room temperature. Fibrinogen was diluted in 0.05 M Tris-HCl buffer pH 7.4 with 0.13 M NaCl with 0.001 M $CaCl_2$ in the range of concentrations from 0.06 to 0.32 mg/mL. Final volume was 0.25 mL. Clotting was initiated by adding 0.25 NIH/ml of thrombin.

Effect of fibrinogen digestion by protease on architecture of polymeric fibrin

The polymerization of partially hydrolyzed fibrin (by the fungal protease) compared to native fibrin polymerization was studied by transmission electron microscopy of negatively contrasted samples on H-600 Transmission Electron Microscope ("Hitachi", Japan); 1% water solution of uranyl acetate ("Merck", Germany) was used as a negative contrast. For sample preparation, in sterile glass tubes were sequentially added 0.32 mg/mL human fibrinogen, 0.025 M $CaCl_2$ in 0.05 M ammonium formiate buffer (pH 7.9), and a total sample volume was 0.22 mL. The polymerization of fibrin was initiated by the introduction of thrombin to a final concentration of 0.25 NIH/mL.

After 180 s, aliquots were taken from the polymerization medium. Each aliquot was diluted to a final fibrinogen concen-

tration of 0.07 mg/mL; 0.01 mL probes of fibrinogen solution were transferred to a carbon lattice, which was treated with a 1% uranyl acetate solution after 2 minutes. Investigations were performed using an H-600 electron microscope at 75 kV. Electron microscopic images were obtained at magnification of 20, 000 - 50, 000.

Effect of fibrinogen digestion by protease on aggregation of washed platelets

Washed platelets were prepared as described above. Truncated fibrinogen *vs* native fibrinogen were added to the washed platelet suspension at final fibrinogen concentration of 1.5 mg/mL. Aggregation started by addition of 0.0125 mM ADP and the extent of aggregation was monitored by SOLAR aggregometer (Belarus).

Statistical analysis

Statistical processing of the results was carried out on a personal computer using standard statistical programs "Microsoft Exel".

Results

Protease action on fibrinogen

Previously, a protease that possesses fibrinogenase activity was isolated and purified from the culture fluid of *Pleurotus ostreatus*.[16] For a detailed study of the specificity of the protease with respect to fibrinogen, the composition of fibrinogen hydrolysates obtained after prolonged incubation with the enzyme was investigated by SDS-PAGE in the presence of 0.2% β-mercaptoethanol. According to electrophoresis data (Figure 1), protease hydrolyzes the Aα-chain of fibrinogen with the appearance of a product with an apparent molecular weight of about 40 kDa. Traces of the native Aα-chain of fibrinogen visible after 2 hours of incubation with fibrinogenase disappeared after 6 hours of incubation, but the Bβ chain and γ chains were not splitted.

To determine what part of fibrinogen Aα-chain was digested by the protease and to confirm the integrity of Bβ-chain, Western-Blotting analysis was performed using II5-C or 1-3C antibody correspondingly.

Western-blotting with antibody 1-3C specific towards Bβ26-36 fragment of fibrinogen molecule proved the results of SDS-PAGE and confirmed that incubation up to 6 hours did not lead to the digestion of Bβ-chain of fibrinogen. The use of anti- Aα20-78 antibody II-5C allowed us to detect the proteolysis of Aα-chain by the enzyme and to identify the hydrolytic products, which contained amino acid residue 20-78.

It was found that step-by-step cleavage of fibrinogen Aα-chain leaded to the formation of products with apparent molecular weights 54 and 47 kDa. It means that the protease from the culture fluid of *Pleurotus ostreatus* leads to the formation of truncated form of fibrinogen lacking C-terminal portions of αC-regions with molecular weight approximately 25 kDa (Figure 2).

Polymerization of fibrin derived from the truncated form of fibrinogen

Protease from the culture fluid of *Pleurotus ostreatus* was incubated with fibrinogen and thrombin-induced polymerization of resulting fibrin was studied. Turbidity study demonstrated that polymerization of truncated fibrin was much impaired (Figure 3).

Measurements of turbidity changes during fibrin polymerization showed typical curves consisting of three stages: lag-stage (the formation of protofibrils), stage of exponential growth (their lateral association) and maximal turbidity (Figure 3A).

We demonstrated that cleaving off the 25 kDa fragment of fibrinogen αC-regions leaded to the substantial decrease of the speed of lateral association of protofibrils and had no action on the time of their formation (lag-stage) (Figure 3B). Speed of lateral association of protofibrils was much decreased from 1.5 to 2.2 times in the case of truncated fibrinogen when compared to native one dependently on the initial concentration of fibrinogen (Figure 3C).

Also, the final turbidity of the clot was much affected in the

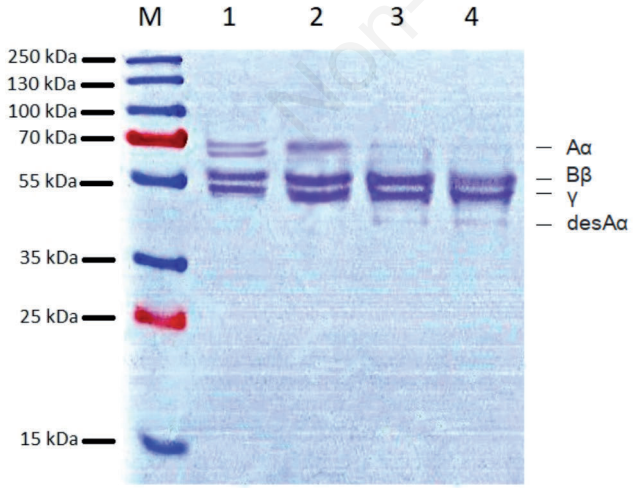

Figure 1. SDS-PAGE of fibrinogen hydrolysis products using the protease from culture fluid of *Pleurotus ostreatus*. M – molecular weight markers (250, 130 ,100, 70, 55, 35, 25, 15 kDa); 1 – native fibrinogen; 2, 3, 4 – 2, 4, 6 hours of proteolysis. Samples were prepared in the presence of 0.2% β-mercaptoethanol). desAα – cleaved form of Aα-chain.

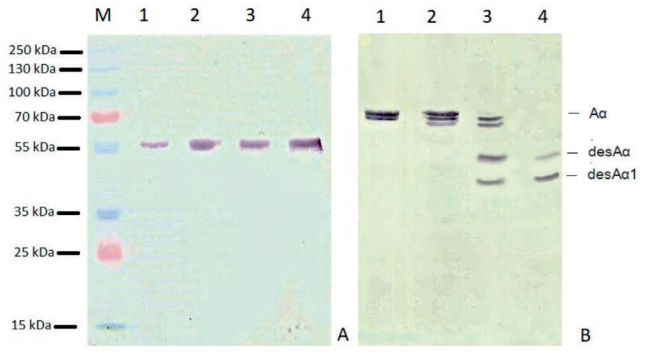

Figure 2. Western-blotting analysis of fibrinogen digested by the protease from the culture fluid of *Pleurotus ostreatus*. Incubation time 2, 4 and 6 hours. Samples were analyzed under reducing conditions and immunoprobed by anti-fibrinogen Bβ26-42 antibody (A) and anti-fibrinogen Aα20-78 antibody (B). desAα, desAα1 – cleaved form of Aα-chain.

case of truncated fibrin, that was shown on panel D (Figure 3). This change indicated the prominent disturbance the architecture of three-dimensional fibrin web. So, we studied it directly using electron microscopy (Figure 4).

We examined the structure of clots formed by native *vs* truncated fibrins on the stage when they had maximal turbidity (Figure 5). We showed the formation of fibrin strands that branching forming the web in the case of native fibrin (Figure 5A). However, in the case of truncated fibrin we observed only individual fibrils, no branching was found (Figure 5B).

Platelet aggregation in the presence of truncated fibrinogen

Fibrinogen provides platelet aggregation, forming bridges between activated platelets, while simultaneously binding to GPIIbIIIa-receptors of several platelets. Here we aimed to examine how the proteolysis by the protease from the culture fluid of *Pleurotus ostreatus* influence the ability of fibrinogen to support platelet aggregation.

Washed human platelets were re-suspended in the HEPES-buffer containing 1.5 mg/mL of native *vs* truncated forms of fibrinogen. It was shown that platelet aggregation in the presence of fibrinogen lacking 25 kDa fragments of αC-regions was less effective than in the presence of native fibrinogen (Figure 5).

Discussion

As it was reported earlier the protease from the cultural fluid of *Pleurotus ostreatus* was calcium-dependent metalloprotease with molecular mass 45 kDa and had the best specificity to chromogenic substrate Leu-pNa.[22] Here we proved that protease belongs to α-fibrinogenase, *i.e.* such enzymes that have the greatest specificity towards the Aα-chain of fibrinogen. At the beginning this protease cleaved off a small fragment of the fibrinogen molecule. Then, due to the prolonged action of the fungal enzyme, approximately 25 kDa part of the αC region of the fibrinogen molecule was cleaved off.

The resulting partially hydrolyzed form of fibrinogen was tested for its ability to support platelet aggregation and polymerization. The obtained data showed a significant decrease in the rate and degree of polymerization of hydrolyzed fibrin, as well as an

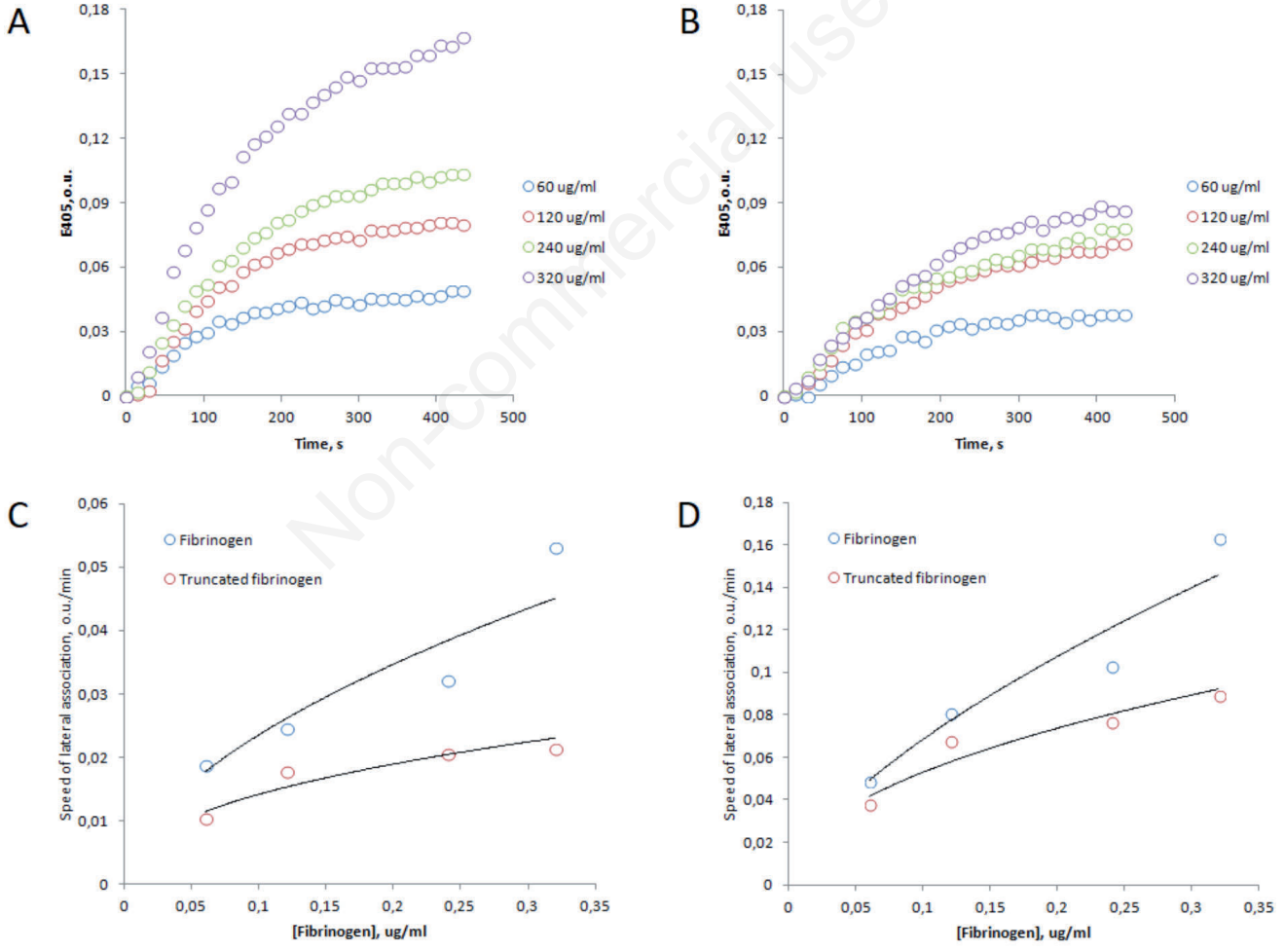

Figure 3. The results of turbidity study of polymerization of different concentrations (60, 120, 240, 320 μg/mL) of native fibrin and fibrin truncated by protease from the culture fluid of *Pleurotus ostreatus*. A) Polymerization of native fibrin; B) Polymerization of truncated fibrin; C) Comparison of speed of polymerization of native and truncated forms of fibrin; D) Comparison of final turbidity of polymerization of native and truncated forms of fibrin.

almost complete inhibition of platelet aggregation in the presence of hydrolyzed fibrinogen, and indicated the important role of the C-terminal 25 kDa fragment of αC-regions in protein-protein and protein-cell interactions of the fibrinogen molecule.

It is known that αC-regions of fibrinogen contain sites of the lateral association of protofibrils.[23] In addition, αC-regions play an important role in the recognition of monomeric fibrin molecules and the formation of primary contacts between them, which facilitate subsequent binding using a system of more specific polymerization centers, thus promoting the process of self-assembly of fibrin and its branching.[24]

Our studies using protease from the culture fluid of *P. ostreatus* allowed us to confirm both postulates: lateral association of protofibrils (but not their self-assembly) was inhibited for the truncated fibrin that finally formed fibrils without active branching.

αC regions also contain areas of interaction with activated platelet receptors, in particular RGD and KGD sequences, with which GPIIbIIIa receptors are connected.[25] These residues are located in 572-574 and 418-420 fragments of Aα-chains of fibrinogen,[26,27] but at the moment, the participation of individual sections of the αC domain in particular RGD and KGD remains unknown. Under the action of the studied protease, a fragment is splitted off, which in addition to the RGD also has a sequence of KGD. We suppose that prominent pronounced impairment of platelet aggregation in the presence of fibrinogen cleaved by protease from *Pleurotus ostreatus* indicates the importance of the KGD 418-420 fragment in platelet aggregation. This phenomenon must be studied more precisely.

Another interesting point concerning fibrinogen-specific proteases is their possible use as a promising antithrombotic and fibrinolytic agent.[28,29] Selectively hydrolyzing the Aα-chain of fibrinogen, the protease from the culture fluid of *Pleurotus ostreatus* reduces the ability of both fibrinogen and fibrin to participate in the formation of the fibrin-platelet thrombus.

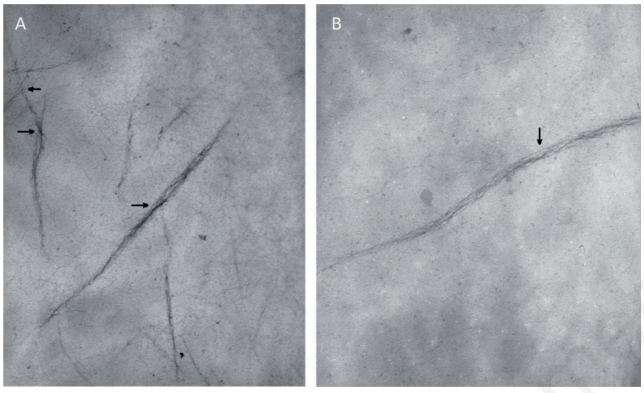

Figure 4. Electron microscopic photos of polymer fibrin clots formed under the action of thrombin from native and partially hydrolyzed fibrinogen. A) Fibrils formed by the native fibrin; B) Fibrils formed by fibrin after proteolysis of the protease from the culture fluid of *Pleurotus ostreatus*. Arrows indicate the junctions or branching points of fibrils on the panel A and individual long fiber on the panel B.

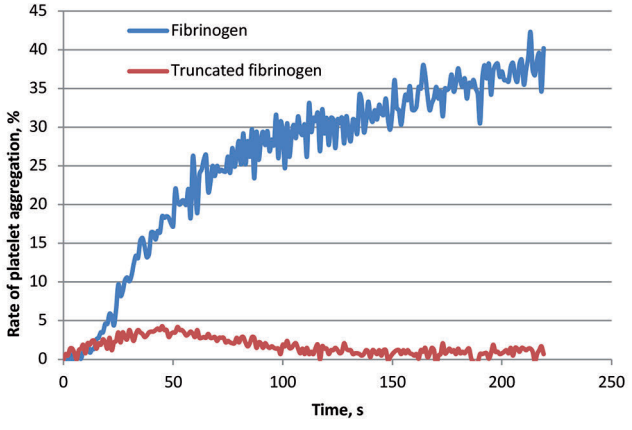

Figure 5. The results of ADP-induced aggregation of washed platelets in the presence of native or truncated forms of fibrinogen (1.5 mg/mL).

Conclusions

Protease from culture fluid of *Pleurotus ostreatus* was shown to be a promising tool to study protein and cellular interactions of fibrinogen. We characterized the peculiarities of its action on the fibrinogen molecule and found that it preferentially cleaves C-terminal 25-kDa fragment of Aα-chains. Truncated form of fibrinogen obtained using this protease allowed us to demonstrate the exceptional role of this fragment in lateral association of protofibrils, branching of fibrils and platelet aggregation.

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