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MAPPING OF RESIDUES OF FIBRINOGEN CLEAVED BY PROTEASE II OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* IMV B-7465

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The limited proteolysis of macromolecules allows obtaining the fragments that preserve the structure and functional properties of the whole molecule and could be used in the study of proteins structure and function. Proteases targeted to fibrinogen and fibrin are of interest as the tool for obtaining of functionally active fragments of fibrin(ogen) and for the direct defibrination in vivo. That is why the aim of the present work was to study the proteolytic action of Protease II (PII) purified from Bacillus thuringiensis var. israelensis IMV B-7465 on fibrinogen.

Hydrolysis products of fibrinogen by PII were analysed by SDS-PAGE under reducing conditions with further immunoprobing using the mouse monoclonal 1-5A (anti-Aα509-610) and II-5C (anti-Aα20-78) antibodies. It was shown that PII cleaved preferentially the Aα-chain of fibrinogen splitting off the peptide with apparent molecular weight of 10 kDa that corresponded the C-terminal part of Aα-chain of fibrinogen molecule.

MALDI-TOF analysis of hydrolysis of fibrinogen was performed using a Voyager-DE. Results analyzed by Data Explorer 4.0.0.0 allowed to detect the main peak occurring at mass/charge (M/Z) ratio of 11 441 Da. According to «Peptide Mass Calculator» this peptide corresponded to fragment Aα505-610 of fibrinogen molecule. The result showed that PII cleaves the peptide bond AαAsp-Thr-Ala504-Ser505.

Thus, PII can be used for the obtaining of unique fragments of fibrinogen molecule. As far as αC-domain contains numerous sites of fibrin intermolecular interactions we can consider PII as a prospective agent for their study and for the defibrination.

K e y w o r d s: protease, Bacillus thuringiensis, fibrinogen, αC-domain, limited proteolysis.

Proteases could be found in pathogenic and nonpathogenic species of microorganisms could be targeted to proteins of human and other mammals [1-4]. Fibrinogen as big and labile molecule could be a cleaved by proteases more or less specifically. Fibrinogen-specific metalloproteases were purified from *Serratia* sp. [5] and *Pseudomonas aeruginosa* [6], serine proteases from *Brevibacillus brevis* [7], *Bacillus* sp. [8], *Bacillus cereus* [9], *Vibrio metschnikovii* [10], *Aeromonas sobria* [11]. Their molecular weight ranges from 30 to 60 kDa. Some of them preferentially degrade the Aα-chain of fibrinogen [9, 11], others are targeted to both Aα- and Bβ-chains of fibrinogen [6, 8] or even all three chains of fibrinogen molecule [9]. Most of bacterial fibrinogenases cleave both fibrinogen and polymeric fibrin [10, 11]. These properties allowed authors to conclude the potential anti-thrombotic use of the enzymes in peptide-based cardiovascular drug development [7-9, 12]. Some of them were already tested in animal models [8] or were cloned for these purposes [13].

Proteases targeted to fibrinogen and fibrin also are of interest as the source for obtaining of physiologically active fragments of fibrin(ogen) and for direct defibrination in vivo. That is why the aim of our work was to study the proteolytic action of PII purified from *Bacillus thuringiensis* var. *israelensis*

IMV B-7465 on human fibrinogen and detection the site(s) of proteolysis on fibrinogen molecule.

Materials and Methods

Materials. PII was purified from *B. thuringiensis* var. *israelensis* IMV B-7465 [14]. Fibrinogen used in this study was purified from human citrated blood plasma according to the method described by Varetskaya [15] and was further plasminogen depleted on a Lysine-Sepharose affinity column. Chromogenic substrates used in the study were S2238 (H-D-Phe-Pip-Arg-pNA), S2251 (D-Val-Leu-Lys-pNA), S2765 (Z-D-Arg-Gly-Arg-pNA), S2236 (pyro-Glu-L-Pro-L-Arg-pNA), S2302 (H-D-Pro-Phe-Arg-pNa) and S1040 (Glp-Ala-Ala-Leu-pNa) (Chromogenics, Sweden). Mouse monoclonal 1-6B (anti-Aα509-610) and II-5C (anti-Aα20-78) antibodies were designed and purified in Protein Structure and functions department of Palladin Institute of biochemistry, NAS of Ukraine.

SDS-PAGE/Western blot. The molecular weights and purity of proteins were determined by SDS-PAGE using 10 or 12% gels accordingly to Laemli [16]. Hydrolysis products of fibrinogen and fibrin obtained by PII action were also analyzed by SDS-PAGE under reducing conditions. The separated proteins were further transferred to a nitrocellulose membrane in order to specify the bands by immunoprobing. The membrane was blocked with 5% milk in PBS for an hour, incubated with a mouse monoclonal antibody to Bβ26-42 or to Aα20-78 for another hour and then developed with a secondary HRP-labeled goat anti-mouse antibody. The bands were visualized using 0,001 M 4-chloro-1-naphtol solution in 0.5 M Tris-HCl) pH 7.5 and 0.03% H_2O_2.

Mass-spectrometry. MALDI-TOF analysis of purified fibrinogenase was performed using a Voyager-DE (Applied Biosystems, USA). H^+-matrix ionization of polypeptides under sinapic acid (Sigma-Aldrich) was used. Results were analyzed by Data Explorer 4.0.0.0 (Applied Biosystems) [17].

Fibrinogenolytic activity of protease. Fibrinogen (1.5 mg/ml) was mixed with PII (0.005 mg/ml) in TBS-buffer pH 7.4. The molar enzyme/substrate ratio was 1:30. The mixture was incubated during 5, 10, 15, 20, 25, 30, 40, and 60 minutes at 25 °C for SDS-PAGE or for 30 and 60 minutes for the Western blot analysis. The hydrolysis was terminated by the addition of electrophoresis sample buffer containing 2% SDS, 5% glycerine and 2% β-mercaptoethanol. Solubilised samples were separated by SDS-PAGE and immunoprobed in Western blot analysis.

Chromogenic substrate assay. Cleavage of chromogenic substrates was studied in microtiter plates by mixing of 0.05 M Tris-HCl buffer of pH 7.4 containing 0.13 M NaCl with chromogenic substrates in the concentration range from 25 to 160 μM, and PII (0.0005 mg/ml), at 25 °C. Amidase activity of PII was continuously monitored at 405 nm. The amount of hydrolyzed substrate was calculated using a molar extinction coefficient of 10.500 $M^{-1} \cdot cm^{-1}$ for free pNA on reader Multiskan EX [18].

Results and Discussion

Previously was shown that PII purified from the *B. thuringiensis* var. *israelensis* IMV B-7465 could effectively degrade fibrinogen [19]. To identify the region of fibrinogen molecule attacked by PII, the digestion mixture was analyzed by SDS-PAGE under reduced conditions. Plasminogen-free human fibrinogen was incubated with PII at ambient temperature. Upon incubation of fibrinogen with PII, the Bβ- or γ-chains of the molecule appeared to be the same as those of control fibrinogen whereas the Aα-chain gradually disappeared, resulting in the formation of a truncated form of about 58 kDa (Fig. 1). The low-molecular weight fragment of Aα-chain cleaved-off by PII was detected using SDS-PAGE in 15% polyacrilamide gel without β-mercaptoethanol as a polypeptide with molecular weight of about 12 kDa (Fig. 2).

To localize the PII-sensitive area within the fibrinogen Aα-chain, we used specific monoclonal antibody to the C-terminal Aα509-610 (1-5A) [20] and to the N-terminal Aα20-78 (II-5C) [21] portions of fibrinogen Aα-chain for immunoprobing of SDS-PAGE separated proteins of the digestion mixture (Fig. 3, 4). In the case of I-5A antibody use the traces of the native fibrinogen Aα-chain which was slightly visible after 30 minutes of incubation with PII and almost completely disappeared after 60 minutes while the low-molecular weight fragment of Aα-chain appeared (Fig. 3). On the other hand, when II-5C antibody was used we observed high-molecular weight fragment of Aα-chain that contain N-terminal portions of the Aα-chain (Fig. 4).

Thus we can conclude that PII selectively degrades Aα-chain of fibrinogen releasing a polypeptide with average molecular weight of about 12 kDa. This molecular weight was checked by MALDI-TOF

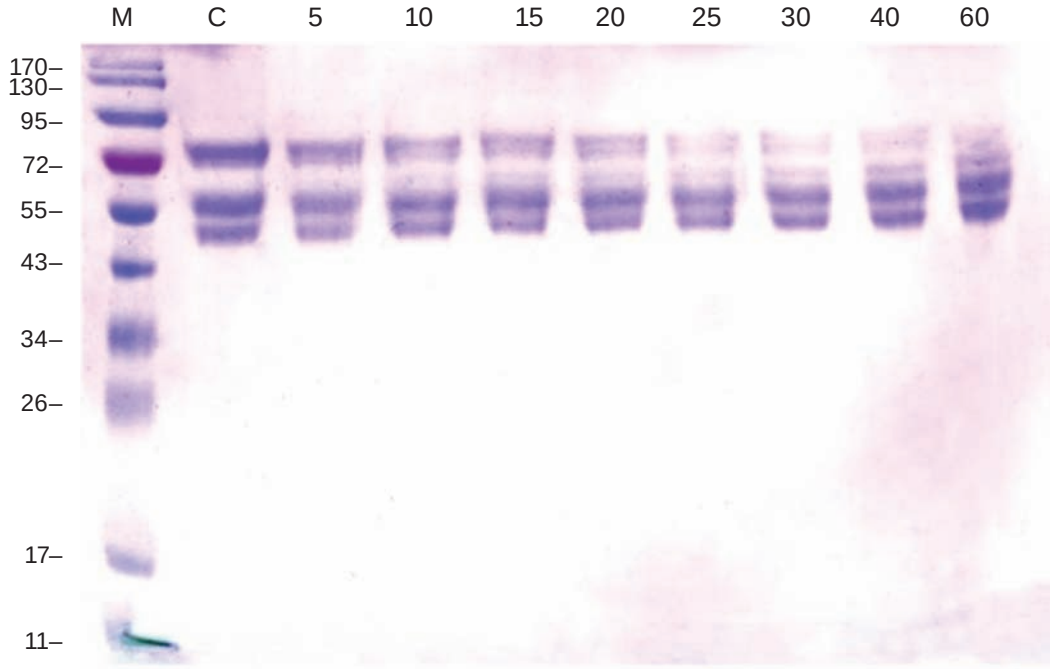

Fig. 1. SDS-PAGE of fibrinogen (1.5 mg/ml) digested by PII (0.005 mg/ml). Incubation time 5-60 min. C – native fibrinogen; M – molecular weight markers. Samples were analyzed under reducing conditions (0.2% β-mercaptoethanol)

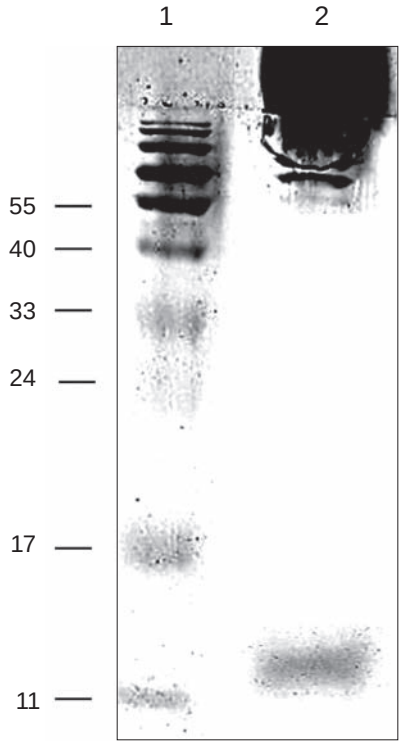

Fig. 2. SDS-PAGE of fibrinogen digested by PII (0.005 mg/ml) during 60 min. 1 – molecular weight markers; 2 – hydrolyzed fibrinogen

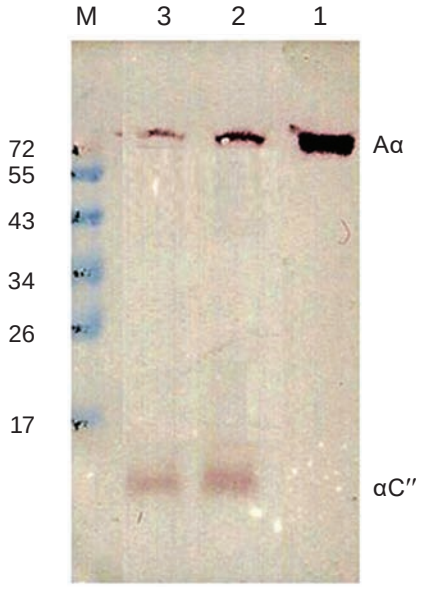

Fig. 3. Western blot analysis of fibrinogen digested by PII (0.01 mg/ml). M – molecular weight markers; 1 – native fibrinogen; 2 – fibrinogen after 30 min of hydrolysis; 3 – fibrinogen after 60 min of hydrolysis. Samples were analyzed under reducing conditions and immunoprobed by anti-fibrinogen antibody 1-5A (anti-Aα509-610) targeted to the C-terminal parts of αC-domains

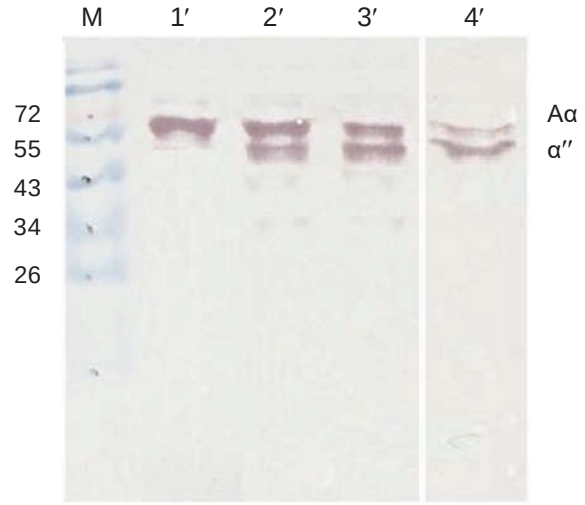

Fig. 4. Western blot analysis of fibrinogen digested by PII (0.01 mg/ml). M – molecular weight markers; 1 – native fibrinogen; 2 – fibrinogen after 30 min of hydrolysis; 3 – fibrinogen after 60 min of hydrolysis. Samples were analyzed under reducing conditions and immunoprobed by anti-fibrinogen antibody II-5C (anti-Aα20-78) targeted to the N-terminal part of α-chain

analysis. We compared the spectra of the mixture of fibrinogen with PII before the incubation (A) and after 60 min of incubation (B). The main peak that appeared after hydrolysis occurred at mass/charge (M/Z) ratio of approximately 11441 (Fig. 5) generated by a polypeptide of 11.441 kDa bearing one charge. Other peaks were minor and did not repeat themselves across multiple spectra.

Analysis of C-terminal portions of the finbrinogen Aα-chain in "Peptide Mass Calculator" (http://www.peptidesynthetics.co.uk/tools/) showed that peptide with molecular weight of 11445.317 Da corresponded to the peptide "stgktfpgffspmlgefvsetesrgsesgiftntkessshhpgiaefpsrgksssyskqftsstsyn rgdstfesksykmadeagseadhegthstkrghaksrpv", that could be formed after cleavage of the peptide bond AαAsp-Thr-Ala504-Ser505.

Surprisingly this observation showed that PII predominantly cleaves fibrinogen at the carboxyl side of the non-polar hydrophobic amino acid Alanine. To approve this conclusion we performed the chromogenic substrate assay and compared the activity of PII towards chromogenic substrates S2238 (H-D-Phe-Pip-Arg-pNA), S2251 (D-Val-Leu-Lys-pNA), S2302 (H-D-Pro-Phe-Arg-pNa), S1040 (Glp-Ala-Ala-Leu-pNa). We showed that the PII was more specific to peptide bonds formed by C-group of hydrophobic Leucine (as in S1040 substrate) and was less specific to the bond formed by C-group of positively charged Lysine proceeded by the hydrophobic Leucine (as in S2251 substrate) (Fig. 6). Proteases targeted to peptide bonds formed by C-groups of hydrophobic amino acids are not numerous but Leucine-specific proteases were reported [22] and one of such proteases was purified from *Bacillus* sp. [23]. It is also known that substrates containing Alanine or Valine in the P1 position are specific for elastase [24]. So the data on specificity of PII confirmed the data of MALDI-TOF analysis of fibrinogen-derived peptide formed by this enzyme.

Fibrinogen αC-regions are distant C-terminal parts of Aα-chains (Aα392-610) that binds tothe central portion of the molecule. After the conversion of fibrinogen to fibrin αC-regions dissociate from the central region and are available for intermolecular interaction [25-27]. These parts of molecule take part in polymerisation of fibrin [28], they contain Arg-Gly-Asp residues (Aα572-574) that interacts with platelet receptors [29] and support endothelial cells migration and proliferation [30]. Using the forms of fibrinogen with removed αC-regions is known as an approach for the study of their role in biological processes [31, 32]. That is why characterisation of new proteases targeted to the residues of αC-regions is of interest for the study of fibrinogen structure and functions.

On Fig. 7 are shown C-terminal residues of fibrinogen Aα-chain with points of proteolysis by several enzymes. Plasmin that is serine protease involved in fibrinolysis has numerous proteolytic sites at the αC-regions [33]. Among them Lys509-Thr510 and Lys584-Met585 are located in distant C-terminal parts of the Aα-chains [34].

There are the list of reports on proteases that attack αC-regions but the specificity is not revealed for most of these enzymes [35-37]. Recently described serine protease from the venom of *Echis multisquamatis* cleaves Arg491-His492 peptide bond releasing peptide Aα492-610 [38]. Macrophage elastase characterised in the work [39] attacks Glu520-Phe521. *Hementin purified* from the posterior salivary glands of the leech Haementeria ghilianii is able to cleave the peptide bond Ala498-Ala499 [40]. Remarkably hementin is specific to the bond formed by C-terminal group of the hydrophobic Alanine. According to our results PII of *B. thuringiensis* var. *israelensis* IMV B-7465 cleaved-off peptide AαAla505-Ser610 from fibrinogen molecule.

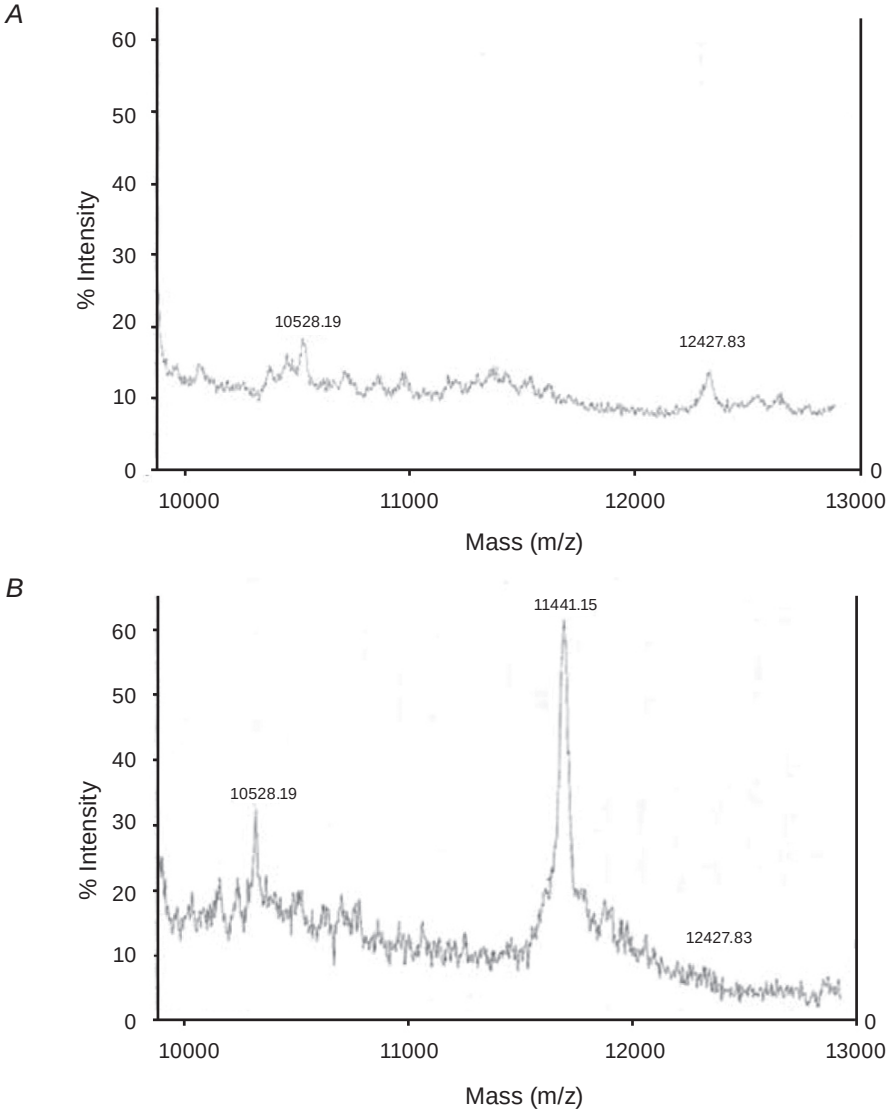

Fig. 5. MALDI-TOF spectrum of fibrinogen hydrolyzed by PII. A – MALDI-TOF spectrum before hydrolysis; B – MALDI-TOF spectrum after hydrolysis

A new fibrinogen-specific protease from the *B. thuringiensis* var. *israelensis* IMV B-7465 was described. It was established that the target of its proteolytic action on fibrinogen is AαAla494-Ser495 peptide bond. As far as proteases with such specificity have not been described previously we could suggest its potential use for obtaining of the unique digested forms of fibrinogen. Fibrinogen desAα505-610 could be used in the study of the role of distant C-terminal portions of fibrinogen αC-regions in the protein and cellular interactions. Further studies of coagulability of digested fibrinogen as well as the study of PII action *in vivo* will allow to assume the possible use of PII as anticoagulant agent.

Addendum

All authors contribute to the work equally. Eugene Stohniy performed SDS-PAGE and Western Blot experiments, Volodymyr Chernyshenko contributed to the study design and acquisition of data. Natalya Nidialkova purified and characterized the enzyme. Andriy Rebriev performed MALDI-TOF analysis. Tamara Chernyshenko purified and characterized human fibrinogen used in the study. Veronika Hadzhynova and Iryna Kolesnikova constructed the monoclonal antibody that was used in study. Eduard Lugovskoy and Liudmyla Varbanets contributed to the analysis and interpretation of data, as well as the drafting of the article. All authors approved the final version of the article to be published.

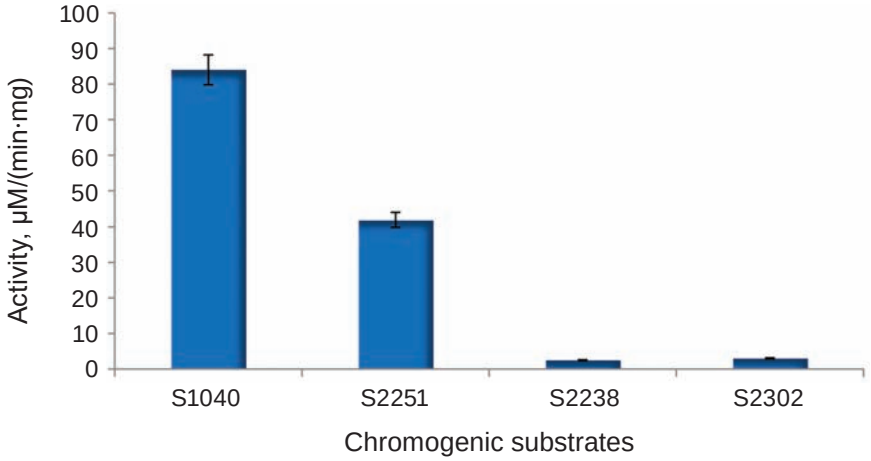

Fig. 6. Amidase activity of PII towards some chromogenic substrates: S2238 (H-D-Phe-Pip-Arg-pNA), S2251 (D-Val-Leu-Lys-pNA), S2302 (H-D-Pro-Phe-Arg-pNa), S1040 (Glp-Ala-Ala-Leu-pNa)

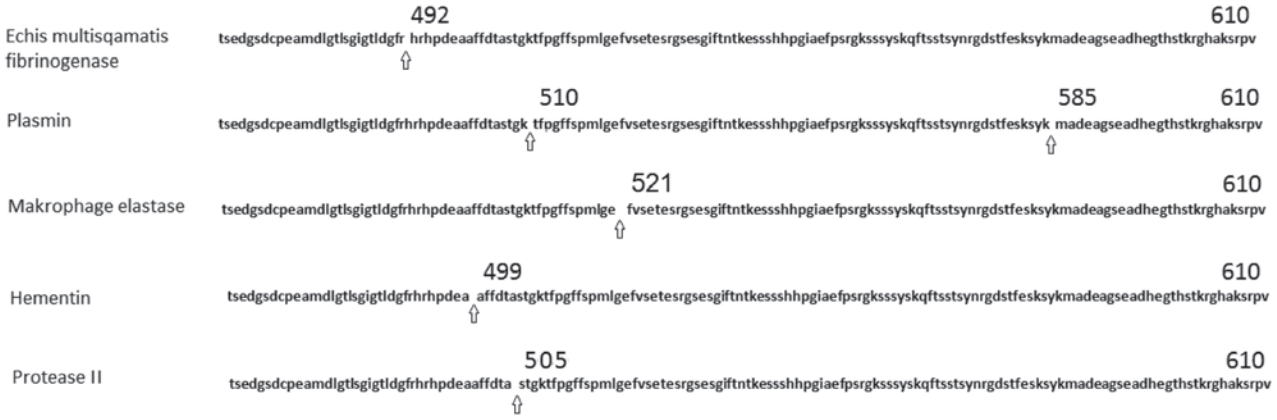

Fig. 7. Amino acid residue of C-terminal points of Aα-chain of fibrinogen. Points of hydrolysis by different proteases are marked with the spaces. Terminal amino acids of resulting peptides are marked

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