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Separation of Monomerizing and Lysozyme Activities of Destabilase from Medicinal Leech Salivary Gland Secretion

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Abstract—Destabilase, endo-ε-(γ-Glu)-Lys-isopeptidase, was prepared from the salivary gland secretion of the medicinal leech (*Hirudo medicinalis*). The secretion prepared by the known method of Rigbi et al. (1987) (secretion-K) lacks the destabilase-characteristic highly specific isopeptidase activity (the D-dimer-monomerizing activity) because of its degradation by proteolytic activity (the substrate of Glp-Ala-Ala-Leu-pNA) due to contamination with leech intestinal channel contents. Therefore, we have elaborated a new technique for preparation of a true leech secretion (secretion-I). This secretion is characterized by the complete absence of the leech intestinal channel contents and has no proteolytic activity. For the first time the destabilase-specific D-dimer-monomerizing and lysozyme activities were separated by fractionation of secretion-I by HPLC gel filtration through Superose S-12. For the purified destabilase preparation, these activities were separated by reversed-phase chromatography in an acetonitrile gradient (0-60%) in the presence of 0.1% trifluoroacetic acid. The monomerizing activity of destabilase is responsible for the ability of secretion-I to dissolve stabilized fibrin via isopeptidolysis of α-α and γ-γ fibrin chains bound by ε-(γ-Glu)-Lys-isopeptide bonds.

Key words: medicinal leech, salivary gland secretion, destabilase, isopeptidase, lysozyme, stabilized fibrin

Destabilase, a highly specific endo-ε-(γ-Glu)-Lys-isopeptidase produced by medicinal leech *Hirudo medicinalis* [1-3], is the only enzyme known to hydrolyze endo-isopeptide bonds formed by transglutaminases between the γ-carbamide group of glutamine and the ε-amino group of lysine [4]. In addition to stabilized fibrin with its ε-(γ-Glu)-Lys-isopeptide bonds, its fragment D-dimer with similar bonds is also a suitable substrate for destabilase. Monomerization of the D-dimer suggests the hydrolysis of the isopeptide bonds [2]. In addition to the D-dimer-monomerizing activity, we have recently found lysozyme activity in highly purified destabilase preparations from medicinal leech extracts [5]. Therefore, it was interesting to elucidate if the monomerizing and lysozyme activities are functions of the same or of different proteins. This work was designed to separate the monomerizing and lysozyme activities in a highly purified destabilase preparation and in a specially prepared salivary gland secretion in comparison with the secretion prepared by the known method [6]. In the fractions of leech salivary gland secretion, the proteolytic activity was also determined as a parameter determining the difference between the two secretion preparations taken from leeches by two methods. In secretion-I the isopeptidolysis products were also analyzed, using as the substrate the insoluble stabilized fibrin which is known to become soluble under the influence of the leech secretion [1].

MATERIALS AND METHODS

The medicinal leech salivary gland secretion contaminated with the leech's intestinal channel contents (secretion-K) was prepared as described in [6]. To obtain the true secretion (secretion-I), we have modified this method. The essence of our modification was the enrichment with the salivary gland secretion of 0.15 M NaCl solution containing 1 mM arginine, which was used to feed the leeches; to do this, the solution was intermixed constantly, its greater fraction (0.5 ml) was taken, and fresh solution was added. During the feeding, 12-15 samples were taken from every leech. For each sample containing secretion-I the absorption spectrum was recorded in the range 200-320 nm. Solutions containing secretion-

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K and those containing secretion-I were evaporated and dialyzed against water using a Spectra/Por CE (Cellulose Ester) Membrane (MWCO:500, USA). The solutions under dialysis and the dialyzates were collected and concentrated. The salivary gland secretion was fractionated by HPLC gel filtration through Superose S-12 in 0.02 M Tris-HCl buffer (pH 7.4). Destabilase was isolated from the medicinal leech extracts as described in [3] and then fractionated by reversed-phase chromatography on a Vydac 300-5C4 column (4.6 × 150 mm) equilibrated with 0.1% trifluoroacetic acid; the protein was eluted with a linear gradient (0-60%) of acetonitrile containing 0.1% trifluoroacetic acid. The D-dimer-monomerizing activity was determined as described earlier [3], with D-dimer as the substrate [7] and expressed in nkat/mg protein (0.02 M Tris-HCl buffer (pH 7.4), incubation for 24 h at 37°C) or in percent of the D-dimer monomerized, with the initial D-dimer taken as 100%. The lysozyme activity to *Micrococcus lysodeikticus* cells (Sigma, USA) (0.25 mg/ml) was determined turbidimetrically at 450 nm in 0.02 M Tris-HCl buffer (pH 7.4) at 25°C and expressed in conventional units [5]. The lysozyme activity unit corresponds to a decrease of 0.001 in A_{450} for 1 min of incubation. The proteolytic activity was determined using the chromogenic substrate Glp-Ala-Ala-Leu-pNA synthesized as described in [8] which we were kindly given by Doctor of Chemistry G. N. Rudenskaya (School of Chemistry, Moscow State University). An increase of 0.001 in optical density at 405 nm for 10 min of incubation at 25°C in 0.02 M Tris-HCl buffer (pH 7.4) supplemented with 5% dimethyl sulfoxide, at the substrate concentration of 0.25 mg/ml was taken as the optical density unit. Polyclonal antibodies to recombinant destabilase were used which were prepared as described in [5] and were active in immunoblotting at the dilution of 1 : 1000 [9]. The destabilase concentration was determined by enzyme immunoassay (ELISA). The degree of the stabilized fibrin dissolution by the leech salivary gland secretion was determined by the method of Astrup [10] using unpurified commercial fibrinogen preparations from bovine blood (Bakpreparaty, Kaunas, Lithuania) and expressed in cm^2/mg protein. These preparations contained plasminogen and factor XIII admixtures. Thrombin was from the same production. The protein concentration was determined as described in [11]. Electrophoresis in 12.5% SDS polyacrylamide gel was carried out by the Laemmli method [12].

RESULTS

Characteristics of the secretion-I. Portions (0.5 ml) of the above-described membrane solution (secretion-I) were used to obtain UV absorption spectra in the range 200-320 nm. Figure 1 (a-d) presents the characteristic spectra of the first three and of the last (fifteenth) portions. The peptide–protein spectrum of the secretion was the same during the period of the leech's sucking of the blood substituting solution. However, the absorption maximum changed depending of the portion order: the clear maximum at 210 nm in the first and second portions was shifted to 230 nm in the third portion as an asymmetric peak up to the very last portion. And a differently expressed absorption maximum at 280 nm also remained (Fig. 1, a-d).

Fractionation of secretion-I and of secretion-K. After dialysis (see "Materials and Methods"), secretion-I and secretion-K preparations were fractionated by HPLC gel filtration through Superose S-12 (Figs. 2 and 3). The figures show different elution profiles of both secretions and the distribution of D-dimer-monomerizing, lysozyme, and proteolytic activities. Their specific activities are presented in the table. The fractionation results of secretion-I are especially interesting. Figure 2 (a and b) clearly shows the separation of the fractions with the monomerizing and lysozyme activities. Secretion-K was specified by the fraction with the proteolytic activity (Fig. 3, a and b), which was absent in secretion-I (table, Fig. 2b).

Fractionation of a highly purified destabilase preparation. In the next step of the work a highly purified (95% purity) destabilase preparation was used (after HPLC gel filtration through Superose S-12 as the final purification

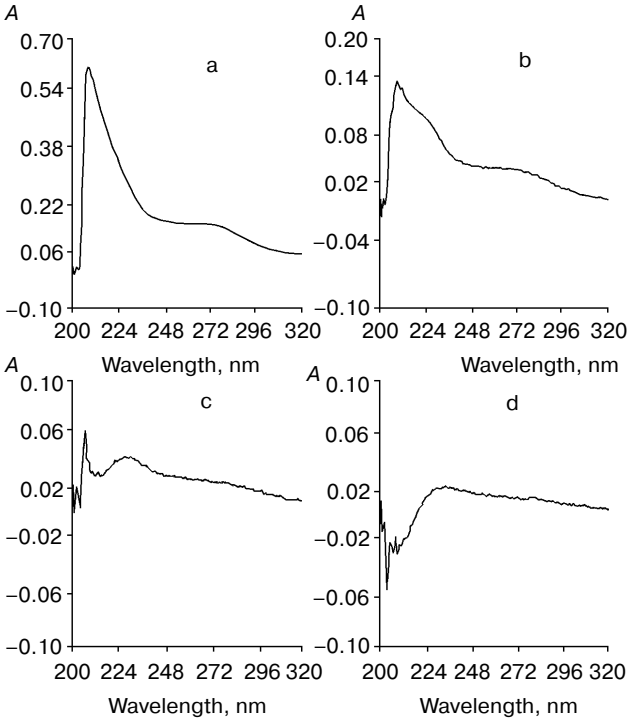

Fig. 1. Absorption spectra of the secretion-I portions taken from the same leech during the whole period of its sucking of 0.15 M NaCl containing 1 mM arginine: a) first portion; b) second portion; c) third portion; d) fifteenth portion.

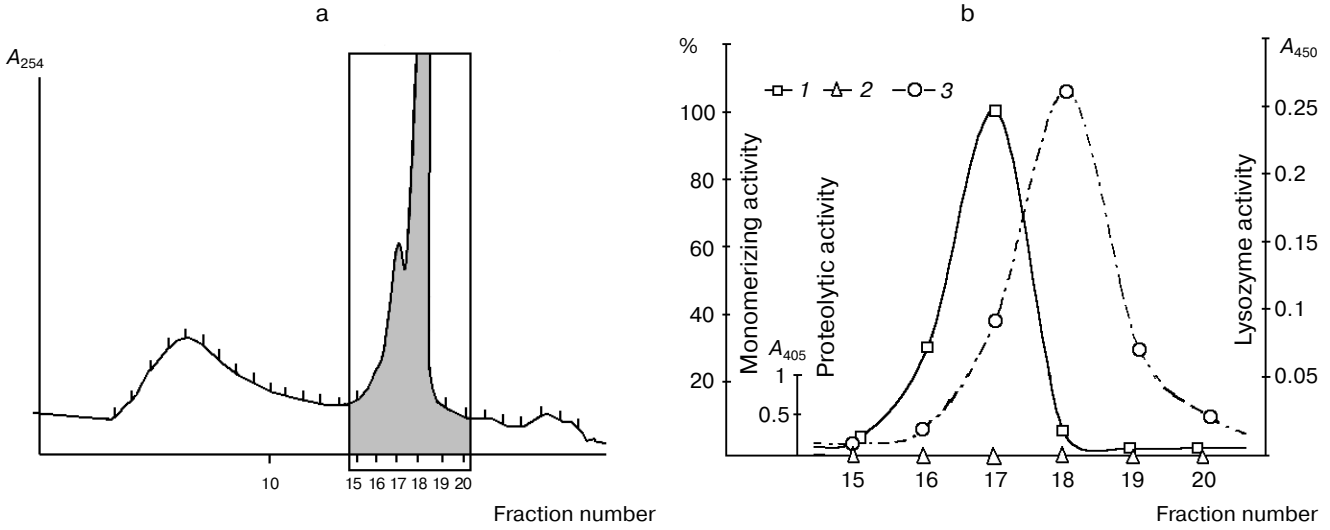

Fig. 2. Fractionation of secretion-I by HPLC gel filtration through Superose S-12 in 0.02 M Tris-HCl buffer (pH 7.4). a) Protein elution profile (active fraction zone is crosshatched, see (b)); b) distribution of activities: *1*) D-dimer-monomerizing activity; *2*) proteolytic activity; *3*) lysozyme activity.

step); it had monomerizing and lysozyme activities. This preparation was fractionated by reversed-phase chromatography in a gradient of acetonitrile (0-60%) containing 0.1% trifluoroacetate. All fractions were analyzed for the presence of lysozyme and D-dimer-monomerizing activities. In addition, polyclonal antibodies to recombinant destabilase were used to detect the corresponding antigen in the protein fractions under analysis. The fractionation results are presented in Fig. 4. One can see that the fractions with the lysozyme activity were eluted with 46-49% acetonitrile. The fractions with the D-dimer-monomerizing activity were eluted with 56-59% acetonitrile. All fractions with the lysozyme activity were antigen-positive, whereas the antigen was not detected in the fractions with the D-dimer-monomerizing activity (Fig. 4).

Analysis of products of stabilized fibrin isopeptidolysis with secretion-I. The endo-ε-(γ-Glu)-Lys-isopeptidase activity of secretion-I was detected by the presence of a liquid zone in the stabilized fibrin plate on the application place of the preparation under analysis compared to saline used as the control. The isopeptidase activity of the secretion-I preparation was $1.5 \cdot 10^3$ cm^2/mg protein. The liquid zone was collected and analyzed by 12.5% SDS polyacryl-

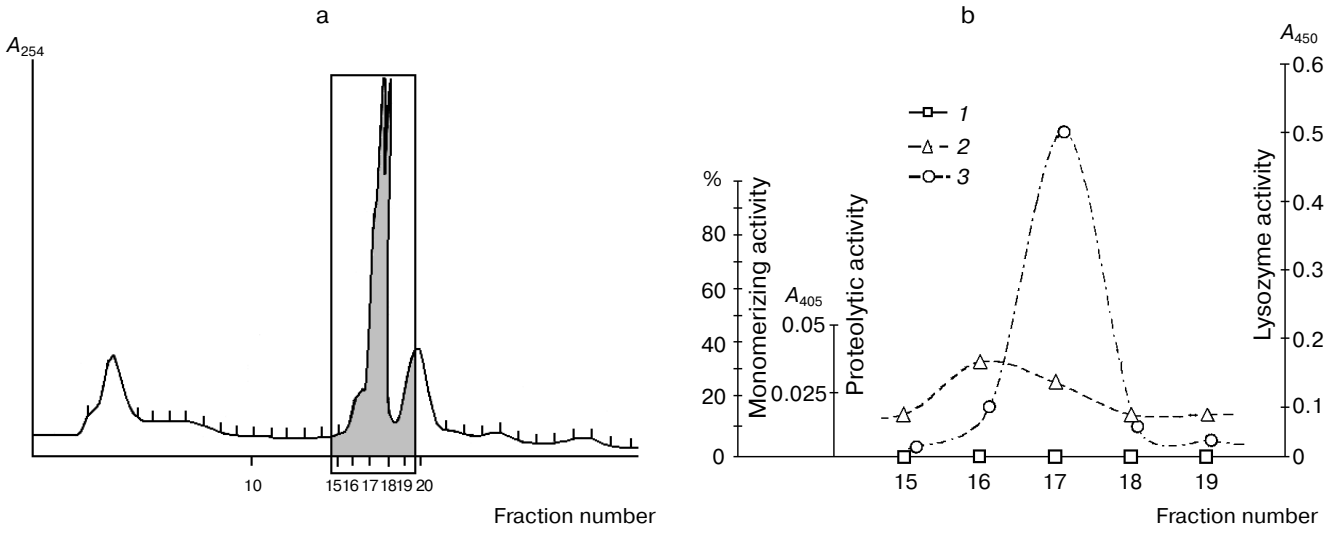

Fig. 3. Fractionation of secretion-K by HPLC gel filtration through Superose S-12 in 0.02 M Tris-HCl buffer (pH 7.4). a) Protein elution profile (active fraction zone is crosshatched, see (b)); b) distribution of activities: *1*) D-dimer-monomerizing activity; *2*) proteolytic activity; *3*) lysozyme activity.

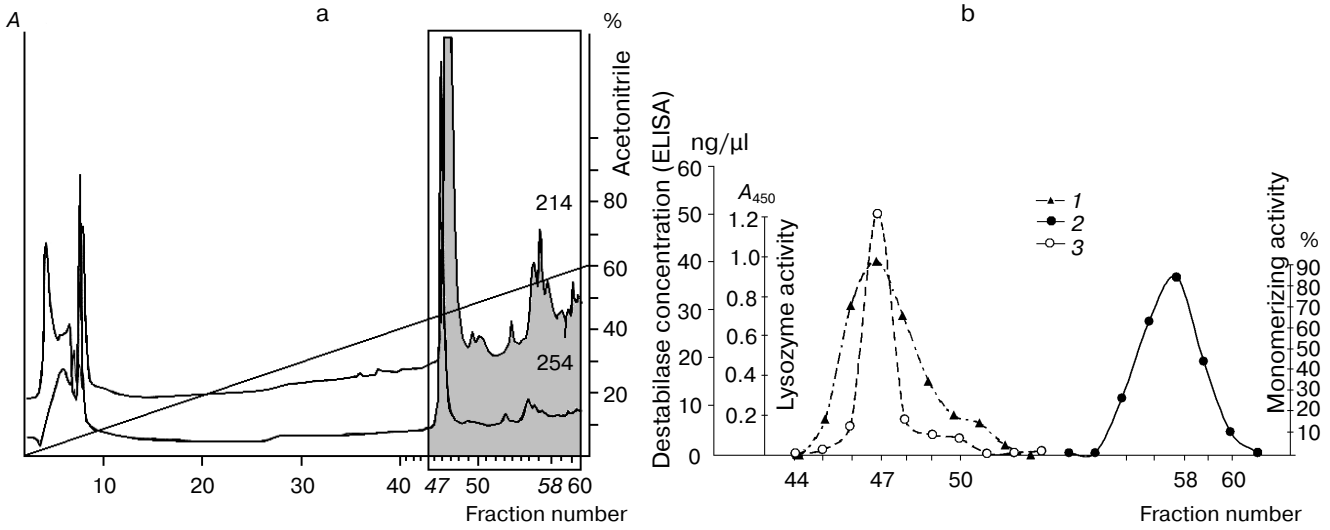

Fig. 4. Fractionation of highly purified destabilase preparation by reversed-phase chromatography on a C4 column in a gradient of acetonitrile (0-60%) containing 0.1% trifluoroacetate. a) Protein elution profile (active fraction zone is crosshatched, see (b)); b) distribution of activities: *1*) lysozyme activity; *2*) D-dimer-monomerizing activity; *3*) amount of protein determined by enzyme immunoassay using antibodies against recombinant destabilase.

amide gel electrophoresis in the presence of mercaptoethanol. A stabilized fibrin plate incubated with saline for 48 h was used as the control sample (Fig. 5, lane *1*). The figure shows the protein band distribution that is characteristic for stabilized fibrin: the α-chain multimers have the least mobility and remain in the application zone; they are followed by a protein corresponding to fibrin γ-γ-chains (94 kD), the α-chain outside of the cross-linking (68 kD), and the β-chain (55 kD) [13]. Among products of the stabilized fibrin incubation for 48 h with secretion-I (Fig. 5, lane *2*) the band corresponding to the γ-γ-chain virtually disappeared, but a band appeared which corresponded to the γ-chain. Multimers of the less polymerized α-α-chains appeared (in some experiments they were completely absent). The β-chain position was unchanged, and two new proteins with molecular weights of 43 and 25 kD appeared.

Characteristics of secretion-K and secretion-I preparations (average results of analysis of three preparations of each secretion)

Activity	Secretion-K	Secretion-I
Specific D-dimer-monomerizing activity (D-dimer as substrate)	0	0.1 nkat/mg
Specific lysozyme activity (*Micrococcus lysodeikticus* as substrate)	127 000 units/mg	135 000 units/mg
Specific proteolytic activity (Glp-Ala-Ala-Leu-pNa as substrate)	200 units/mg	0

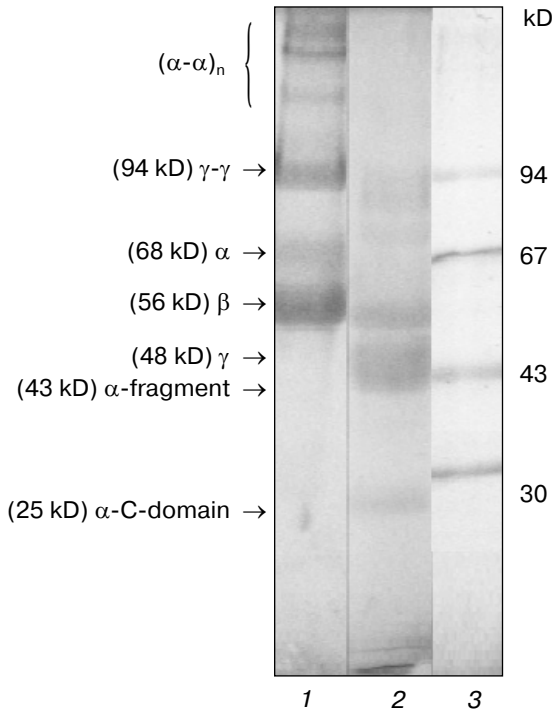

Fig. 5. Electrophoresis in 12.5% SDS polyacrylamide gel in the presence of mercaptoethanol of stabilized fibrin incubated for 48 h with saline (lane *1*) and with secretion-I (lane *2*). Lane *3* contained protein markers.

DISCUSSION

Unlike the known method of preparation of the secretion-K [6] contaminated with the leech intestinal channel contents, the present method results in only the secretion which is injected by the leech into a wound bitten through animal or human skin. Our method of secretion taking is a modification of method [6] of Rigbi who proposed to use an impermeable membrane to be bitten by the leech and to allow it to suck 0.15 M NaCl with 1 mM arginine instead of blood. The salivary gland secretion is swallowed by the leech together with this solution. To prepare the secretion by the Rigbi method [6], the solution swallowed should be pressed out from the leech's intestinal channel, and this solution is enriched not only with the leech's secretion but also with the intestinal channel contents (secretion-K). The essence of our modification is the absence of contact between secretion-I prepared by us and the intestinal channel. To provide this, repeatedly renewed portions of the solution placed into the above-membrane space of the cylinder are taken with constant stirring during the sucking by the leech. This prevents the occurrence of the intestinal channel eructation into the above-membrane solution, whereas this cannot be absolutely prevented when the concentrated secretion is prepared in response to stimulation of the leech head end receptors with NaCl crystals, as we proposed earlier [14].

The presence of peptides and proteins in the UV absorption spectra in all portions of the secretion-I taken from the leech during the sucking of the given salt solution indicates first of all that the leech is ejecting the salivary gland secretion during the whole blood sucking period. The difference in the absorption spectra of the secretion from the same leech (Fig. 1) suggests certain changes in the composition of the secretion ejected by the leech during the blood sucking.

In the present work, we used secretion-I and secretion-K preparations deprived of components with molecular weights below 500 daltons by dialysis using a special membrane (see "Materials and Methods"). The fractionation through Superose S-12 revealed significant difference between these secretions (Figs. 2 and 3, table). Secretion-K was characterized by proteolytic activity due to contamination with the leech intestinal channel contents (Fig. 3b). The use of the chromogenic substrate of Glp-Ala-Ala-Leu-pNa sensitive to many proteolytic enzymes (subtilisins, chymotrypsin-like enzymes, elastase-type enzymes, exo- and endo-leucine aminopeptidases [8, 15]) allowed us to detect the proteolytic activity which was not identified in secretion-K by Rigbi et al. [6], who used hemoglobin and bovine serum albumin as substrates. However, one cannot exclude the presence of specific collagenases [6] in secretion-K. The proteolytic activity of secretion-K correlates with its inability to catalyze D-dimer monomerization. This seems to be associated with the proteolytic degradation of destabilase by one or some proteases of this secretion.

The absence of proteolytic activity is an absolutely specific feature of secretion-I. Such activity was also absent in the medicinal leech salivary gland secretion obtained in response to stimulation of the leech head end receptors with NaCl crystals [1, 14]. The secretion prepared by the last method inhibited trypsin and thrombin activities [16] and also the activity of blood plasma kallikrein [17].

We were first to separate the D-dimer-monomerizing and lysozyme activities not only by gel filtration of secretion-I (Fig. 2, a and b) but also by reversed-phase chromatography of highly purified destabilase preparation (Fig. 4). This finding is especially important because earlier we failed to separate proteins with the monomerizing and lysozyme activities by fractionation of such destabilase preparation isolated from extracts of the medicinal leech homogenate by HPLC gel filtration through Superose S-12 [5].

The findings presented here indicate that destabilase, or endo-isopeptidase, is a component of the true medicinal leech salivary gland secretion prepared by the modification of the known method and that it is precisely the secretion that is injected by the leech into the host's (animal or human) body and which is responsible for the humoral effect of hirudotherapy and, in particular, for the destabilase-stimulated thrombolytic effect [18]. Destabilase is a protein secreted by salivary glands, and this is consistent with the signal sequence found earlier in the destabilase gene [3], which is a specific feature of secreted proteins [19].

The clear separation of the lysozyme and D-dimer-monomerizing activities of destabilase suggests that these two activities either should belong to two different proteins or be determined by the same protein, which acts as an oligomer with two active sites expressed depending on the reaction conditions. The molecular weight of destabilase is about 12 kD [20]; however, in some cases electrophoresis revealed its dimerization and even tetramerization. It should not be ruled out that during the fractionation the destabilase molecule can be changed with production of its two forms, each with its inherent specific activity. We have observed changes in destabilase molecule when the enzyme was exposed to trace amounts of various detergents that resulted in the appearance of the pronounced proteolytic activity [21]. Each of the hypotheses is viable and needs further studies.

The monomerizing activity of destabilase is responsible for the ability of secretion-I to dissolve stabilized fibrin via isopeptidolysis of α-α and γ-γ fibrin chains bound by isopeptide bonds, which were produced under stimulation by factor XIIIa [4]. This dissolving of fibrin is accompanied by an irreversible depolymerization of destabilized fibrin [1]. The distribution of protein fractions in the electrophoregram (Fig. 5) supports the above-described mechanism. Two new proteins with molecular weights of 43 and 25 kD appear to be fragments of α-chain resulting

from its limited proteolysis with plasmin. The peptide bond (Lys219–Ser220) of fibrin α-chain [22] is known to be sensitive to plasmin. This bond binds the α-chain C-terminal domain, which is an independent domain of the fibrin molecule (43 kD) with the main pivot of fibrinogen or of fibrin-monomer molecule. Obviously, plasmin is produced due to spontaneous activation of plasminogen during a long-term incubation of fibrin plates containing plasminogen admixtures. No doubt the production of the α-C-domain and of the α-fragment is secondary; it occurs only after the fibrin has been dissolved by the secretion and is not observed in the control (Fig. 5, lane *1*). This is obviously because the C-terminal region of α-chains of the corresponding multimers are inaccessible for plasmin. Isolated preparations of destabilase, endo-ε-(γ-Glu)-Lys-isopeptidase, cause a similar monomerization of α-α multimers and of γ-γ dimers of stabilized fibrin chains and of fibrin fragment D-dimer [2, 20, 23].

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REFERENCES

1. Baskova, I. P., and Nikonov, G. I. (1985) *Biokhimiya*, **50**, 424-431.
2. Zavalova, L. L., Kuzina, E. V., Levina, N. B., and Baskova, I. P. (1993) *Thromb. Res.*, **71**, 241-244.
3. Zavalova, L., Lukyanov, S., Baskova, I., Snezhkov, E., Berezhnoy, S., Bogdanova, E., Barsova, E., and Sverdlov, E. (1996) *Mol. Gen. Genet.*, **253**, 20-25.
4. Aeschlimann, D., and Paulsson, M. (1994) *Thromb. Haemostasis*, **71**, 402-415.
5. Zavalova, L. L., Baskova, I. P., Lukyanov, S. A., Sass, A. V., Snezhkov, E. V., Akopov, S. B., Artamonova, I. I., Archipova, V. S., Nesmeyanov, V. A., Kozlov, D. G., Benevolensky, S. V., Kiseleva, V. I., Poverenny, A. M., and Sverdlov, E. D. (2000) *Biochim. Biophys. Acta*, **1487**, 69-77.
6. Rigbi, M., Levy, H., Iraquy, F., Teitelbaum, M., Orevi, M., Alajoutsijarvi, A., Horovitz, A., and Galun, R. (1987) *Comp. Biochem. Physiol. [B]*, **87**, 563-573.
7. Belitser, V. A., Pozdnjakova, T. M., Platonova, T. N., and Vovk, E. V. (1981) *Thromb. Res.*, **21**, 565-572.
8. Lyublinskaya, L. A., Khaidu, I., Balandina, G. N., Filippova, I. Yu., Markaryan, A. N., Lysogorskaya, E. N., Oksenoit, E. S., and Stepanov, V. M. (1987) *Bioorg. Khim.*, **13**, 748-753.
9. Harlow, E., and Lane, D. (1988) *Antibodies. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 479-511.
10. Jespersen, J., and Astrup, T. (1983) *Haemostasis*, **13**, 301-315.
11. Sedmak, J. J., and Grossberg, S. E. (1977) *Analyt. Biochem.*, **79**, 544-552.
12. Laemmly, U. K. (1970) *Nature*, **227**, 680-685.
13. Budzynski, A. Z., Marder, V. J., and Shainoff, J. R. (1974) *J. Biol. Chem.*, **249**, 2294-2302.
14. Baskova, I. P. (1994) *The Method for Preparation of Bloodsucking Leech Salivary Gland Secretion*, Patent No. 2045954.
15. Romanova, T. V., Rudenskaya, G. N., Tebloeva, L. T., Zverev, D. V., and Emirova, Kh. M. (1998) *Vopr. Med. Khim.*, **44**, 296-304.
16. Baskova, I. P., and Nikonov, G. I. (1984) *Folia Haematol.*, **111**, 131-137.
17. Baskova, I. P., Khalil, S., Nikonov, G. I., Rabinovich, S. E., Nartikova, N. F., and Paskhina, T. S. (1989) *Biokhimiya*, **53**, 1467-1473.
18. Baskova, I. P., Nikonov, G. I., and Khalil, S. (1988) *Folia Haematol.*, **115**, 166-170.
19. Dalbey, R. I., and von Heijne, G. (1992) *Trends Biochem. Sci.*, **17**, 474-478.
20. Baskova, I. P., Nikonov, G. I., Zavalova, L. L., and Larionova, N. I. (1990) *Biokhimiya*, **55**, 674-679.
21. Baskova, I. P., Zavalova, L. L., and Kuzina, E. V. (1994) *Bioorg. Khim.*, **20**, 492-497.
22. Medved, L. V., Gorkun, O. V., and Privalov, P. L. (1983) *FEBS Lett.*, **160**, 291-295.
23. Zavalova, L. L., Nikonov, G. I., Popova, G. Yu., and Baskova, I. P. (1991) *Biokhimiya*, **56**, 115-124.