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# CARP FIBRINOGEN AND ITS TERMINAL PLASMIN DEGRADATION PRODUCTS

The isolation and characterization of carp (Cyprinus carpio) plasma fibrinogen and its plasmin degradation products are described. Alike other vertebrate species, carp fibrinogen is a dimeric protein and consists of three different pairs of disulfide-bonded polypeptide chains. Aα, Bβ, and γ. In contrast to mammalian fibrinogen, the Bβ chain of carp fibrinogen has apparently a higher molecular weight than Aα chain. The relatively large size of the carp Bβ chain results from the unusually large size of the $NH_2$-terminal B fibrinopeptide released by thrombin cleavage of fibrinogen. As in mammalian species, plasmin digestion of carp fibrinogen produced as main terminal end- products two classes of fragments D and E. It was found that fragment D ($D_1$) inhibited fibrin monomer polymerization not only in homo- but in heterologous system as well.

## 1. INTRODUCTION

Human fibrinogen is a soluble glycoprotein ($M_r$ 340 000) built up of three pairs of non-identical polypeptide chains (Aα, Bβ, γ) which form two-half molecule units held together by three disulfide bonds at the $NH_2$-terminal portions of polypeptides [2, 3, 17, 18]. In native molecule there exist different functional domains (two D and one E) important for protofibril formation [4, 17, 18]. Following thrombin-mediated removal of two pairs of fibrinopeptides, A and B, from the Aα and Bβ chains of the fibrinogen, respectively, activated fibrin monomers appeared which spontaneously polymerize. The general mechanism of fibrin formation has been suggested to be based on the interaction of preexisted binding sites located in the COOH-terminal region (domain D) of the fibrinogen molecule with polymerizing sites situated in the $NH_2$-terminal part (domain E) of the neighboring molecule [16, 25] and polymer assembly commences with formation of double-strended fibrils. The fibrils subsequently associate laterally, forming thick fibers of fibrin [7, 29–32, 37, 39].

Plasmin digestion of human fibrinogen gives as main terminal end-products two classes of carbohydrate-containing fragments D and E representing the COOH-terminal and $NH_2$-terminal ends (central nodule) of molecule, respectively [5, 15, 28, 35]. Depending on the degree of degradation of the $\gamma$-chain, fragment D may exist as $D_1$ (94 000), $D_2$ (88 000) or $D_3$ (83 000). Digestion of human fibrinogen in the presence of calcium ions results in fragment $D_1$. When proteolysis is carried out in the presence of EDTA (EGTA) $D_3$ is the main product of hydrolyzate [38]. It has been shown that fragment $D_1$ but not $D_3$ strongly inhibited fibrin monomers polymerization [26, 27, 39]. We found in a comparative investigation that fragment $D_1$ of duck fibrinogen also exerts inhibitory effect on the above process [23].

The aim of this study was to characterize submolecular composition and some physico-chemical properties of carp fibrinogen as well as its plasmin degradation products ($D_1$, $D_3$) and compare the effect of $D_1$ and $D_3$ fragments on polymerization of carp fibrin monomers (homologous system) and human fibrin monomers (heterologous system).

## 2. MATERIAL AND METHODS

*Blood collection.* Nine parts of fish blood obtained by direct cardiac puncture after the heart was surgically exposed, were taken into one part of 10% sodium citrate containing 0.1 M $\varepsilon$-aminocaproic acid. 50 u KI/ml of trasylol and 15 $\mu$g/ml phenylmethanesulphonyl fluoride.

*Fibrinogen preparation.* Fibrinogens (fish and human) were isolated from fresh blood plasma according to the method of Doolittle et al. [12]. Fibrinogen preparations were dissolved in 0.14 M NaCl buffered with 0.01 M sodium phosphate. pH 7.4 and passed through a Sepharose-lysine column to remove plasminogen. Both isolated fibrinogens were 93–95% coagulable with bovine thrombin. Fg preparations were characterized by polyacrylamide gel electrophoresis according to the method of Weber and Osborn [41].

*Protein concentration* was determined by the method of Itzhake and Gill [21].

*Isolation of fragments $D_1(D_{Ca^{+2}})$ and $D_3(D_{EDTA})$.* Human or carp fibrinogens (15 mg/ml) in 1 mM Tris/HCl buffer, pH 7.4, containing 0.15 M NaCl and 5 mM $CaCl_2$ were digested with human plasmin at 0.05 cesein units/mg of fibrinogen for 18 h at 37°C. Fragments $D_1$ were isolated from the digest by chromatography on Lys-Sepharose by the method of Rupp et al. [36]. $D_3$ remnants obtained from fragments $D_1$ by further plasmin hydrolysis in the presence of 5 mM EDTA were purified on Sephadex G-100. Molecular weights of fish and human fragments were estimated by SDS-PAGE.

*Inhibition of fibrin monomers polymerization.* Thrombin fibrin monomers were prepared from carp and human fibrinogen according to the method of Belitser et al. [1]. Polymerization of fibrin monomers was measured spectrophotometrically at 350 nm [6]. In typical experiment, 0.1 ml aliquit of the fragments $D_1$ or $D_3$ in 0.15 M Tris/HCl buffer was mixed with 0.8 ml of the same buffer in a 10 mm long quartz cell and the base line was recorded at 350 nm. At zero time the recorder was started, 0.1 ml of fibrin monomers (1.5 mg/ml in 0.02 M acetic acid) was added, mixed well and the increase of absorbance was recorded for 15 min. To measure the rate of polymerization of fibrin monomers alone, 0.1 ml of fibrin monomers was added to 0.9 ml of the buffer. The maximum rate of polymerization was calculated from the slope of the steepest part of the curve and expressed as percent of that for fibrin monomers alone.

## 3. RESULTS

SDS-polyacrylamide gel electrophoresis of unreduced and reduced samples of carp fibrinogen are presented in Fig. 1. The results indicate that this protein is built up of 3 pairs of non-identical subunits ($M_r$ 48 000, 50 500, 55 500) bound by S-S bridges, corresponding to 3 pours of mammalian fibrinogen chains. Molecular weight of native carp fibrinogen was calculated to be about 310 000.

*Identification of carp fibrinogen subunits.* In order to relate the three observed polypeptides to the three subunits of carp fibrinogen, we investigated which polypeptide bands are sensitive to digestion with specific proteolytic enzymes known to selectively digest particular subunits of fibrinogen. Thrombin splits off the A and B fibrinopeptides from the Aα and Bβ subunits of fibrinogen, giving rise to the α and β chains of fibrin. The γ subunit is not digested by this enzyme. The snake venom protease- batroxobin selectively digests Aα subunit of fibrinogen but not of the Bβ and γ.

In our electrophoresis gel system undigested both human and carp fibrinogens were resolved each of them into three bands corresponding to Aα, Bβ and γ chains (Fig. 2, line F). The same figure presents polyacrylamide gel electrophoresis pattern of both above mentioned proteins after

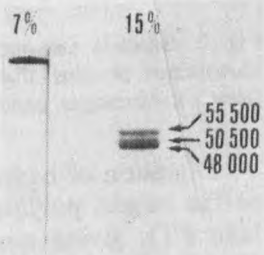


Fig. 1. SDS polyacrylamide gels after electrophoresis of unreduced (7% gel) and reduced (15% gel) carp fibrinogen stained with Coomasie brillant blue

digestion with batroxobin. As we can see proteolysis of human fibrinogen with the enzyme, results in cleavage of largest polypeptide only and in the appearance of new band of slightly lower molecular weight. In case of carp fibrinogen the middle band was attacked by batroxobin and new faster migrating band overlopping $\gamma$ chain appeared (Fig. 2, lane FB).

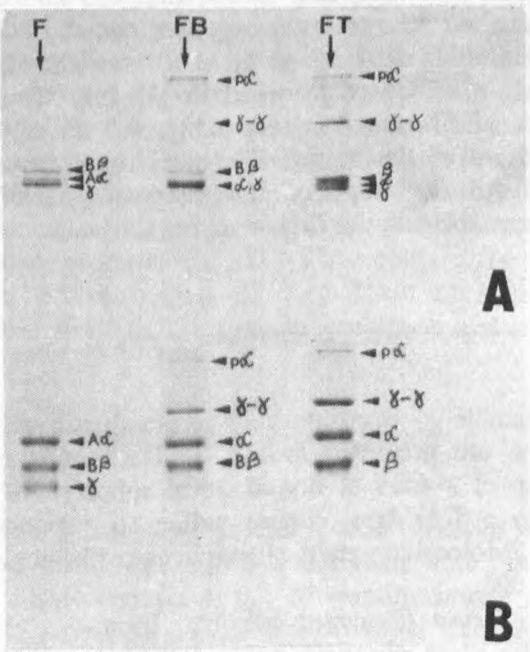


Fig. 2. Selective enzymatic digestion of carp (A) and human (B) fibrinogens. Coomassie blue-stained proteins after SDS-polyacrylamide gel electrophoresis of: undigested fibrinogen (lane F), fibrinogen digested with batroxobin (lane FB) fibrinogen digested with thrombin (lane FT)

Digestion of human and carp fibrinogens by thrombin results in cleavage of the largest polypeptides as well as the middle one in both cases (Fig. 2, lane FT), giving rise to faster migrating bands. As it was shown above (Fig. 2) batroxobin fails to digest the largest polypeptide of carp fibrinogen. Since this chain is digested by thrombin, we can deduct that it must be the B$\beta$. Molecular weights of polypeptide subunits of carp fibrinogen and fibrin estimated by SDS-polyacrylamide gel electrophoresis are given in Tab. 1.

Table 1

Molecular weights of polypeptide subunits of carp fibrinogen and fibrin

| Subunits | | | | | | |
|---|---|---|---|---|---|---|
| A$\alpha$ | $\alpha$ | B$\beta$ | $\beta$ | $\gamma$ | FpA | FpB |
| 50 500 | 48 500 | 55 500 | 49 000 | 48 000 | 2 000 | 6 500 |

Apart from that visible on the top of the gels additional slow migrating bands (lanes FB and FT) designed as $\gamma$-$\gamma$ and p-$\alpha$ correspond to very well known gamma dimer and polymers $\alpha$ chains, respectively. It is the result of crosslinking of monomers by fibrin stabilizing factor [34].

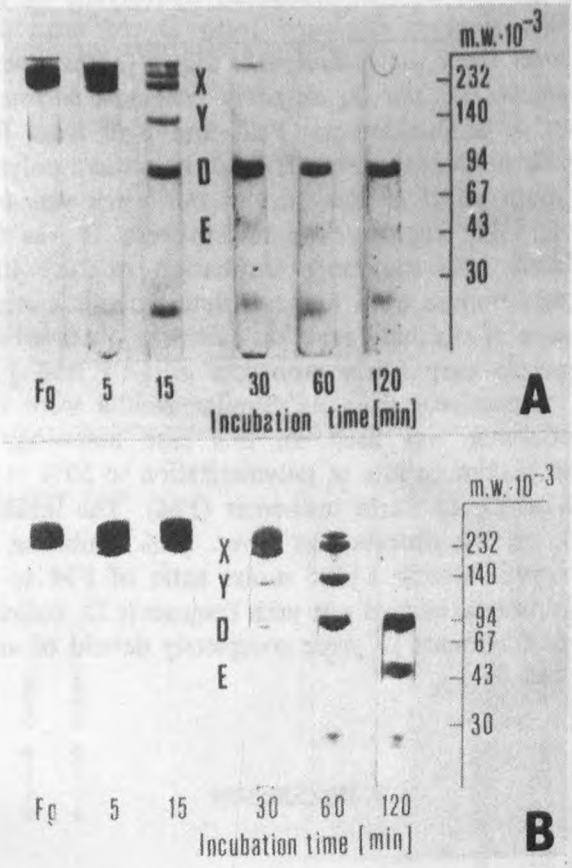


Fig. 3. SDS-polyacrylamide gels after electrophoresis of unreduced samples of plasmin digest of carp (A) and human (B) fibrinogen (Fg). The same type and size of plasmic degradation products in both cases are seen (X,Y, D, E). However, carp fibrinogen appears to be degraded by plasmin at faster rate

*Plasmin degradation products of carp fibrinogen.* Fig. 3 shows typical SDS polyacrylamide gel electrophoresis pattern of both unreduced human and carp fibrinogen digest with plasmin with respect to time. Carp fibrinogen shows significant increase in mobility already at 15 min. and becomes a mixture of intact fibrinogen and X, Y, D, E fragments. At this time human fibrinogen indicates a very small degree of conversion to degradation products. Similar pattern was practically reached after 60 min of digestion

(appearance of D and E fragments on the gel). This analysis indicates that fibrinogen isolated from carp plasma is inherently less ressistant to plasmin than human. However, it is worth to notice that both fragments (called $D_1$) occupy almost the same position on the gel and have mol. weight of 94 kDa. When fragment $D_1$ was repeatedly hydrolyzed by plasmin in the presence of EDTA a more degraded form $D_3$ of molecular weight of 80 kDa was isolated from the hydrolyzate digest (not shown).

*Effect of fragments $D_1$ and $D_3$ on fibrin monomer polymerization.* After thrombin removal of amino-terminal FpA and FpB from fibrinogen new binding sites appear in central nodul (E) and monomers polymerize to form fibrin clot. The main effort of this part of our work was to evaluate the influence of $D_1$ and $D_3$ fragments on this process. It was found that $D_1$ fragment introduced into monomer incubation mixture inhibited fibrin monomer polymerization in both homo- i heterologous system (Fig. 4 and 5). A 50% decrease of maximal reaction rate was observed at molar ratio of these fragments to carp fibrin monomer of 1 : 1 and 1 : 2.5 for carp and human $D_1$, respectively (Fig. 4). Similar results were obtained when human fibrin monomer was used. In this case homology fragment $D_1$ (human) decreased maximum rate of polymerization to 50% at approximately 1 : 1 molar ratio of $D_1$ to fibrin monomer (FM). The inhibitory effect of carp fragment $D_1$ on this process was lower. 50% inhibition was estimated to occur at an approximately 1 : 3.5 molar ratio of FM to carp $D_1$ (Fig. 5). The same experiments carried out with fragments $D_3$ indicated that these degraded forms of fragments $D_1$ were completely devoid of antipolimerizing activity (Fig. 4 and 5).

## 4. DISCUSSION

In all mammalian species which have been studied up to now fibrinogen has dimeric structure with a total molecular weight of 340 000 and the relative sizes of polypeptide chains from largest to smallest are $A\alpha$, $B\beta$ and $\gamma$. Fibrinopeptides removed from fibrinogen of mammalas are relatively constant in size with nearly all of them comprising 13–21 amino acids and thus having molecular weight in the range of 1000–2000 [9, 10].

Carp fibrinogen possesses high degree of similarity to mammalian fibrinogen in respect to molecular weight (310 000), submolecular structure (3 pairs nonidentical polypeptide chains) and amino acid composition (not shown). In contrast to mammalian coagulable protein $B\beta$ chain of carp fibrinogen has a higher apparently molecular weight than the $A\alpha$ chain. The $\gamma$-chain has the lowest molecular weight in the carp protein, as in that

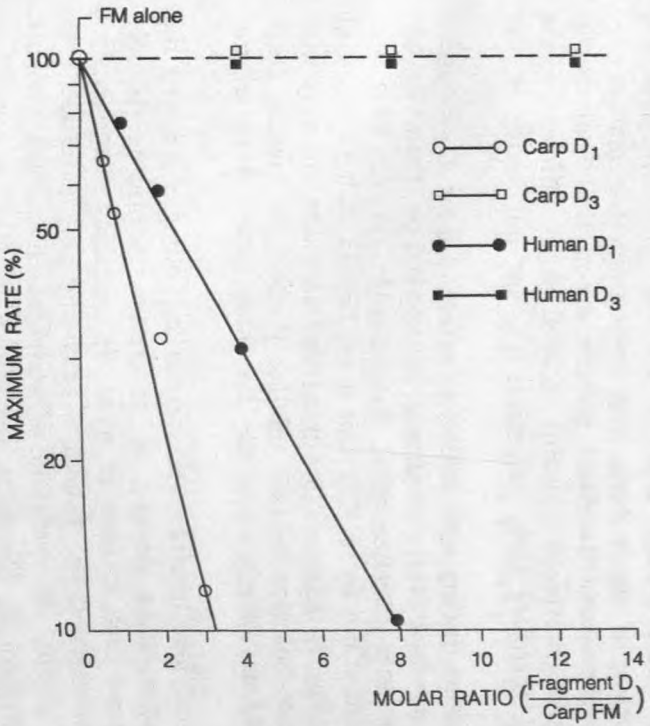


Fig. 4. The effect of fragments $D_1$ and $D_3$ of carp and human fibrinogen on the rate of carp fibrin monomer polymerization. Various ratios of the fragments to fibrin monomers (abscissa) were tested. The formation of fibrin polymers was monitored in a spectrophotometr at 350 nm. For each run the maximum reaction rate was calculated from the slope of the steepest part of the curve and expressed as percent of that for fibrin monomer alone (100% on the ordinate)

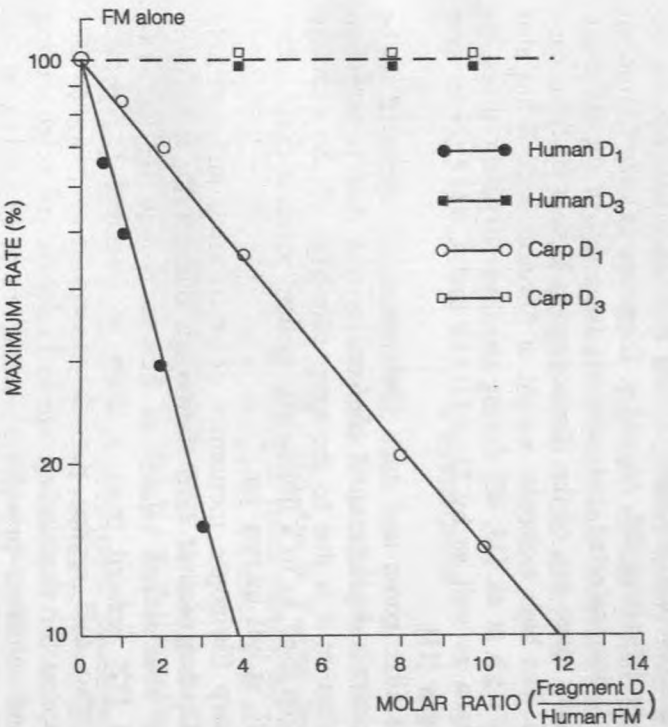


Fig. 5. The effect of fragments $D_1$ and $D_3$ of human and carp fibrinogen on the rate of human fibrin monomer polymerization

of other species. Therefore the sequence of carp fibrinogen subunits on the gel (Fig. 1) should be following: B$\beta$, A$\alpha$ and $\gamma$. Digestion of carp fibrinogen by thrombin (Fig. 2) demonstrated that relatively large size of the B$\beta$ chain results from unusually large size of the fibrinopeptide B split of from carp fibrinogen (Tab. 1). The high molecular weight of fibrinopeptide B has also been found by Wangh et al. [19, 40] during selective enzymatic digestion of *Xenopus* fibrinogen as well as by Doolittle and Cottrell in case of lamprey fibrinogen [11].

Carp fibrinogen alike goose and duck fibrinogens [23] appeared to be more sensitive to plasmin degradation in comparison with than mammalian fibrinogens [24]. Whether it is due to the some properties of the structure of the carp fibrinogen itself or to a fibrinolytic system, which is more active in carp plasma [22], is not known yet.

Inhibition of carp fibrinogen monomers polymerization and assembly of fibrin clot by $D_1$ fragment of carp fibrinogen, but not $D_3$ is in agreement with earlier observations related to human protein [13]. Olexa and Budzynski [32] isolated from $\gamma$ chain of fragment $D_1$, peptide built up of 38 amino acid residue peptide encompassing positions 374 to 411, which compited for the binding site in E domain of neighbouring fibrin monomer and inhibited protofibril formation. Horowitz et al. [20] found that 23-residue peptide originating from the carboxy-terminal region of $\gamma$-chain inhibits fibrin monomer polymerization and contains polymerization site within $\gamma$-chain segment 374–396. This site does not overlap with segments of the $\gamma$-chain that responsible for platelet aggregation ($\gamma_{400-411}$). The above described polymerization site is absent in all the $D_3$ remnants (shortened $\gamma$-chain) isolated from different kinds of fibrinogens and therefore these fragments $D_3$ are not active in the process.

Similar observations dealing with inhibitory effect of duck $D_1$ fragment but not $D_3$ both on duck fibrin monomer polymerization (homologous) and pig fibrin monomer polymerization (heterologous system) have also been successfully carried out by us [23]. Our latest finding with $D_1$ remnant of carp fibrinogen (Fig. 5) indicated that this fragment reacts not only with carp fibrin monomer but also exhibits inhibitory effect on human fibrin monomer (heterologous system), however, this was observed to smaller extend.

Evidence presented here together with our earlier finding concerning the homology of polymerization domain in vertebrate fibrinogens strongly support the hypothesis that, in spite of some distinguishing characteristics for each of the fibrinogen types, structures providing polymerization sites remain unchanged during the evolution of vertebrate fibrinogen and are uncommonly conservative [8, 33]. As it was proved by Cierniewski et

al. [7] the polymerization site is formed simply both a linear sequence of amino acid residues in a segment of the γ-chain and native tertiary structure. It means therefore that two elements are essential for the expression of polymerization sites in the structural D domain.

## 5. REFERENCES


[1] Belitser V. A., Tolstykn T. V., Tsarjuk V. M., Pozdnjakova T. M. (1975), Thromb. Res., 7, 797–806.
[2] Blomback B., Blomback M. (1972), Ann N. Y. Acad. Sci., **202**, 77–97
[3] Blomback B., Hessel B., Hogg D. (1976), Thromb, Res., **8**, 639–658.
[4] Blomback B., Hessel B., Hogg D., Therkildsen L. (1978), „Nature", **275**, 501–505.
[5] Budzynski A. Z., Marder V. J. (1977), Thromb. Haemostas., **38**, 793–800.
[6] Budzynski A. Z., Olexa S. A., Pandya B. V. (1983), Ann N. Y. Acad. Sci., **408**, 301–314.
[7] Cierniewski C. S., Budzynski A. Z. (1992), „Biochemistry", **31**, 4248–4253.
[8] Cierniewski C. S., Krajewski T., Janiak A. (1980), Thromb. Res., **19**, 599–607.
[9] Doolittle R. F. (1976), Federation. Proc., **35**, 2145–2149.
[10] Doolittle R. F. (1983), Ann N. Y. Acad. Sci., **408**, 13–26.
[11] Doolittle R. F., Cottrell B. A. (1974), Biochem Biophys. Res. Commun., **60**, 1090–1096.
[12] Doolittle R. F., Schubert D., Schwartz S. A. (1967), Arch. Biochem Biophys., **118**, 456–467.
[13] Dray-Attali L., Larrieu M. J. (1977), Thromb. Res., **10**, 575–586.
[14] Furlan M., Rupp C., Beck E. A. (1983), Biochem Biophys. Acta, **742**, 25–32.
[15] Gaffney P. J., Brasher M. (1973), Biochem Biophys. Acta, **295**, 308–313.
[16] Hantgan R., McDonagh J., Hermans J. (1983), Ann N. Y. Acad. Sci., **408**, 344–367.
[17] Henschen A., Lottspeich F., Kehl M., Southan C. (1983), Ann N. Y. Acad. Sci., **408**, 28–43.
[18] Hoeprich P. D., Doolittle R. F. (1983), „Biochemistry", **22**, 2049–2055.
[19] Holland L. J., Wangh L. J., Spolski R. J., Weisel J. W. (1984), J. Biol. Chem., **259**, 3757–3762.
[20] Horowitz B. H., Varadi A., Scheraga H. A. (1984), Proc. Natl. Acad. Sci. USA, **81**, 5980–5984.
[21] Itzhake R. F., Gill D. M. (1964), Anal. Biochem, **9**, 401–410.
[22] Krajewski T., Cierniewski C. S., Wrona U. (1981), IRCS. J. Med. Sci., **9**, 806–807.
[23] Krajewski T., Nowak P., Cierniewski C. (1980), Biochem Biophys. Acta, **622**, 94–104.
[24] Krajewski T., Nowak P., Cierniewski C. S. (1985), Acta Biochim. Pol., **32**, 145–154.
[25] Kudryk B., Collen D., Woods K. R., Blomback B. (1974), J. Biol. Chem., 429, 3322–3325.
[26] Larrieu M. J., Dray L., Ardaillou N. (1972), Br. J. Haematol., **22**, 719–733.
[27] Larrieu M. J., Dray L., Ardaillou N. (1975), Thromb. Diathes. Haemorrh., 34, 686–692.
[28] Marder V. J., Budzynski A. Z. (1974), Prog. Hemostasis Thromb., **2**, 141–174.

[29] Medved L. V., Litvinovich S. V., Ugarova T. P., Lukinova N. I., Kalikhevich V. N., Ardemasova Z. A. (1993), FEBS. Lett., **320**, 239–242.
[30] Mosesson M. W. (1990), J. Lab. Clin. Med., **116**, 8–17.
[31] Olexa S. A., Budzynski A. Z. (1980), Proc. Natl. Acad. Sci., USA, **77**, 1374–1378.
[32] Olexa S. A., Budzynski A. Z. (1981), J. Biol. Chem., **256**, 3544–3549.
[33] Pan Y., Doolittle R. F. (1992), Proc. Natl. Acad. Sci. USA, **89**, 2066–2070.
[34] Pisano J. J., Finlayson J. S., Peyton M. P. (1968), „Science", **160**, 892–893.
[35] Pizzo S. V., Schwartz M. L., Hill R. L., McKee P. (1972), J. Biol. Chem., **247** 636–645.
[36] Rupp C., Sievi R., Furlan M. (1982), Thromb. Res., **27**, 117–121.
[37] Ugarova T. P., Budzynski A. Z. (1992). J. Biol. Chem., **267**, 13687–13693.
[38] Van Ruijven-Vermees J. A. M., Nieuwenhuizen W., Haverkate F., Timan G. (1979), Hoppe-Seyler's Z. Physiol. Vhem., **360**, 633–637.
[39] Varadi A., Scheraga H. A. (1986), „Biochemistry", **25**, 519–528.
[40] Wangh L. J., Holland L. J., Spolski R. J., Aprison B. S., Weisel J. W. (1983), J. Biol. Chem., **258**, 4599–4605.
[41] Weber K., Osborn M. (1969), J. Biol. Chem., **244**, 4406–4410.

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## FIBRYNOGEN KARPIA I JEGO KOŃCOWE PRODUKTY PLAZMINOWEJ DEGRADACJI

W pracy opisano sposób izolowania fibrynogenu karpia (Cyprinus carpio) oraz produktów jego plazminowej degradacji, a także dokonano charakterystyki tych białek. Stwierdzono, że podobnie jak u innych gatunków kręgowców, fibrynogen karpia składa się z trzech par nieidentycznych łańcuchów polipeptydowych, Aα, Bβ i γ. W przeciwieństwie jednak do fibrynogenu ssaków, łańcuch Bβ fibrynogenu karpia posiada wyższą masę cząsteczkową niż łańcuch Aα. Stosunkowo duży rozmiar łańcucha Bβ fibrynogenu karpia wynika z wysokiej masy cząsteczkowej $NH_2$-końcowego fibrynopeptydu B, odłączanego z fibrynogenu przez trombinę. Tak jak u ssaków, trawienie fibrynogenu karpia plazminą prowadzi do otrzymania dwóch głównych końcowych produktów degradacji: fragmentów D i E. Wykazano, że fragment D ($D_1$) hamuje polimeryzację monomerów fibryny nie tylko w układzie homo-, ale także w heterologicznym.