ances arise in physically untrained animals. Repetition of stress with intensive physical exertion in physically untrained animals is accompanied by exhaustion of the adrenal cortex against the background of persistent and marked hyperlipemia and can be regarded as an atherogenic factor [8]. It will be clear that the use of physical activity as a means of preventing atherosclerosis demands exact criteria of the degree of change of the individual. The use of considerable physical exertion after stress not only restores normal indices of lipid metabolism, but also considerably enhances the hyperlipemia in physically untrained individuals.

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PROTEOLYSIS OF PROTHROMBIN BY β/γ-THROMBIN

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KEY WORDS: α- and β/γ-thrombins; prothrombin; proteolysis

The principal function of the serine proteinase of thrombin is specific cleavage of four bonds between Arg-Gly amino acid residues in the fibrinogen molecule, which triggers the process of fibrin formation. Thrombin also catalyzes proteolysis of various secondary substrates. Prothrombin is hydrolyzed by thrombin at the Arg^{156}-Ser^{157} bond with the formation of prethrombin 1 and fragment I of prothrombin [6, 9, 11]. Prethrombin 1, which has no calcium binding sites, which are located in fragment I, cannot be effectively converted into thrombin and may accumulate in the blood, thus producing self-regulation of thrombin formation [9, 10]. The existence of numerous forms of thrombin, arising through partial proteolysis or autolysis of the enzyme, has recently been demonstrated [2, 5, 8, 11]. Conversion of α-thrombin into the β- and γ-forms is accompanied by loss of coagulating, but preservation of esterase, activity. Inclusion of the γ-form into the stage of fibrin stabilization through activation of factor XIII has been demonstrated [7]. There is no evidence that low-molecular-weight forms of thrombin participate in the regulation of thrombin formation. The object of this investigation was to study the action of β/γ-thrombin on prothrombin, and it was found that proteolytic

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TABLE 1. Characteristics of Preparations of α- and β/γ-Thrombins

Preparation	Activity			
	esterase, μmoles BAME/mg/min	amidase, as BPVA-NA, $\Delta A \cdot 10^4$/mg·min	Prothrombinolytic, μmoles/mg, x clotting, min, NIH units/mg	
α-thrombin	6,8	28	0,11	2500
β/γ-thrombin	7,5	7,6	0,005	0—0,5

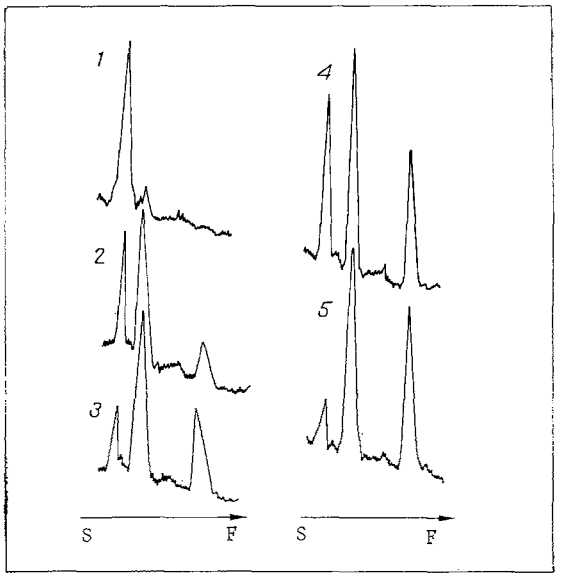


Fig. 1. Electrophoresis of prothrombin (1) and products of its 60-min (2) and 120-min (3) hydrolysis by β/γ-thrombin and 10-min (4) and 30-min (5) hydrolysis by α-thrombin. Here and in experiments illustrated in Fig. 2, the reaction was carried out in 0.05 M Tris-HCl buffer, containing 0.145 M NaCl at 37°C; prothrombin concentration 0.47 mg/ml, activity 1200 NIH units/mg, activity of β/γ- and α-thrombin 0.2 and 0.02 μmole BAME/ml · min, respectively; S) start, F) finish.

activity is preserved, whereas clotting activity is almost completely absent.

EXPERIMENTAL METHOD

Prothrombin with activity of 1200 NIH units/mg protein, uncontaminated with factor X, was obtained by the method in [1]. The β/γ-thrombin was obtained from α-thrombin, purified on SP-Sephadex C-50 by the method in [2], by limited proteolysis with trypsin immobilized by ourselves on activated CH-Sepharose 4B. Immobilization was carried out in 0.05 M borate buffer, pH 8.0, for 12 h at 4°C. The trypsin-Sepharose had activity of 170 μmoles (BAME)/min/g Sepharose. Esterase activity was determined by the method described in [13] based on hydrolysis of BAME. Amidase activity was determined relative to N-benzoyl-phenylalanyl-valyl-arginine p-nitroanilide (BPVA-NA) [12]. The molecular weight (mol. wt.) was determined by electrophoresis [4] in 8% polyacrylamide gel in the presence of sodium dodecylsulfate (SDS).

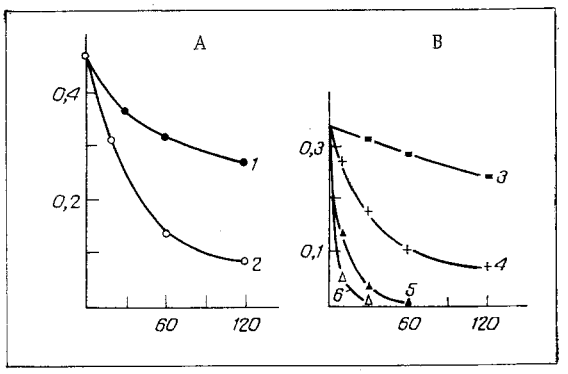


Fig. 2. Kinetics of hydrolysis of prothrombin by β/γ-thrombin (A) in concentrations of 0.05 (1) and 0.2 (2) μmole BAME/ml · min and by α-thrombin (B) in concentrations of 0.002 (3), 0.008 (4), 0.02 (5), and 0.08 (6) μmole BAME/ml · min. Abscissa, incubation time of reaction mixture (in min); ordinate, prothrombin concentration (in mg/ml).

EXPERIMENTAL RESULTS

Electrophoretic analysis showed that the β/γ-thrombin preparations were uncontaminated with α-thrombin and contained 25% of β-thrombin with mol. wt. 28,000 ± 1000 and 75% γ-thrombin, represented by fragments with mol. wt. 16,000 ± 500 and 13,000 ± 600. Removal of peptides from α-thrombin during its conversion into the β/γ-form was accompanied not only by preservation, or even an increase, of esterase activity, but also by the almost total loss of coagulating activity (Table 1), evidently as a result of disturbance of the conformation of the substrate-binding site of the active center [3]. Meanwhile, β/γ-thrombin exhibited fairly high amidase activity toward the highly specific substrate of α-thrombin, BPVA-NA. The ability of β/γ-thrombin, virtually without clotting activity, to attack the prothrombin molecule was investigated. The results of electrophoresis of prothrombin, incubated with β/γ- and α-thrombins, are given in Fig. 1. A decrease in the concentration of the main component, corresponding in molecular weight to prothrombin (mol. wt. 76,000 ± 2000) during hydrolysis by β/γ-thrombin was accompanied by accumulation of only two degradation products in the reaction mixture, identified as prethrombin 1 (mol. wt. 58,000 ± 2000) and fragment I of prothrombin (mol. wt. 25,000 ± 2000), identical with the products of proteolysis of prothrombin by α-thrombin. Evidently, β/γ-thrombin, like α-thrombin, ruptures one bond (Arg^{156}-Ser^{157}) in the prothrombin molecule. Evidence of removal of fragment I of prothrombin by β/γ-thrombin is given by the results of analysis of the biological activity of the prothrombin degradation products. The digest exhibited high antithrombin and anti-activator activity, reducing the activity of thrombin generated from prothrombin in the two-stage prothrombin analysis test [14], by 56%. The kinetics of hydrolysis of prothrombin by different concentrations of enzymes is illustrated in Fig. 2. With β/γ-thrombin and prothrombin present in molar proportions of 1:10, hydrolysis of 50% of the substrate took place within 30 min (Fig. 2A); proteolysis with α-thrombin took place at the same reaction velocity if the molar proportions were 1:300. Hydrolysis of prothrombin by β/γ-thrombin is due to the proteolytic activity of the enzyme itself and not to contamination with α-thrombin, for addition of α-thrombin with activity equal to the residual coagulating activity of β/γ-thrombin to the prothrombin did not induce proteolysis. The two forms were not equally specific toward prothrombin. The values of the prothrombinolytic activity of β/γ- and α-thrombin are given in Table 1. Dependence of the velocity of hydrolysis of prothrombin on enzyme concentration is illustrated in Fig. 3. The α-form was more specific than the β/γ-form; the latter preserved about 5% of the prothrombinolytic activity of α-thrombin, 27% of amidase activity, and only about 0.02% of its fibrinogen-coagulating activity (Table 1), i.e., the difference between the specificities of β/γ- and α-thrombins toward prothrombin is much less (250 times) than the difference in the specificity of these enzymes toward fibrinogen.

It can be concluded from these results and data in the literature [7] on the ability of α- and β/γ-thrombins to activate factor XIII, that the absence of coagulating activity of β/γ-thrombin is due to a change in specificity of the enzyme on conversion into the β/γ-form and not to a loss of its proteolytic properties.

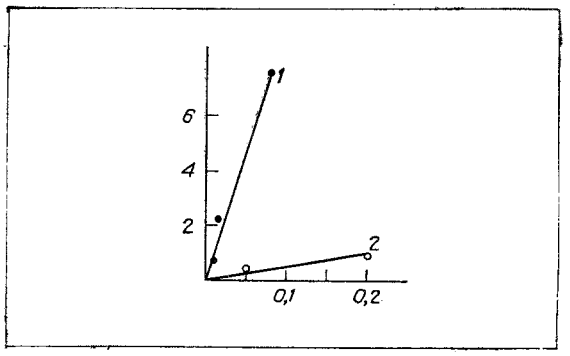
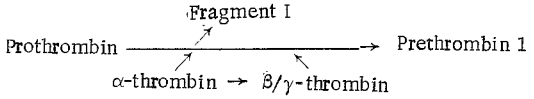


Fig. 3. Velocity of hydrolysis of prothrombin as a function of concentration of α-thrombin (1) and β/γ-thrombin (2). Abscissa, enzyme concentration (in μmoles BAME/ml · min); ordinate, velocity of hydrolysis of prothrombin, in μmoles/min $\times 10^4$.

Because of the discovery that β/γ-thrombin has proteolytic activity, corrections can be introduced into the scheme representing proteolysis of prothrombin:



Although β/γ-thrombin has no fibrin-coagulating activity, it thus preserves its regulating function, for degradation of prothrombin to prethrombin 1 prevents its physiological activation, and this in turn prevents the formation of excessive quantities of thrombin.

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