Colorimetric Determination of Three Forms of Plasma Kallikrein and Its Adsorption on Kaolin

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The proposed method allows one to determine the nature and degree of disturbances of kininogenesis and to assess conformational changes in the kallikrein structure in therapeutic (infectious and chronic hepatitis in children) and surgical (osteomyelitis and peritonitis) pathologies.

Key Words: *kallikrein; colorimetry; microamount of plasma; pathology*

The functional state of blood kallikrein-kinin system (KKS) is usually assessed by measuring the activities of kallikrein and prekallikrein, i.e., a kinin-forming enzyme and its precursor, using the esterase method [12] with some modifications [1,2] and tube-chromatographic method [6]. In brief, 0.02-0.08 ml of native plasma (serum) is incubated at 25 or 37°C for 15-60 min in the presence of 5-10 μmol N-benzoyl-L-arginine ethyl ester (BAEE) or 40-53 μmol N-tosyl-L-arginine methyl ester (TAME) per one milliliter of the incubation mixture. The enzyme activity is evaluated from the single final point and expressed in various non-SI units: μmol/ml/h, mU/ml, μU/ml, mmol/h, etc. [4,6-8].

Analysis of the published data on the state of the KKS in various pathologies in adults and children revealed some discrepancies and contradictions in prerequisites, interpretations, and conclusions [4-7,10].

The aim of the present study was to modify the colorimetric determination of various forms of plasma kallikrein to define the nature of the kininogenesis disturbances occurring during therapy of some diseases and during the postoperation period.

MATERIALS AND METHODS

The BAEE (TAME)-esterase reaction was optimized *in vitro*, which was necessary for the determination of specific kallikrein activity but not of the total activity of serine proteases occurring in native plasma. Plasma volume, incubation time, and the amount of activator (kaolin) were chosen so that to determine the enzyme activity from the linear fragment of kinetic curve. The esterase activity of native plasma as well as the esterase and kininogenase activities of heat-inactivated plasma (61°C, pH 3) containing no serine proteases except for kallikrein and prekallikrein [9] were assayed.

Blood plasma from 20 healthy individuals, 94 donors, and 149 patients of therapeutic and surgery departments was used.

Kallikrein activity was evaluated by a decrease in BAEE (TAME) after incubation with the enzyme. The BAEE (TAME) level was measured by the method described elsewhere [13] with some modifications [11,14]. These modifications have been used for the determination of TAME-esterase plasmin activity [14], BAEE-esterase prekallikrein and kallikrein inhibitor activity [2,12], serum BAEE-esterase activity [11], and coagulation factors XI and XII [3].

BAEE×HCl, TAME×HCl, and bradykinin triacetate (Reanal) and Russian-manufactured tris(hydroxymethyl)aminomethane and kaolin were used.

RESULTS

In vitro experiments showed that kallikrein and prekallikrein of the heat-inactivated blood plasma (serum) (61°C, pH 3, 18-20 min) exhibit the same order of

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BAEE (TAME)-activity as the whole proteinase complex (thrombin, plasmin, trypsin, and kallikrein) of native plasma. Experiments with plasma from 20 healthy individuals showed that biochemical reaction between BAEE (TAME) and native plasma proceeding in a volume of 0.017-0.08 ml (the aliquot usually used by others) and single-point analysis after a 20-30 min incubation with the optimal quantity of the substrate (16.6 μmol BAEE/ml mixture) yield ambiguous results which depend on the time of the enzyme incubation with the substrate. The greater the sample volume and the longer incubation time, the lower proenzyme activity. For instance, prekallikrein activity measured after a 1-min incubation of 0.08, 0.033, 0.0016, 0.0007, and 0.00035 ml plasma/ml mixture at 37°C was 300±26, 625±58, 8105±690, 30,967±2116, and 52,353±4284 mmol/sec×liter, respectively, while after a 20-min incubation these values were 25±2.1, 365±21.5, 1145±98.6, 1875±162.4, and 4375±287 mmol/sec×liter. Similar relationships were noted when heat-inactivated plasma samples from 26 donors were incubated with BAEE: the kallikrein activity was 4000±200, 15,000±7500, 44,000±2200, 60,000±3400, and 300,000±16,000 μmol/ml×h for aliquots of 3×10^{-4}, 1.5×10^{-4}, 0.75×10^{-4}, 3×10^{-5}, 3×10^{-6} ml plasma/ml incubation mixture.

Our findings suggest that a 20-30-min incubation of 0.003-0.08-ml plasma aliquots in one milliliter of incubation mixture yields kinetic curves (Fig. 1) from which the intrinsic enzyme activity cannot be calculated, since their linear fragments lay within a 5-sec initial interval. We measured the BAEE-esterase activity of prekallikrein and kallikrein in intact and heat-inactivated plasma samples obtained from 36 patients with gastric and duodenal ulcers, 26 children with protracted viral hepatitis A, 15 children with chronic active hepatitis, and 37 workers with occupational exposure to dust (employment more than 20 years). We failed to establish any correlation between esterase and kininogenase activities when plasma aliquots of 0.06-0.08 ml/ml incubation mixture were used; consequently, the nature of the kininogenesis disturbances (enhanced or weakened) remains unclear. In patients with protracted viral hepatitis A and gastric and duodenal ulcer, the same values of TAME-esterase activity corresponded to both enhanced (total kallikrein activity >492 pmol/sec×liter) and weakened (<246 pmol/sec×liter) kininogenesis. It was impossible to pinpoint pathogenetic alterations occurring during the treatment of chronic active hepatitis and estimate the effectiveness of pyrogenal therapy on the basis of the BAEE esterase activity of three forms of kallikrein in heat-inactivated plasma. Thus, our findings suggest that the determination of the BAEE-esterase activity of plasma

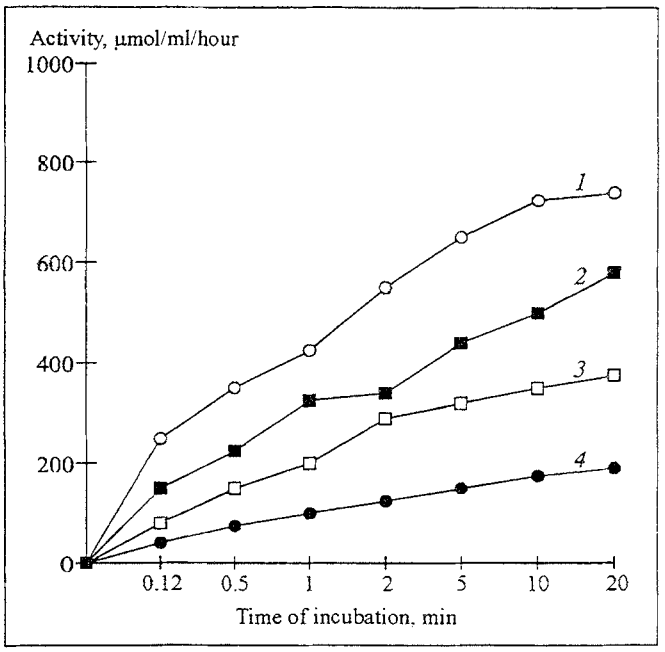

Fig. 1. BAEE-esterase activity of total kallikrein for different plasma amounts during incubation at 37°C. *1*) 0.003 ml; *2*) 0.008 ml; *3*) 0.016 ml; *4*) 0.08 ml.

kallikrein ignoring the kinetics of the enzyme reaction does not reflect the intrinsic kallikrein activity and the state of kininogenesis.

Using plasma samples from 68 donors, we have chosen the optimal quantity of plasma and kaolin which converts prekallikrein into active enzyme. This allowed us to measure the maximum BAEE-esterase activity corresponding to the linear segment of the kinetic curve and reflecting direct dependence between the enzyme activity and incubation time. It was found that without kaolin the plasma BAEE-esterase activity was detected at plasma concentrations corresponding to 3×10^{-9} ml/ml mixture and reached the maximum at 1.5×10^{-9} ml/ml mixture. After the addition of kaolin, the optimal plasma aliquot for the determination of the maximum kallikrein activity was a microamount corresponding to 0.75×10^{-8} ml/ml mixture. A 2-min incubation of such aliquots (n=20) with a saturating concentration of the substrate (16.6 μmol BAEE/ml mixture) at 37°C provided direct dependence between the rate of substrate hydrolysis and plasma kallikrein activity. The optimal kaolin concentration of prekallikrein activation was 0.05 μg/ml mixture, while at higher concentrations (0.1, 0.2, and 0.4 μg/ml mixture) the kininogenase activity of kaolin was lower (by 20, 38, and 75%, respectively) due to adsorption of kallikrein onto kaolin.

Based on these findings, we propose the following modification of the plasma kallikrein colorimetric assay. Physiological saline (0.2 ml) and 1 N HCl (0.05 ml, pH 3) are added to native plasma (0.2

TABLE 1. Changes in BAEE-Esterase Activity of Three Forms of Plasma Kallikrein in Patients with Peritonitis During Laser Therapy (*M±m*)

Time	Kallikrein, mol/sec×liter			Adsorption kallikrein on kaolin, %
	total	inhibitor-bound	prekallikrein	
Before therapy	11000.05±980.0	4000.02±284.0	7000.04±640.0	67
After laser therapy:				
five sessions	17400.09±1236.0	9000.04±780.0	8400.04±680.0	40
ten sessions	21000.10±1670.0	12660.06±820.0	8400.04±710.0	21
Healthy donors	24000.12±1200.10	6000.03±360.03	18000.09±900.06	20

ml). The mixture is heated to 61°C for 18-20 min, cooled, neutralized with 1 N NaOH (0.04 ml), and adjusted to 1 ml with Tris-HCl (pH 7.6-7.8, 0.55 ml). Plasma is diluted with physiological saline to a concentration of 0.25×10^{-8} ml/0.1 ml (7 serial dilutions). Three equal aliquots of this plasma are transferred to separate test tubes: the first test tube contains 0.45 ml Tris-HCl, the second test tube contains 0.3 ml Tris-HCl and 0.15 ml kaolin (0.016 μg in 0.05 ml Tris-HCl), and the third test tube contains 0.3 ml Tris-HCl and 0.15 ml kaolin (0.032 μg in 0.05 ml Tris-HCl). Kaolin (4 mg) is diluted with 1 ml Tris-HCl, and the necessary concentrations are prepared by 5 or 6 serial dilutions. The tubes are incubated in a water bath at 37°C for 2 min, and 0.15 ml BAEE solution (5 μmol/0.05 ml Tris-HCl buffer) is then added. Two 0.3-ml aliquots of the incubation mixture are taken precisely after 30 and 60 sec (using a stop-watch) and transferred to test tubes containing 0.3 ml 3.5 N NaOH and 0.3 ml 2 M hydroxylamine hydrochloride. The tubes are left at room temperature for 25 min, after which 6% trichloroacetic acid in 4 N HCl (0.3 ml) is added. After 5 or 10 min, a 0.96-ml aliquot is withdrawn from the reaction mixture (total volume 1.2 ml), and 1.8% $FeCl_3$ in 0.04 N HCl is added (4 ml). A yellow-brown coloration develops within a 20-min period. The intensity of this coloration is measured in a photoelectrocolorimeter at 260 nm against the reagent-containing control [1]. Two controls are processed in parallel with the samples: the first control contains 0.3 ml Tris-HCl and 0.6 ml of the mixture consisting of 2 M hydroxylamine hydrochloride (0.3 ml) and 3.5 N NaOH (0.3 ml) and the second control consists of 0.25 ml Tris-HCl, 0.05 ml BAEE (5 μmol) and 0.6 ml of the analogous hydroxylamine alkaline mixture.

The difference in light absorbance between the second control and experimental sample (E_{540}/min) is calculated taking into account the kinetics of the enzyme reaction (30- and 60-sec samples). The amount of BAEE hydrolyzed by kaolin-containing and kaolin-free plasma samples is calculated using a calibration curve constructed within the BAEE concentration range of 0-10 μmol. The results are expressed in SI units using a plasma dilution coefficient (under the chosen conditions it was equal to 6666.7). This method can be used for the determination of the activity of three kallikrein forms: inhibitor-bound kallikrein (1 — kaolin-free samples), total kallikrein (2 — samples containing 0.016 μg kaolin), and prekallikrein (3 — the difference between 2 and 1), and adsorption of kallikrein on kaolin [4 — the difference between samples containing 0.016 μg (100%) and 0.032 mg, %]. The latter parameter may serve as an additional criterion characterizing conformation changes in kallikrein molecules. Thus, determination of the four parameters of plasma kallikrein activity requires 6 test tubes with 0.75×10^{-8} ml plasma/ml incubation mixture. In the norm, parameters 1, 2, and 3 are equal to 6000.03±360.03, 24000.12±1200.10, and 18000.09± 9006.06 mol/sec×liter, respectively, and parameter 4 (absorption on kaolin) varies within 15-20%. In native plasma, these forms of kallikrein activity are lower by 33.3, 16.7, and 11.1%, respectively.

The proposed modification of plasma kallikrein colorimetric assay correlated with the previously developed kininogenase method [9] and allowed us to establish the nature of the disturbances of kininogenesis (enhanced or weakened) in pathology. This method proved a positive therapeutic effect of dimephosphone in protracted viral hepatitis A in 29 children and normalizing effect of laser therapy on kallikrein activity in peritonitis and chronic osteomyelitis accompanied by suppressed kininogenesis in 12 children (Table 1). Normalization of kallikrein adsorption by kaolin implies a beneficial effect of laser therapy. The use of native plasma in these pathologies was ineffective.

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