AN INVESTIGATION OF THE KALLIKREINS AND THE KININASES AND PROTEINS BOUND WITH THEM IN THE VENOMS OF CENTRAL ASIAN SNAKES

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The activities of the kallikreins and the kininases and the peptides bound with them in the venoms of Central Asian snakes have been studied. It has been found that when the whole venoms are used an interference of the enzymes with one another and also with potentiating or inhibiting peptides takes place. Bradykinin-potentiating activity the nature of which is connected with an inhibiting action on kininase II has been found in the low-molecular-mass fractions of cobra and viper venoms. The inhibiting activities of the venoms have been characterized in relation to the angiotension-converting enzyme from bovine kidneys and also purified dipeptidyl carboxypeptidase from karakurt (black widow) venom.

The kallikrein-kinin system (KKS), which includes biologically active kinin peptides and kinin-forming and kinin-degrading enzymes, plays an important role in physiological regulation [1]. Substances structurally and functionally similar to the components of the KKS have been detected in many zootoxins, including snake venoms [2]. Snake venoms may contain all the components of the KKS or only some of them; together with inhibitors of other proteinases, the venoms contain peptides suppressing the activity of the kallikreins and kininases [3]. Each venom has a strictly determined combination of all these interacting factors the investigation of which is important both for characterizing the venom itself and in connection with its possible use. For this reason, the appropriate analysis of the venoms of the Central Asian snakes V. lebetina turanica C. and E. multisquamatus Ch. (Vipera and Echis genera, Viperidae family), Ag. halys halys Schn. (Agkistrodon genus, Crotalidae family), and N. oxiana Eichw. (Naja genus, Elipadae family) is an independent problem for the solution of which, in addition to performing biotests factually evaluating myotropic activity, we have investigated the hydrolysis of various protein and nonprotein substrates. The results obtained are summarized in Tables 1 and 2.

The amount of kininogenase (kallikrein) is usually judged from the capacity of the material under investigation for hydrolyzing BAEE [4] and some specific chromogenic substrates, and also from the myotropic activity of the kinin peptides formed by the enzyme from the precursor – kininogen [5]. Neither of these methods separately gives sufficiently complete and, consequently, reliable information on the kininogenase activity of the material, but a comparison of the results of investigation performed with the aid of different methods and approaches permits more unambiguous conclusions to be drawn (Table 1).

The BAA esterase activities in the whole venoms were somewhat higher than in the "unabsorbed fractions" obtained from them. It is known that kallikreins can exist in the form of precursors – kallikreinogens – which, after appropriate activation by proteinases, together with kallikreins, hydrolyze BAEE [6]. Therefore it may be assumed that the venoms investigated contain, in addition to kininogenases, a prekallikreian and other proteinases (Table 2) capable, together with kallicrein and its precursor, of hydrolyzing BAEE. Only the E. multisquamatus venom is an exception in this respect.

The hydrolysis of specific chromogenic substrates describes the kininogenic activity of the venoms more reliably, but here it must be borne in mind that the specificity of the substrates is not absolute: they are cleaved not only by kininogenases but also by other proteinases.

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TABLE 1. Kininase Activities of the Venoms of Central Asian Snakes

Venom	Enzymatic activity (units/mg) in the hydrolysis of				
	BAEE		chromogenic subsrates		kininogenic (myotropic activity of the kinins formed)
	whole venom	unabsorbed fraction	Chromozym-PK	S-2302	
V. Lebetina turanica	17,75	6,01	—	20,70	0 0076
E. multisquamatus	3.40	5.46	8,90	9,15	0,0066
Ag. halys halys	5,46	4,81	—	7,96	0,0065
N. oxiana	4,90	4,73	—	4,02	0,0053

TABLE 2. Proteolytic Activities of the Venoms of Central Asian Snakes

Venom	Enzymatic activity (units/mg of proteins) in the hydrolysis of			
	casein	hemoglobin	hippuryl-L-arginine	bradykinin (myotropic activity) preincubated with the venon
V. Lebetina	5,60	0,60	0,29	$13,488\times10^{-3}$
E. multisquamata	4,60	0,20	0,27	—*
Ag. halys halys	3,50	0,40	0,56	$8,920\times10^{-3}$
N. oxiana	1,10	0,60	0,55	—*

*Absence of kininase II activity. A bradykinin potentiating effect was observed.

It is still more difficult to comment on the result of a biotest, since it is necessary to take into account the variability of the organ used in the biotest (the neck of the rat uterus), the nonstandard nature of the kininogen fraction obtained independently from blood serum, and the presence in the venom under investigation of a kininogen-decomposing enzyme that therefore interferes with kallikrein, and also of substances causing the contraction of a smooth muscle independently of kallikrein.

Summarizing all three approaches, it may be concluded that the venoms investigated possess kininogenase activity and differ with respect to it in the following decreasing sequence: V. lebetina turanica – E. multisquamatus – Ag. halys halys – N. oxiana. The lack of agreement of the results obtained by the use of the individual methods for tests is explained by their inadequate suitability for the problems to be solved and the complex composition of the materials investigated – snake venoms.

In this connection, it is interesting to consider the myotropic activities of snake venoms which independently provide amines causing rapid contraction and slow-contracting kinins; membrane-active cytolytics and phospholipases A_2 may lead to the appearance in the incubation medium of other contractile agents. The nature of action of the venoms on the isolated neck of the rat uterus and the guinea-pig ileum corresponds to the complex mosaic of contactile factors in the venoms: a considerable similarity to the kinins (slow effect) is observed but, in contrast to these, spontaneous muscular contractions subsequently develop the nature of which has not yet been established. The venoms investigated differed from one another with respect to the efficiency of their action: thus, the V. lebetina turanica and E. multisquamatus venoms had a contractile action in a dose of 100 μg, while for the Ag. halys halys and N. oxiana venoms the dose was 200 μg. In biotests connected with evaluating the activity of the kininases, the venoms were used in substantially lower concentrations, which excluded their independent contractile effect.

The results of our investigations, including an analysis of the hydrolytic action of the venoms on the protein-peptide substrates are summarized in Table 2. Of the results presented, only the efficiency of the hydrolysis of hippuryl-arginine and the residual myotropic activity of the bradykinin formed by the venoms can demonstrate the level of kininases which, as is well known, are carboxypeptidase N and dipeptidyl carboxypeptidase; the degree of hydrolysis of casein and hemaglobin illustrate the activity of the endopeptases to a greater extent, and these include, among other substances, kallikrein.

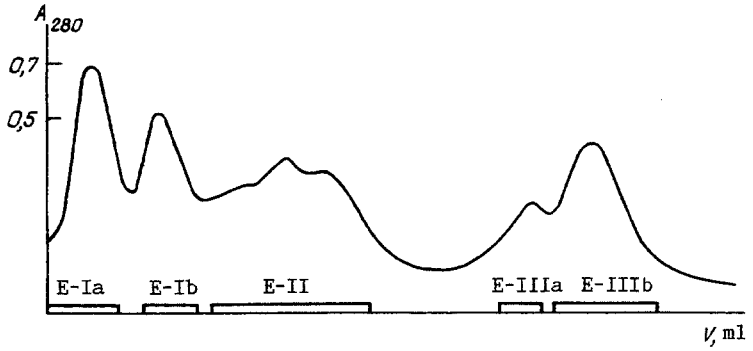

Fig. 1

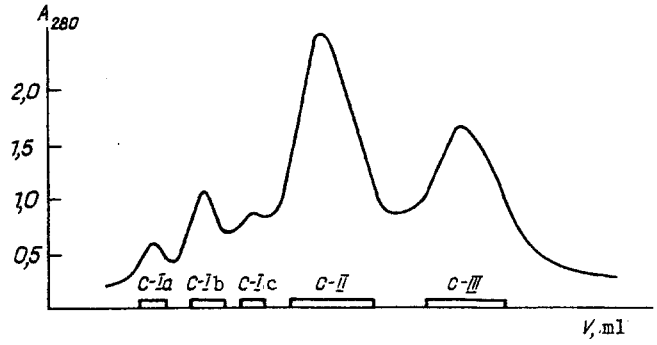

Fig. 2

Fig. 1. Graph of the gel filtration of E. multisquamatus venom on Sephadex G-75. Column 2.5 × 180 cm equilibrated with ammonium acetate (0.05 M, pH 7.9) elution with the same solution at the rate of 30 ml/h: E-I—E-III— fractions of the protein collected preparatively.

Fig. 2. Graph of the gel filtration of N. oxiana venom on Sephadex G-75. Chromatography in 0.005 M ammonium acetate, pH 7.9. Along the axis of abscissa are shown the protein fractions collected preparatively.

In a comparison of the results of the hydrolytic action of the venoms on various protein-peptide substrates, it was impossible to detect correlations with respect to the substrates, which showed the presence in the venoms of a multiplicity of proteinases with different specificities. This also relates to the carboxypeptidases, since the results on the residual myotropic activity of the bradykinin and the hydrolysis of hippuryl-arginine likewise do not correlate in almost all cases. The absence of correlations may also be connected with, in addition to the multiplicity of enzymes — endopeptidases and carboxypeptidases, the influence of such regulating factors as peptide inhibitors of these enzymes. Thus, in an analysis of the N. oxiana and E. multisquamatus venoms it was not a suppression but a stimulation of the myotropic effect of bradykinin (bradykinin-potentiating action) that was observed, which may be connected with the suppression of a bradykinin-decomposing enzyme (kininase II). Such peptide inhibitors of kininases possessing a bradykin-potentiating action have previously been described in venoms [7]. Below, we give our results characterizing the corresponding components of the E. multisquamatus and N. oxiana venoms.

When whole E. multisquamatus venom was separated on Sephadex G-75, it was possible to obtain the low-molecular-mass components in the form of two fractions, E-IIIa and E-IIIb (Fig. 1). By electrophoresis, these fractions were found to contain peptides with molecular masses between 1 and 8 kDa. Testing the fractions obtained on preparations of rat uterus neck and guinea pig ileum showed that, while they did not possess an intrinsic spasmogenic effect, they enhanced the action of bradykinin, i.e., they contained bradykinin-potentiating peptides. The specificity of the bradykinin-potentiating action of the fractions was confirmed by the fact that they did not affect the spasmogenic effect of other contractile sub-

stances: acetylcholine, histamine, serotonin. The mechanism of the bradykinin-potentiating action of the fractions is connected with the suppression of a bradykinin-decomposing kininase; thus, in suppression experiments it was possible to show that in concentrations of 4.0 and 2.5 μg/ml, respectively, they caused a 50% suppression of the activity of the angiotensin-converting enzyme from bovine kidney and of purified kininase (dipeptidyl carboxypeptidase) from karakut (black widow) venom.

The analogous fractionation of N. oxiana venom on Sephadex G-75 permitted us to obtain a fraction of low-molecular-mass components containing, in addition to amino acids, a considerable amount of peptides with molecular masses of 1-5 kDa. In the functional respect, this fraction was analogous to the low-molecular-mass fraction III from E. multisquamatus venom.

EXPERIMENTAL

Venoms of Agkistron halys halys Schn., Echis multisquamata, Naja oxiana Eichw., and Vipera lebetina turanica C. obtained from the Central Asizn Zonal Uzbek Zoocombine and dried over calcium chloride were used.

The proteolytic activities of the whole venom were determined with the aid of the methods of Kunitz and Anson, respectively, in the hydrolysis of casein and hemoglobin [8].

BAEE-Esterase activity was determined by a method described in [9], using N-benzoyl-L-arginine ethyl ester as substrate.

The kininogenase activities of the venoms were investigated by independent methods for the amounts of kallikrein and kallikreinogen. To distinguish the total esterase activity and the activity of the kallikreins, their amounts were determined in the "nonadsorbed" fractions of the venoms. The nonadsorbed fractions were obtained by chromatographing the venoms on a column of DEAE-Sephadex A-50 under conditions of low ionic strength [6].

The amount of kallikrein was determined from its BAEE-esterase activity [4] and also by a biological method [5]. The direct determination of the kallikrein activities in the venoms was made with the use of the chromogenic substrates Chromozym; and S-2302 according to the recommendations of the manufacturers (Boehringer Mannheim GmbH, FRG, and Kabi Diagnostika, (Sweden).

Amounts of kallikreinogen were determined in the nonadsorbed fractions of the venoms after its conversion into active kallikrein [4].

The activities of the kininase I (carboxypeptidase N) in the whole venoms were determined from the cleavage of hippuryl-L-arginine with the formation of hippuric acid, which has a high molar extinction coefficient at 254 nm [10].

The activities of the kininases were determined by a biological method [9] in which the residual activity of bradykinin after its incubation with the venom was measured. In this determination we used amounts of venoms 3-4 orders of magnitudes smaller than those at which their intrinsic myotropic activities were exhibited.

The myotropic activities of the venoms were determined from their capacity for causing the contraction of the isolated neck of the rat uterus that had previously been simulated with diethylstilbestrol, with bradykinin as standard [1]. Myotropic activities were measured in cooperation with Z. Golubenko and A. A. Akhunov in the Institute of Bioorganic Chemistry, Uzbekistan Academy of Sciences (Tashkent).

The bradykinin-potentiating activities of the venoms and their fractions were investigated from the capacity of enhancing the contractile effect of kinins [3], which was characterized by the dose (μg) at which the myotropic action was doubled.

The inhibiting actions of the low-molecular-mass fractions of the venoms were evaluated from their capacity for blocking the activity of kinase II [carboxycathespin from bovine kidney and of dipeptidyl-carboxypeptidase from karakurt (black widow) venom] [9, 11].

The fractionation of the venoms of E. multisquamatus and N. oxiana was carried out on Sephadex G-75 columns (2.5 × 180 cm) in 0.05 M ammonium acetate buffer, pH 7.9.

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SOLID-PHASE SYNTHESIS OF PEPTIDES ON THE POLYMERIC SUPPORT TRILAR®

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The solid-phase synthesis of peptides has been achieved using the polymeric support TRILAR® proposed by the Biolar Scientific–Production Association (Olaine).

The improvement of the solid-phase method of synthesizing peptides presupposes the finding of new polymeric supports and suitable anchoring groupings and the possibility of performing the synthesis and splitting off the peptide from the polymer under various conditions.

Recently, polyamide polymeric supports [1], which, unlike cross-linked polymers of the polystyrene type, swell well in both polar and nonpolar solvents, has been used ever more widely in solid-phase peptide synthesis.

In the present work we have studied the applicability for the solid-phase synthesis of peptides of the domestic polymeric support TRILAR® [1]. This consists of a cross-linked polyamide copolymer based on vinylpyrrolidone. The presence of an alkali-labile α-bromopropionyl anchoring group permits N^{α}-protected peptides with a free carboxy group suitable for further synthesis to be obtained under mild conditions.

The α-bromopropionyl group is readily introduced into the amino groups of the polymer by the action of a 1.5-fold excess of the symmetrical anhydride of α-bromopropionic acid at room temperature for 1 h (Table 1). Various analytical tests have shown a high degree of modification of the amino groups in the polymer by bromopropionyl residues. The observed slight excess bromine introduced above the initial amount of amino groups (see Table 1), as shown by the results of elementary analysis for residual bromine (0.05%), does not lead to any complications whatever in the course of further synthesis.

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