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BRADYKININ-POTENTIATING PEPTIDES FROM *Echis multisquamatus* VENOM

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Eight bradykinin-potentiating peptides have been isolated in the pure form from the low-molecular-mass fraction of the venom of the Central Asian viper Echis multisquamatus *Ch. The peptides isolated differed in the efficacy of their bradykin-potentiating activity and their capacity for inhibiting the angiotensin-converting enzyme (ACE, EC 3.4.15.1); however, complete correlation of the two activities was established for only six of them.*

Bradykinin (BK) is a known physiological blood-pressure regulator possessing a depressor activity. Peptides potentiating the depressor activity of BK by suppressing ACE or affecting receptor binding have been detected in snake venoms [1-5]. Interest in bradykin-potentiating peptides (BPPs) is due to the possibility of creating highly effective and specific antihypertensive drugs on the basis of a structural-functional investigation of them.

A bradykin-potentiating (BP) activity interfering with the contractile effect has been found previously in the venom of the Central Asian viper *Echis multisquamatus* Ch. In the present paper, we consider the isolation of individual BPPs from this venom and give the characteristics of some of them. A scheme of the fractionation of the venon with the isolation of individual BPPs and other peptides is given in general form in Fig. 1.

It has been established [7] that by gel chromatography on Sephadex G-50 the whole venom can be separated into five fractions — casein-hydrolyzing and prothrombin-activating proteinases, callicrein, BAEE esterase, and phospholipase A-2 —, while, in addition, hemorrhagic and contractile activities are concentrated in the high-molecular-mass fractions I, Ia, and II and are separated from the low-molecular-mass fractions E-IIa and E-IIIb, in which ACE and BP inhibitors and part of the contractile activity are found. The latter is probably due to contamination with high-molecular-mass components from the fractions mentioned above.

Chromatography on a TSK HW-40F column permitted further purification of the BPPs, and it was finally possible to eliminate traces of other contractile agents; thus, phospholipase A-2 was separated as a component of fraction E-111aa, and the kinin E-IIIa-1 that had been detected was isolated in the individual state (Figs. 1 and 2,B). However, with a slight exception (E-IIIb-2), all the other peptide fractions possessing BP activity remained heterogeneous after the first chromatographic stage on TSK HW-40F. Their separation and purification were carried out by various methods.

We collected fraction E-IIIa-1 as the sum of the individual components represented by three inadequately separated absorption "peaks" at 280 nm (Fig. 2A). Rechromatography on the same sorbent made possible a better separation of these components, which were designated as E-IIIa-1-1, E-IIIa-1-2, and E-IIIa-1-3. They all contained BPPs, and they were purified by chromatography on Butyl-TSK-650M. According to the conditions of adsorption and desorption on this sorbent (elution was conducted with isopropanol), all the peptides separated were hydrophobic. A total of seven of them were obtained: two each from E-IIIa-1-1 and E-IIIa-1-2, and three from E-IIIa-1-3 (see Fig. 1). The results of TLC showed the absolute individuality of the peptides isolated, but only three of them (E-IIIa-1-1-1, E-IIIa-1-2-1, and E-IIIa-1-3-1) possessed a BP action. The peptides of fraction E-IIIa-2 also interacted with Butyl-TSK-650M, which showed their hydrophobicity. Nevertheless, we were unable to achieve a complete separation of the components of this fraction in this way.

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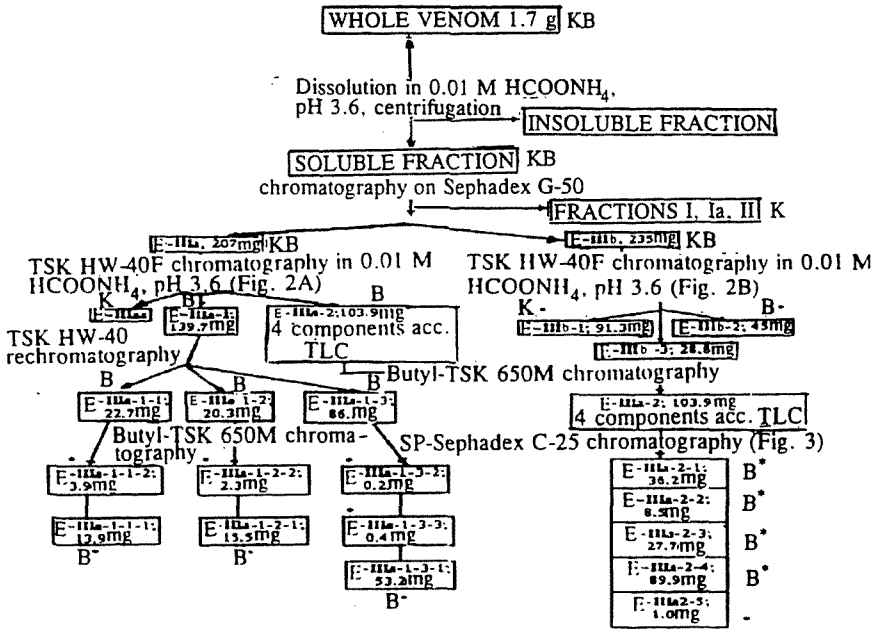

Fig. 1. Scheme of the isolation of bradykinin-potentiating peptides from the venom of the viper *E. multisquamatus*: *K*) contractile activity; *B*) bradykinin-potentiating activity; ≠) ACE inhibitors; *) homogeneity (acc. TLC).

TLC showed the presence of at least four peptides in fraction E-IIIa-2, each with a molecular mass of 1.2 kDa, according to their elution volumes on gel filtration. The further separation of the components of fraction E-IIIa-2 was achieved with the aid of chromatography on SP-Sephadex C-25 (Fig. 3): as a result, we isolated a total of five individual peptides, of which four (E-IIIa-2-1, E-IIIa-2-2, E-IIIa-2-3, and E-IIIa-2-4) possessed BP activity. Peptide E-IIIa-2-5, the last to be desorbed, was characterized, like the other peptides isolated, by complete homogeneity on TLC; however, it has not been investigated further as an ACE inhibitor because of its very low yield.

In ion-exchange chromatography, the first BPP, E-IIIa-2-1, was not adsorbed on a cation-exchange resin at pH 3.6, while all the other peptides were bound to the sorbent fairly strongly, which may indicate that they were highly positively charged. In combination, these results may indicate the presence in the venom of BPPs with a fairly wide range of pI values.

The separation of fraction E-IIIb on TSK HW-40F made it possible to isolate a contractile agent, which proved to be a pure kinin, E-IIIb-1, and also to obtain in the individual state the BPP E-IIIb-2 (Fig. 1). The homogeneity of the latter was shown by TLC; according to its elution volume in gel chromatography, it was a penta- or hexapeptide.

The amount of peptide components in the venom was fairly considerable — about 25% (~12.2% for E-IIIa and ~12.8% for E-IIIb); however, the amounts of the individual peptides varied within wide limits. As a result of the fractionation of the viper venom we obtained in homogeneous form eight BPPs with different physicochemical properties (hydrophobicity, molecular mass, effective charge), in agreement with which their functional properties also varied regularly.

None of the potentiators isolated caused contraction of isolated preparations of smooth muscles in the absence of bradykinin. The potentiating effect of the peptides in relation to BK was specific and was not observed in the presence of other contractile agents: acetylcholine, histamine, and serotonin. In view of the difference in the action of the BPPs on various smooth-muscle preparations [4, 5], we investigated the effects of the peptides isolated in parallel on isolated preparation of rat uterus and guinea pig ileum. The results obtained were compared with those of an investigation of the inhibiting action of the peptides on ACE (Table 1).

The peptides most active in terms of inhibiting effect, E-IIIa-2-3 and E-IIIb-2, exhibited a high efficiency on smooth-muscle preparations and can be compared with peptide P from *Bothrops jararacussu*, which is characterized by an inhibitory activity (IC-50) of 1.4 μg/ml [8]. We also detected peptides inferior to those mentioned above in inhibitory activity but fairly effective on one of the smooth-muscle preparations. A comparison of the results obtained shows the specificity of the peptides in relation to the muscle preparations used as test systems. Thus, some peptides — for example, E-IIIa-2-3 and E-IIIb-2 — were more active on the uterus preparation, while others (E-IIIa-2-4 and E-IIIa-2-2) attacked the ileum more effectively. An analog-

TABLE 1. Bradykinin-potentiating Activity (PU) and Inhibitory Properties of the Viper Venom Peptides

Peptide	PU (μg of substance in ml) in action on preparations of		IC-50, μg/ml
	uterus	ileum	
E–IIIa–1–1–1	16.7 ± 0.3	13.3 ± 0.3	33.0
E–IIIa–1–2–1	None	16.0 ± 0.3	44.0
E–IIIa–1–3–1	None	65.3 ± 0.3	210.0
E–IIIa–2–1	8.7±0.2	8.7 ± 0.1	21.0
E–IIIa–2–2	>400	27.0 ± 0.3	265.0
E–IIIa–2–3	1.07 ± 0.02	2.13 ± 0.02	1.6
E–IIIa–2–4	4.7 ± 0.1	3.9 ± 0.1	10.8
E–IIIb–2	6.5 ± 0.1	10.7 ± 0.2	1.7

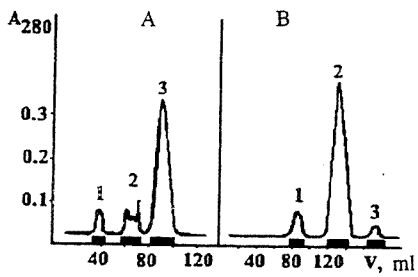

Fig. 2

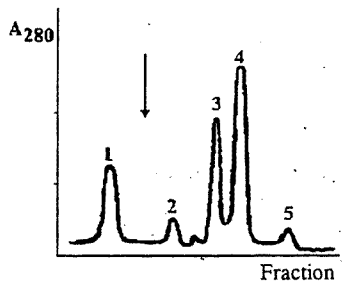

Fig. 3

Fig. 2. Chromatography of the BP-active fractions E-IIIa (*A*) and E-IIIb (*B*) on a TSK HW-40F column: *A*) 1, 2, 3 — fractions E-IIIaa, E-IIIa-1, and E-IIIa-2, respectively; *B*) 1, 2, 3 — fractions E-IIIb-1, E-IIIb-2, and E-IIIb-3, respectively.

Fig. 3. Ion-exchange chromatography of the BP-active fraction E-IIIa-2 on an SP-Sephadex C-25 column: 1, 2, 3, 4, 5) fractions E-IIIa-2-1, E-IIIa-2-2, E-IIIa-2-3, E-IIIa-2-4, and E-IIIa-2-5, respectively.

ous specificity for muscle preparations has been demonstrated for BPPs from the venom of the mamushi *Ag. halys blomhoffii* [4]. This phenomenon is characteristic for BPPs and obviously reflects a link between the structure of the peptides and the nature of the factors interacting with them.

The main mechanism of the potentiation of the depressor action of bradykinin is considered to be suppression of the angiotensin-converting enzyme (ACE) [1, 4, 5]. In actual fact, all the BPPs that we have studied inhibited the activity of ACE to some degree or other, but the question of the interrelationship of the potentiating and inhibitory actions of the BPPs remains open. Correlation analysis of the PU and IC-50 values of a number of peptides (see Table 1) shows an uncertainty of the mechanisms of the potentiating action. If we exclude peptides E-IIIb-2 and E-IIIa-2-2 from consideration, there is practically perfect correlation of the magnitudes characterizing the two effects ($r = 0.86 \pm 0.14$). The relatively high mean error in the calculation of the correlation coefficient is substantiated theoretically and is due to the small set of results ($n = 6$). In other words, for this series of peptides the predominating mechanism of potentiation is inhibition of the enzyme.

Inclusion of the peptides E-IIIb-2 and E-IIIa-2-2 in the analysis led to a marked decrease in the correlation coefficient ($r = 0.68 \pm 0.2$) and showed the existence of additional, most probably receptor, mechanisms in addition to the inhibition of ACE. An example of this is the peptide E-IIIa-2-2, which is inferior to all the other peptides investigated in inhibitory activity but superior to some of them in the efficiency of its action on the ileum and, to an even greater degree, on the uterus. BPPs with a weak inhibiting action on ACE (E-IIIa-2-2, E-IIIa-1-3-1, E-IIIa-1-2-1) differed most profoundly in their activity on smooth-mucle preparations of the rat uterus and guinea-pig ileum, which may be considered in connection with tissue-specific features of the structures of the corresponding receptors and their environment.

The existence of receptor mechanisms in the realization of the potentiating action of BPPs has already been reported. Thus, it has been shown by direct measurements that BPPs from the venom of *B. jararaca* (a competitive ACE inhibitor, SQ 20881) increases the binding of a labeled analog of BK with receptors from cow myometrium but does not affect receptor binding with the kidney medullary layer [2]. In this connection, great interest is presented by the structure of the peptide E-IIIa-2-3, which had a very high activity on both smooth-mucle preparations.

EXPERIMENTAL

We used: the venom of the viper *Echis multisquamatus* obtained from the Central Asian Zonal Zoological Combine that had been dried over calcium chloride; bradykinin from Reanal (Hungary); Sephadexes from Pharmacia (Sweden); and TSL gels from Toyo-Soda (Japan).

The viper venom was fractionated on a column of Sephadex G-50 (2.9 × 100 cm) in 0.01 M ammonium formate, pH 3.6.

The peptides were chromatographed on a column of TSK HW-40F (1.6 × 58 cm) in 0.01 M ammonium formate, pH 3.6, with elution by the same buffer. To prevent sorption of the peptides on the glass, the columns and vessels were siliconized.

The adsorption chromatography of the peptides was conducted on a column (1.4 × 20.5 cm) of Butyl-TSK-650M in 10% isopropanol (w/w), with elution by a stepwise concentration gradient of isopropanol.

The ion-exchange chromatography of the peptides was conducted on a column (1.4 × 16 cm) of SP-Sephadex C-25 in 0.01 M ammonium formate buffer, pH 3.6. The column was eluted with a concentration gradient of ammonium formate (0.01 M, pH 3.6 — 1.0 M, pH 6.6).

The desalting of the peptides obtained was achieved by repeated lyophilization to eliminate the volatile salts of the buffer solutions.

ACE was isolated from ox kidneys as in [9].

The TLC and PC analysis of the peptides was performed on silica gel plates (Silufol-R) and FN-16 chromatographic paper in the solvent systems: butan-1-ol–water–pyridine–acetic acid (15:12:10:3) and butan-1-ol–acetic acid–water (63:10:27), with staining by ninhydrin or the peptide reagent (*tert*-butyl hypochlorite–*o*-toluidine–KI).

The contractile activities of the venom and its fractions were determined from their capacity for shortening isolated muscle preparations of rat uterus and guinea-pig ileum, with bradykinin as standard [10].

The bradykinin-potentiating activities of the venom and its fractions were investigated from their capacity for enhancing the contractile effect of kinins [4], and this was characterized in potentiating units (PU), i.e., the concentrations (μg/ml) at which a doubling of the contractile effect took place.

The inhibitory activities of the peptides in relation to ACE were determined with the use of 4.25 μg of the enzyme, which was incubated with 4.0 μg of substrate in the presence of various concentrations of the BPPs (pH 8.0, 37°C, 60 min). A control sample contained no ACE. The reaction was stopped with the aid of TCAA, and the samples were neutralized with alkali and diluted with oxalic acid (0.0004 M), after which the residual contractile activity of the BK substrate in them was determined. The values of IC-50, i.e., the concentrations of inhibitory peptide (μg/ml) at which the ACE activity was 50% inhibited were calculated on the basis of inhibition curves. Each point of the curve was the mean of three measurements.

The correlation analysis of the results was carried out as described in [11].

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