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ISOLATION AND CHARACTERIZATION OF KININ-RELEASING ENZYME OF *ECHIS COLORATUS* VENOM*

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Abstract—*Echis coloratus* venom releases bradykinin upon incubation with a kininogen-enriched equine plasma fraction. A procedure is described for the purification of kinin-releasing enzyme from the venom. Two kinin-releasing enzyme preparations were obtained: one, having in addition slight kininase activity, electrophoretically heterogeneous, the other, devoid of kininase activity, electrophoretically homogeneous. The electrophoretically homogeneous enzyme preparation possessed capillary permeability increasing activity, but was devoid of the proteolytic, hemorrhagic and fibrinolytic activities of the venom. This preparation, having an estimated molecular weight of 22,000, hydrolysed arginine and tyrosine esters and synthetic lysine peptides, but not arginine amide nor lysine esters. It was thermostable, sensitive to DFP, insensitive to trasylol and to soybean trypsin inhibitor. The properties of the enzyme are compared with other kallikreins and a possible mode of its action on kininogen is discussed.

GLANDULAR tissues in various orders of vertebrates, and snake venoms contain enzymes capable of releasing pharmacologically active peptides from plasma globulin. These peptides, termed kinins, cause pain, edema, leukocyte migration, hypotension and contraction of various smooth muscles. The kinin-releasing enzymes (KRE) from various sources differ in physical characteristics, such as molecular weight and electrophoretic mobility, as well as in chemical properties, determining the type of kinin released, inhibitors and reaction with specific antibody. The KRE are both species specific and organ specific.[1] Rocha e Silva *et al.*[2] demonstrated bradykinin-releasing activity in the venom of Bothrops jararaca. Recently, Suzuki *et al.*[3] partially purified a KRE from the venom of *Agkistrodon halys blomhoffii*. In the present communication we report the purification and some biochemical properties of the KRE from *Echis coloratus* venom.

MATERIALS AND METHODS

Materials. Echis coloratus venom (ECV) was kindly supplied by the Department of Zoology, Tel Aviv University. DEAE-cellulose (1·0 m-equiv./g) was purchased from Whatman, Sephadex G-25 and G-75 from Pharmacia. Reagents for disc electrophoresis were obtained from Eastman Organic Chemicals; *p*-tosyl-L-arginine methyl ester (TAME) and *N*-benzoyl-L-arginine ethyl ester (BAEE) from Calbiochem; L-lysine methyl ester (LME), L-lysine ethyl ester (LEE) and *p*-tosyl-L-lysine methyl ester

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(TLME) from N.B.C.; l-tyrosine ethyl ester (TEE), *N*-acetyl-l-tyrosine ethyl ester (ATEE), polyarginine (Mol. wt. 15,500) and polylysine (Mol. wt. 14,500), from Miles-Yeda Ltd; benzoyl-l-arginine amide (BAA) from Mann; clupein from Fluka. Short synthetic lysine peptides were kindly provided by Dr. I. Schechter of the Weizmann Institute of Science, Israel. Soybean trypsin inhibitor (STI) was purchased from Worthington; pancreatic trypsin inhibitor (iniprol) from Choay; benzamidine from Aldrich; trasylol from Bayer; epsilon amino caproic acid (EACA) from Lederle; 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK) and tosyl phenyl alanine chloromethyl ketone from Calbiochem, mepyramine from Specia. Synthetic bradykinin, B.O.L. (2-brom-lysergic acid diethylamide bitartrate) were purchased from Sandoz; Pontamine Sky blue from E.I. Du Pont de Nemours.

Purification of KRE from ECV. A 5% solution of ECV in 0·005 M sodium acetate acetic acid buffer, pH 6·9 was prepared, and insoluble material removed by centrifugation at 1110 *g* for 15 min at +4°. Ten ml of the clear solution were applied to a DEAE cellulose column (1 × 60 cm) pre-equilibrated with the same buffer. The general procedure for the chromatography on DEAE cellulose was patterned after the method described by Sato *et al.*[4] Gradient elution was carried out in successive steps (as detailed in Fig. 1), whereupon the active fractions were pooled, freeze-dried and

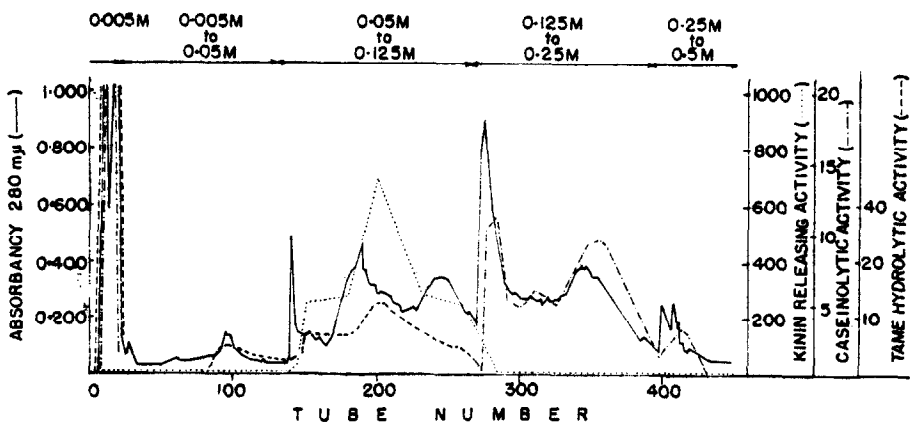

FIG. 1. *Echis coloratus* venom fractionation. 500 mg of ECV were applied to a DEAE-cellulose column (1 × 60 cm). Gradient elution with sodium acetate–acetic acid buffer pH 7·0 from 0·005 M to 0·5 M was carried out in four consecutive steps. 5 ml fractions were collected. Flow rate was 20 ml/hr.

desalted on Sephadex G-25 equilibrated with 0·005 M sodium acetate acetic acid buffer, pH 7·0. The kinin-releasing fraction was rechromatographed on a DEAE-cellulose column (1 × 60 cm) pre-equilibrated with 0·05 M sodium acetate–acetic acid buffer, pH 7·0. A 250-ml gradient (0·05–0·25 M) was applied and the eluted active fractions were concentrated and desalted on Sephadex G-25 equilibrated with 0·05 M ammonium acetate, pH 6·8. The excluded fraction was applied to a Sephadex G-75 column (1·5 × 90 cm) equilibrated and eluted with 0·05 M ammonium acetate, pH 6·8.

Analytical methods. Disc electrophoresis was performed according to Davis[5] on a 7·5% acrylamide gel, pH 9·5. Pre-run for 60 min was carried out in order to eliminate ammonium persulfate. Sedimentation velocity measurements were performed in a

Spinco model E ultracentrifuge operated at 59,780 rpm at 20°. The diffusion measurements were carried out in the ultracentrifuge with a synthetic boundary cell operating at low gear (9430 rpm).[6] Molecular weight was calculated from the sedimentation coefficient and the diffusion coefficient. For amino acid analysis of the peptide released by ECV from equine kininogen substrate, peptide hydrolysates were chromatographed on a Spinco model 120 amino acid analyser. High voltage electrophoresis (53 volts/cm) was carried out using 0·86 M pyridine–acetate buffer, pH 3·5, in the apparatus described by Michl.[7] Protein content of the chromatographed fractions was determined by following the absorbance at 278 mμ ($E^{1\%}_{278} = 12{\cdot}00$). For enzyme assays protein was also estimated according to Lowry[8] with albumin as standard.

Biological and enzymatic activities. Capillary permeability increasing activity (CPIA) was estimated according to Miles.[9] Kinin-releasing enzyme activity was tested on guinea pig ileum as described by Henriques *et al.*[10] The muscle preparation was suspended in Tyrode solution containing atropine sulfate ($1{\cdot}4 \times 10^{-6}$ M) at 33°. Synthetic bradykinin served as a standard. One unit was defined as the amount of enzyme which releases 1 μg of bradykinin equivalent in 1 min. Esterase activity was assayed by Hestrin's colorimetric method, as modified by Roberts.[11] Caseinolytic activity was assayed according to Rick.[12] Peptidase activity was detected by high-voltage electrophoresis (53 volts/cm) using 0·86 M pyridine–acetate buffer, pH 3·5, in the apparatus described by Michl.[7] Kininase activity was estimated by incubating the chromatographic fraction (0·4 ml) with synthetic bradykinin (0·4 ml, 200 μg) and testing the residual kinin activity of 0·5 ml of the mixture on guinea-pig ileum. The kininase activity was evaluated by the incubation time required for complete destruction of bradykinin. Phospholipase activity was detected according to Boman.[13]

Identification of the kinin released from equine kininogen substrate by ECV. ECV solution (1 mg/ml saline) was maintained for 10 min in boiling water in order to destroy its kininase activity. The boiled venom was added to equine Werle's substrate prepared according to Henriques *et al.*[10] in a proportion of 4 ml per 100 ml substrate, and incubated for 1 hr at 37°. The mixture was then poured into boiling absolute ethyl alcohol, and maintained at boiling temperature for 5 min. The precipitate was discarded and the supernatant was dried in a Rotavapor apparatus. The extraction of kinin from the dried powder was performed according to Habermann.[14] The method consisted in extracting the kinin with acetic acid, then precipitating it with diethyl ether. The precipitate was dissolved in 0·05 M ammonium formate buffer, pH 5, and applied to an Amberlite CG-50 column (1 × 20 cm) equilibrated and eluted with two successive ammonium formate gradients: 0·05 M pH 5 to 0·125 M pH 8·2 and 0·125 M pH 8·2 to 0·5 M pH 9·2. The effluent was monitored for its protein concentration by absorbance at 280 mμ and for its kinin activity on guinea-pig ileum (as described above). The active fractions were pooled, freeze dried three times, dissolved on 0·05 M ammonium formate buffer, pH 6, and applied on a Sephadex G-25 column (1 × 60 cm) pre-equilibrated and eluted with 0·05 M ammonium formate buffer. The effluent was monitored for its biological activity. The active fractions were pooled, freeze-dried and rechromatographed on Sephadex G-25. The procedure was repeated once more.

RESULTS

Isolation of KRE from ECV

KRE was isolated from ECV in three consecutive steps. (a) Chromatography on

DEAE-cellulose (Fig. 1). The KRE was eluted at 0·1 M sodium acetate–acetic acid buffer, pH 6·8. This fraction also contained arginine-ester hydrolase, kininase, phospholipase A and CPIA, but was devoid of caseinolytic and hemorrhagic activities. (b) Rechromatography on DEAE-cellulose of the KRE fraction, whereupon phospholipase activity was removed. (c) Gel filtration on Sephadex G-75, whereupon two fractions with KRE activity were obtained (Fig. 2). The first fraction (I) (tube number 35–44) possessed a high KRE activity (8 units/mg protein), high BAEE esterase activity (15·3 μmoles.min^{-1}/mg protein) and slight kininase activity. Three hours incubation of fraction I (0·2 mg/ml) with bradykinin were required to destroy the latter's biological activity, as compared to 40 min required for whole venom at the same con-

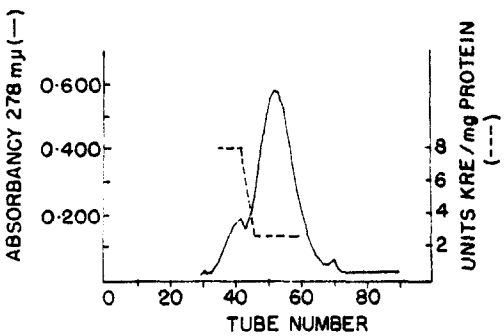

FIG. 2. Gel filtration on Sephadex G-75 column (1·5 × 90 cm) of the DEAE-cellulose KRE fraction. 1·5 ml fractions were collected. Flow rate was 6 ml/hr.

TABLE 1. DATA ON KRE FRACTIONATION

Enzyme preparation	KRE specific activity, (μg bradykinin-equiv. min^{-1}/mg)	Total protein (mg)
Echis coloratus venom	1·1	650
DEAE-cellulose fraction	6·6	47·5
Sephadex—G 75		
Fraction I	8·0	1·25
Fraction II	2·5	11·25

centration. As seen in Table 1, the KRE activity in this fraction was 7·5-fold purified. The second fraction (II) (tube number 45–65) displayed a lower KRE activity (2·5 units/mg protein) and a lower BAEE esterase activity (4·2 μmoles min^{-1}/mg protein). No kininase activity was detected. As seen in Table 1, the KRE activity of this fraction was 2-fold purified.

Characterization of the KRE fractions

Disc electrophoresis of the KRE fraction I (Fig. 3) showed at least three bands, that of KRE fraction II was more homogeneous, showing a major band and a fainter one. Ultracentrifugal studies were carried out on fraction II. The sedimentation coefficient

was S_{20} 2·3 and the diffusion coefficient $D_{20} = 10{\cdot}2 \times 10^{-7}$ cm²/sec. Assuming a partial specific volume of 0·75, the molecular weight was estimated to be about 22,000.

Both KRE fractions I and II were capable of hydrolyzing arginine esters but not arginine amide (benzoyl arginine amide) or lysine ester bonds; Table 2 shows the values obtained with KRE fraction II. Neither of the two purified KRE fractions hydrolyzed clupein (an arginine-rich polypeptide), polyarginine or polylysine, but they did split lysine peptide bonds in short synthetic peptides (Table 3). Both KRE fractions I and II hydrolyzed ATEE (0·3 μmoles min^{-1}/mg protein fraction II).

The KRE fractions were not inhibited by trasylol, pancreatic trypsin inhibitor, STI,

TABLE 2. HYDROLYSIS OF LOW MOLECULAR WEIGHT SYNTHETIC SUBSTRATES BY PURIFIED KRE FRACTION II, AND COMPETITIVE INHIBITION OF KININ RELEASE

Substrate	Hydrolysis, (μmoles min^{-1}/mg)	Ester concentration in inhibition assay, (molar)	Inhibition of KRE (%)
BAEE	4·2	2.10^{-3}	100
BAEE		2.10^{-4}	20
TAME	2·1	4.10^{-3}	100
TLME	0	4.10^{-3}	0
LME	0	4.10^{-3}	0
LEE	0	4.10^{-3}	0
BAA	0	4.10^{-3}	0

The assay system for hydrolysis of esters consisted of 20 μmoles substrate, 100 μg KRE, 250 μmoles tris-HCl buffer pH 8·0 for adjusting the final volume to 1 ml. The reaction was carried out at 37°, and the residual ester determined by Hestrin's colorimetric method (see Methods). When hydrolysis was observed, initial velocities were determined for the evaluation of specific activity. Competitive inhibition of KRE was studied in the system described in the legend of Table 4, using reaction conditions identical to those of hydrolysis of esters.

TABLE 3. PEPTIDASE ACTIVITY OF *ECHIS COLORATUS* VENOM–KRE FRACTIONS I AND II

Substrate	Cleavage
Phe-Lys-Ala NH_2	+
Ala-Phe-Lys-Ala NH_2	+
Phe-Ala-Lys-Ala NH_2	+
Ala-Ala-Lys-Ala NH_2	+
Polylysine	–
Polyarginine	–
Clupein	–

The assay system contained 0·02 M substrate, 0·13 phosphate buffer pH 7·8, KRE 60 μg/ml. The reaction was carried out at 37° for 2 hr, and hydrolysis detected by submitting the reaction samples to high voltage paper electrophoresis at pH 3·5 (see Methods). The arrow denotes the bond cleaved (release of alanine amide).

EACA or TLCK. However, both KRE fractions were inhibited by DFP and benzamidine (Table 4). The pH profile for KRE activity of fraction II showed an optimum at pH 8·3 (Fig. 4). The KRE and BAEE esterase activities of both fractions were found stable on boiling in a water bath for 20 min.

Both KRE fractions possessed considerable CPIA but were devoid of hemorrhagic activity.

Purification and characterization of the peptide released from equine kininogen substrate by ECV

The biologically active principle released by ECV from equine kininogen substrate

TABLE 4. EFFECT OF ENZYME INHIBITORS ON THE ACTIVITY OF ECV–KRE FRACTIONS I AND II

Inhibitor	Concentration in assay system	Inhibition, (%)
DFP	$1 \cdot 10^{-3}$ M	100
Benzamidine	$3 \cdot 10^{-2}$ M	100
Benzamidine	$1 \cdot 10^{-2}$ M	50
Urea	$1 \cdot 10^{-2}$ M	100
EDTA	$3 \cdot 10^{-3}$ M	0
Trasylol	200 U/ml	0
Iniprol	600 U/ml	0
STI	0·3%	0
EACA	0·7%	0
TLCK	0·1%	0

The inhibitor used was preincubated with KRE fractions (40 μg/ml) for 1 hr at room temperature in 250 μmoles of tris-HCl buffer pH 8·0 to a final volume to 1 ml. Subsequently 1 ml of equine Werle's substrate was added and KRE activity tested on guinea-pig ileum as described in Methods.

TABLE 5. AMINO ACID ANALYSIS OF THE PURIFIED KININ RELEASED BY *ECHIS COLORATUS* VENOM FROM EQUINE WERLE'S SUBSTRATE

Amino acid	μmoles	Residues per mole glycine	
		Experimental	Theoretical
Lysine	0·003		
Histidine	0·003		
Arginine	0·506	1·95	2
Aspartic acid	0·003		
Threonine	0·003		
Serine	0·234	0·90	1
Glutamic acid	0·003		
Proline	0·770	2·97	3
Glycine	0·260	1	1
Alanine	0·003		
Half cystine	—		
Valine	0·003		
Methionine	0·003		
Isoleucine	0·003		
Leucine	0·003		
Tyrosine	0·003		
Phenylalanine	0·533	2·05	2

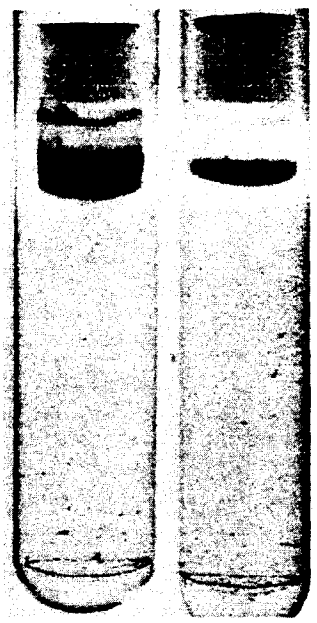

FIG. 3. Disc electrophoretic pattern of the KRE fractions separated by Sephadex G-75 gel filtration. Left: Fraction I; right: Fraction II.

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was identified as kinin by the following criteria: neither the antihistaminic substance mepyramine, nor the antiserotonin agent B.O.L. (2-brom-lysergic acid diethylamide bitartrate) inhibited the activity on guinea-pig ileum of the substance released by ECV. When the guinea-pig ileum was treated with χ-chymotrypsin and then washed, as described by Edery,[15] the response of the muscle to the activity released by ECV was increased at least 20-fold. This increased response was shown by Edery[15] to be specific for kinin peptides.

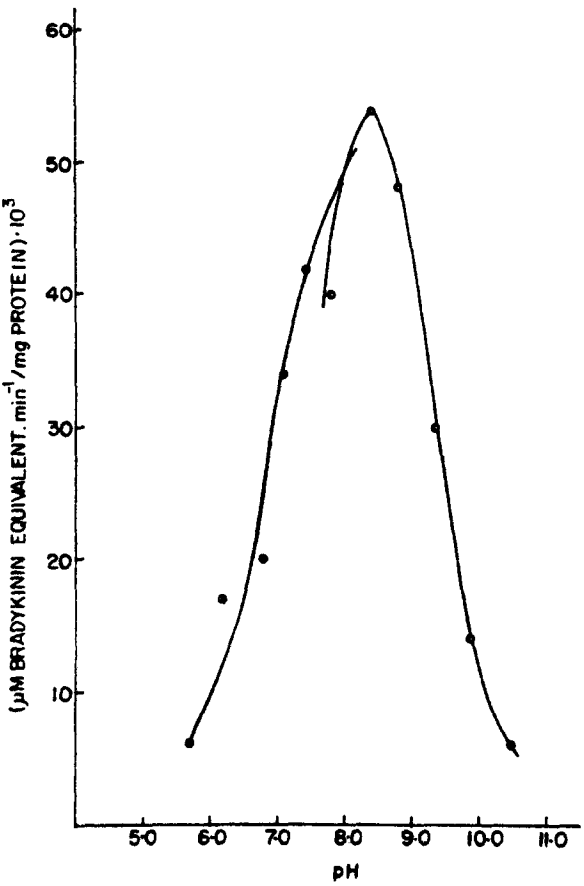

FIG. 4. pH activity curve for the release of kinin by purified ECV-KRE fraction II. The assay system contained 8 μg KRE, 1 ml equine Werle's substrate, 7·56 μm EDTA, and buffer to a total volume of 2·7 ml. After 3 min incubation at 37°, KRE activity was tested on guinea-pig ileum as described in Methods. ●——● 0·25 M phosphate buffer; ○——○ 0·5 M glycine-NaOH buffer.

The kinin was isolated as described in Methods. The purified kinin-active fraction was submitted to high voltage electrophoresis and to amino-acid analysis. The electrophorogram revealed one ninhydrine-sensitive spot and the amino acid composition (Table 5) was identical to that of synthetic bradykinin.

DISCUSSION

Bradykinin is the pharmacologically active peptide released from kininogen substrates by Crotalidae snake venoms such as *Bothrops jararaca*,[2] *Agkistroden halys blomhoffii*,[3] and *Crotalus adamanteus*.[14] In the present study the kinin released by *Echis coloratus* venom from a kininogen-enriched plasma was also identified as bradykinin.

Kinin-releasing enzyme and capillary permeability increasing activity were found associated in each of the two protein fractions purified from ECV. Plasma kallikrein (EC3.4.4.21) similarly has been shown to possess permeability-increasing property.[16] Miles[17] attributed the permeability-increasing activity of plasma kallikrein to the *in vivo* release of kinins. On the other hand, Sato *et al.*[4] separated from *Agkistrodon halys blomhoffii* three enzymatic entities, each possessing arginine esterase activity, the "bradykinin-releasing", "clotting", and "capillary permeability-increasing" enzymes.

Two KRE fractions were isolated from ECV. One of them, having traces of kininase activity, was electrophoretically heterogeneous, and displayed much higher KRE activity than the second fraction which was free of kininase and was homogeneous upon electrophoresis and ultracentrifugation. The relatively low specific activity of the latter fraction may possibly be ascribed to instability of the highly purified material, similarly to the instability of plasma kallikreins beyond a certain level of purification reported by Erdös.[1] The molecular weight of the homogenous ECV–KRE protein was estimated as 22,000, close to the value (24,000) obtained by Haberman[18] for pig pancreatic kallikrein. Higher estimates were given for some human kallikreins: 40,500 for urinary kallikrein, and 31,200 for pancreatic kallikrein.[19] No data pertaining to molecular weights of KRE from snake venoms were found in the literature.

ECV–KRE proved to have both peptidase and esterase activities, the former towards lysine peptides, the latter towards arginine esters. Regarding the peptidase activity, ECV–KRE split short lysine peptides, whereas arginine peptides of various molecular sizes such as BAA, clupein and polyarginine (MW 15,500) were not susceptible. Regarding the esterase activity, ECV–KRE was found to hydrolyze the well-known low molecular weight kallikrein substrates,[20] BAEE and TAME, whereas lysine esters were not susceptible. ATEE was hydrolysed to a slighter extent by the ECV–KRE preparations which, in this respect, were similar to Padutin and the Bothrops jararaca enzyme.[21]

A parallelism between the KRE and the benzoyl-arginine ethyl ester hydrolase activities of the ECV–KRE preparations was suggested by their similar response to various enzyme inhibitors, the only effective ones being DFP and benzamidine. These results are consistent with the hypothesis of Pierce[22] concerning the ester nature of the bond linking the C-terminal arginine of bradykinin to the serine of the kininogen molecule.[23] This view, contested by Kato and Suzuki,[24] was confirmed by Holman *et al.*[25] and Morley.[26] Thus the cleavage by the ECV–KRE preparations of arginine ester bonds but not amide bonds, and of lysine amide bonds but not ester bonds, would conform to the bradykinin being a peptide chain within the kininogen molecule, as proposed by Pierce[22] for human kininogen II.

The sensitivity of the ECV–KRE proteins to inhibitors was found to differ from that of various other kallikreins. KRE and arginine ester hydrolase of the purified ECV fractions were not inhibited by trasylol, STI or pancreatic trypsin inhibitor. In

contradistinction, the KRE of *Agkistrodon halys blomhoffii* is sensitive to trasylol,[27] and pancreatic, plasma and urinary kallikreins are inhibited by trasylol, pancreatic trypsin inhibitor and STI.[28] The inhibition of the KRE and arginine ester hydrolase activity of the purified ECV fractions by DFP seems to indicate involvement of a serine residue in the active center of the enzyme. A similar consideration applies to other kallikreins.[1] The pH profile of the ECV–KRE fractions (optimal activity at pH 8·3) suggests that histidine also participates in the active site of the enzyme. It is noteworthy that TLCK, a histidine alkylating inhibitor, specific for trypsin,[29] did not inhibit ECV–KRE, similarly to the lack of susceptibility of horse and rat urinary kallikrein to this inhibitor.[30] However, as was pointed out by Mares-Guia and Diniz,[30] this lack of interaction with TLCK can not be considered proof of the absence of a histidyl residue in the active center of the KRE. Finally, although ECV–KRE, like trypsin, is inhibited by benzamidine, the lack of response of ECV–KRE, as well as of urinary kallikreins[30] to TLCK indicates a difference between the active center of the kallikreins and that of trypsin

REFERENCES

1. E. G. Erdös, *Adv. Pharmacol.* **4**, 1 (1966).
2. M. Rocha e Silva, W. T. Beraldo and G. Rosenfeld, *Am. J. Physiol.* **156**, 261 (1949).
3. T. Suzuki, S. Iwanaga, N. Nagasawa and T. Sato, in *Hypotensive Peptides* (Eds. E. G. Erdös, N. Back and F. Sicuteri) p. 149. Springer Verlag, New York (1966).
4. T. Sato, S. Iwanaga, Y. Mizushima and T. Suzuki, *J. Biochem. (Tokyo)* **57**, 380 (1965).
5. B. J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
6. E. Daniel and E. Katchalski, in *Polyamino Acids, Polypeptides and Proteins* (Ed. M. A. Stahman) p. 183. University of Wisconsin Press, Madison (1962).
7. H. Michl, *Chromatog.* **1**, 11 (1959).
8. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and L. T. Randall, *J. biol. Chem.* **193**, 265 (1961).
9. A. A. Miles and D. L. Wilhelm, *Br. J. exp. Path.* **36**, 71 (1955).
10. O. B. Henriques, A. A. C. Lavras, M. Fichman and Z. P. Picarelli, *Biochem. Pharmac.* **15**, 31 (1966).
11. P. Roberts, *J. biol. Chem.* **232**, 285 (1958).
12. W. Rick, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer) p. 811, Academic Press, New York (1963).
13. H. G. Boman and U. Kaletta, *Biochim. biophys. Acta* **24**, 619 (1957).
14. E. Habermann, in *Hypotensive Peptides* (Eds. E. G. Erdös, N. Back and F. Sicuteri) p. 116, Springer Verlag, New York (1966).
15. H. Edery, *Br. J. Pharmac.* **24**, 485 (1965).
16. K. D. Bhoola, Calle and J. D. Schachter, *J. Physiol.* **152**, 75 (1960).
17. A. A. Miles, *Ann. N.Y. Acad. Sci.* **116**, 855 (1964).
18. E. Habermann, *Ann. N.Y. Acad. Sci.* **104**, 130 (1963).
19. H. Moriya, J. V. Pierce and M. E. Webster, *Ann. N.Y. Acad. Sci.* **104**, 172 (1963).
20. M. E. Webster and J. V. Pierce, *Proc. Soc. exp. Biol. Med.* **107**, 186 (1961).
21. M. Rocha e Silva, *Ann. N.Y. Acad. Sci.* **146**, 448 (1968).
22. J. V. Pierce, *Fedn Proc.* **27**, 52 (1968).
23. D. F. Elliott, *Ann. N.Y. Acad. Sci.* **104**, 35 (1963).
24. H. Kato and T. Suzuki, *Biochem. biophys. Res. Commun.* **32**, 800 (1968).
25. G. Holman, J. S. Lowe, J. S. Morley and M. J. Smithers, *Biochem. biophys. Res. Commun.* **33**, 140 (1968).
26. J. S. Morley, *Biochem. biophys. Res. Commun.* **33**, 841 (1968).
27. S. Iwanaga, T. Sato, V. Mizushima and T. Suzuki, *J. Biochem. (Tokyo)* **58**, 128 (1965).
28. N. Back and R. Stager, *Fedn Proc.* **27**, 96 (1968).
29. E. Shaw, M. Mares-Guia and W. Cohen, *Biochemistry* **4**, 2219 (1965).
30. M. Mares-Guia and C. R. Diniz, *Archs Biochem. Biophys.* **121**, 750 (1967).