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Purification and Properties of a Kininogenin from the Venom of *Vipera ammodytes ammodytes*

By GRAHAM S. BAILEY*† and RUDOLPH A. SHIPOLINI†
* *Department of Chemistry, University of Essex, Colchester CO4 3SQ, Essex, U.K.,*
and † *Department of Chemistry, University College London, London WC1E 6BT, U.K.*

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A kininogenin (EC 3.4.21.8) was purified from the venom of *Vipera ammodytes ammodytes* (European sand viper) by a combination of gel filtration and ion-exchange chromatography. The enzyme is approximately six times more active than bovine trypsin in its ability to release vasoactive peptides from a plasma precursor. The kininogenin is a glycoprotein containing 18–20% by weight of carbohydrate. It showed a mol.wt. of 40500 on gel filtration. Gel electrophoresis of the reduced sample in the presence of sodium dodecyl sulphate and 2-mercaptoethanol revealed the presence of two major components of mol.wt. 34300 and 31300. The heterogeneity, which was also observed on disc electrophoresis, was removed by incubation with neuraminidase. After incubation with neuraminidase the kininogenin retained full enzymic activity and possessed an isoelectric point of pH 7.2. The carbohydrate content had been decreased to 10% by weight, and the single component seen on electrophoresis in the presence of sodium dodecyl sulphate and 2-mercaptoethanol corresponded to a mol.wt. of 29500.

Certain snake venoms are known to possess peptides which potentiate the contractile response of the guinea-pig ileum to the potent vasoconstrictor, bradykinin (Ferreira *et al.*, 1970; Sander *et al.*, 1972). All such peptides so far isolated have been demonstrated to inhibit the enzymic destruction of bradykinin. Some of the venoms containing the peptides also possess enzymes capable of releasing kinins from plasma precursors (Kato & Suzuki, 1971). The kinin-releasing enzymes, kininogenins, have not yet been well characterized. The present paper reports the isolation and characterization of a kininogenin from a viper venom which is known to possess kinin-potentiating peptides (R. A. Shipolini, unpublished work).

Experimental

Bovine thrombin and fibrinogen were purchased from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.). The venom of *Vipera ammodytes ammodytes* (European sand viper) was obtained from the Institute of Epidemiology, Sofia, Bulgaria. The enzyme neuraminidase (from *Clostridium perfringens*) was purchased from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.). Synthetic bradykinin (BRS 640) was donated by Sandoz Products Ltd. (Feltham, Middx., U.K.).

Enzyme substrates

All enzyme substrates were purchased from Sigma.

Chromatography materials

Sephadex G-10, Sephadex G-50, Sephadex G-75 and SE‡-Sephadex C-25 were obtained from Pharmacia (G.B.) Ltd. (London W5 5SS, U.K.). The microgranular resin Whatman DE 52 was purchased from H. Reeve Angel and Co. Ltd. (London SE1 6BD, U.K.).

Electrophoresis

Disc electrophoresis at pH 3.8 was carried out on 10% (w/v) polyacrylamide gels by the method of Davis (1964). Gel electrofocusing was carried out by the method of Pearce *et al.* (1972), by using Ampholine ampholytes (pH 3.5–10.0, LKB Instruments Ltd., South Croydon, Surrey, U.K.). Electrophoresis gels were stained with Amido Black 10B to reveal protein bands and were stained by the procedure of Clarke (1964) to reveal the presence of carbohydrates.

Amino acid analyses

N-Terminal amino acids were determined as the dansyl derivatives by the method of Gray & Hartley (1963). Protein hydrolysates (24, 48 and 72 h) were prepared by the method of Bargetzi *et al.* (1963) and

† To whom requests for reprints should be sent.

‡ Abbreviations: SE-, sulphoethyl-; Tos-Arg-OMe, *p*-tosyl-L-arginine methyl ester; Bz-Arg-OEt, α-*N*-benzoyl-L-arginine ethyl ester; Ac-Lys-OMe, *N*-acetyl-L-lysine methyl ester; Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; Bz-DL-Arg-NHPhNO$_2$, α-*N*-benzoyl-DL-arginine *p*-nitroanilide.

analysed on a Beckman amino acid analyser. Tryptophan was measured by the spectrophotometric method of Bencze & Schmid (1957).

Carbohydrate analyses

Carbohydrate was determined by g.l.c. by the method of Clamp *et al.* (1972).

Molecular-weight determinations

Estimation of molecular weight by gel filtration was made by the method of Andrews (1965) by using a column (2.5cm × 100cm) of Sephadex G-75 in 0.05M-Tris/HCl buffer, pH 7.75, containing 0.1M-NaCl. The proteins used as standards were myoglobin (mol.wt. 17800), chymotrypsin (25700), pepsin (35000) and bovine serum albumin (68000). Estimation of molecular weight of reduced proteins by gel electrophoresis in the presence of sodium dodecyl sulphate and 2-mercaptoethanol was made by the method of Weber *et al.* (1972). The marker proteins used were cytochrome *c* (mol.wt. 11700), trypsin (23300), ovalbumin (43000) and serum albumin (68000).

Enzyme assays

All enzyme assays were carried out at 25°C. Proteolytic activity was determined by the method of Kunitz (1947) by using a 1% casein solution in 0.05M-Tris/HCl buffer, pH 7.8, containing 0.02M-$CaCl_2$. The unit of proteolytic activity was defined as the amount of enzyme which caused an increase in E_{280} of 0.001 unit/min over the blank value. Kininogenin activity was usually determined in the purification procedure by measurement of the hydrolysis of arginine esters. Esterolytic activity was assayed by a modification of the method of Murata *et al.* (1963). The enzyme solution was added to 15μmol of Tos-Arg-OMe or Bz-Arg-OEt or Ac-Lys-OMe in 4ml of 0.15M-NaCl/0.02M-$CaCl_2$ solution in a pH-stat cell flushed with N_2. The acid liberated was automatically titrated at pH 8.5 with 0.05M-NaOH in the pH-stat assembly of Radiometer (Copenhagen, Denmark). The unit of activity was defined as the amount of enzyme producing 1μmol of acid/min. The ability of various compounds to inhibit the esterolytic activity of the enzyme against Bz-Arg-OEt was tested by incubating the compounds and enzyme for 30min at 25°C. Residual enzymic activity was then assayed by the normal procedure. Chymotrypsin-like activity was determined by the method of Schwert & Takenaka (1955), by using 0.93mM-Ac-Tyr-OEt in 0.05M-Tris/HCl buffer, pH 7.0. Trypsin-like amidase activity was determined by the method of Erlanger *et al.* (1961), by using 1mM-Bz-DL-Arg-$NHPhNO_2$ in 0.05M-Tris/HCl buffer, pH 7.75.

Fibrinolytic activity was measured by using a fibrin clot produced by the action of thrombin on a 1% solution of fibrinogen (Kline, 1955). Coagulant activity was determined by using 1% fibrinogen in 0.05M-Tris/HCl buffer, pH 8.0.

The kininogenin activity of purified samples was measured by the method of Rocha e Silva *et al.* (1949). Heated dog plasma (60°C, 3h), which was shown to be free of kininogenin, kinin and kininase activities was used as the plasma substrate. Activity was measured under conditions where the enzyme was saturated by the substrate but not inhibited by kininogenin inhibitors present in the plasma. The unit of activity was defined as the amount of enzyme which in 1min released kinin-like peptides having an activity equivalent to that of 1μg of synthetic bradykinin.

Results

Purification procedure

Step 1. The crude venom (145g) was processed in 15g batches by gel filtration on a column (5.4cm × 250cm) of Sephadex G-50 by using upward flow at 120ml/h, with 0.1M-ammonium formate/formic acid, pH 4.7, as the elution buffer. Arginine esterolytic activity was confined to fraction 3 in the published elution profile (Bailey *et al.*, 1975). The active fraction was desalted by gel filtration on Sephadex G-10. The freeze-dried fraction represented 13.8% by weight of the crude venom, and showed a specific activity against Tos-Arg-OMe of 0.6μmol/min per mg of protein in the sample.

Step 2. Fraction 3 was fractionated in 10g batches by cation-exchange chromatography on a column (2.5cm × 45cm) of SE-Sephadex C-25, equilibrated with 0.05M-Tris/0.03M-citric acid buffer, pH 7.3, at a flow rate of 13.5ml/h. The arginine esterolytic activity passed straight through the column and was separated from more basic proteins.

Step 3. After desalting and freeze-drying the active fraction from the previous stage was subjected to anion-exchange chromatography on a column (2.5cm × 45cm) of Whatman DEAE-52 resin, equilibrated with 0.005M-Tris/HCl buffer, pH 7.2, at a flow rate of 37ml/h; 18.5ml fractions were collected. Adsorbed material was removed from the resin by application of a linear salt gradient (0–0.30M-NaCl in starting buffer; total volume 925ml). The fractions were tested for activity against Tos-Arg-OMe and casein. The fraction showing the highest activity against Tos-Arg-OMe, fraction 1 on the elution profile (Fig. 1), was collected.

Step 4. Fraction 1 was subjected to rechromatography on the anion-exchange resin, at a flow rate of 40ml/h; 20ml fractions were collected. Adsorbed material was removed from the resin by means of a linear gradient (0.005–0.060M-Tris/HCl, pH 7.2; total volume 1200ml). The fractions produced by the rechromatography were pooled as shown on the elution profile (Fig. 2) and were desalted and freeze-dried.

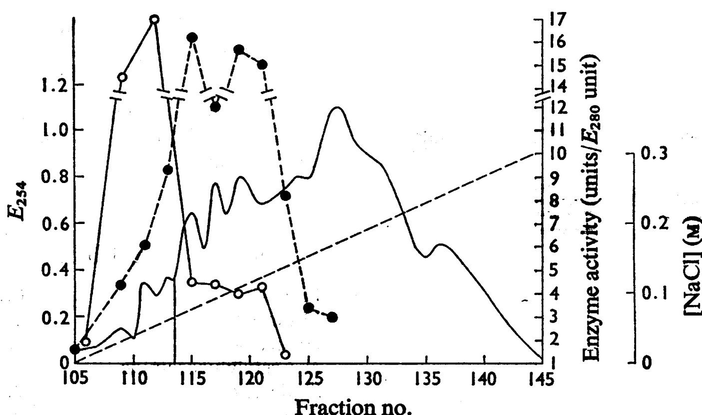

Fig. 1. *Step* 3 *of the purification procedure*

Fractionation of the kininogenin on DEAE-cellulose at pH 7.2. See the text for experimental details. ——, E_{254}; ○, enzyme activity against Tos-Arg-OMe; ●, enzyme activity against casein; ----, salt gradient.

Table 1. *Weights and enzymic activities of fractions obtained in the purification procedure*

For experimental details, see the text. For comparison the kininogenin activity of pure bovine trypsin was 7.0 units/mg. N.D., Not determined.

Fraction	Weight (g)	Yield (%w/w)	Activity against casein (units/mg)	Activity against Ac-Lys-OMe (units/mg)	Activity against Bz-Arg-OEt (units/mg)	Activity against Tos-Arg-OMe (units/mg)	Kininogenin activity (units/mg)	Purification based on activity against Tos-Arg-OMe
Crude venom	145	100	46.0	N.D.	N.D.	0.1	N.D.	1
Step 1	20	13.8	14.0	N.D.	N.D.	0.6	N.D.	6
Step 2	11.9	8.2	10.0	N.D.	N.D.	1.2	N.D.	12
Step 3								
Fraction 1	0.27	0.19	N.D.	N.D.	N.D.	14.4	N.D.	140
Step 4								
Fraction A	0.073	0.05	2.0	1.8	39.0	13.8	43.2	138
Fraction B	0.053	0.04	4.0	1.5	39.0	14.0	42.4	140
Fraction C	0.072	0.05	10.0	—	42.0	16.3	15.7	163

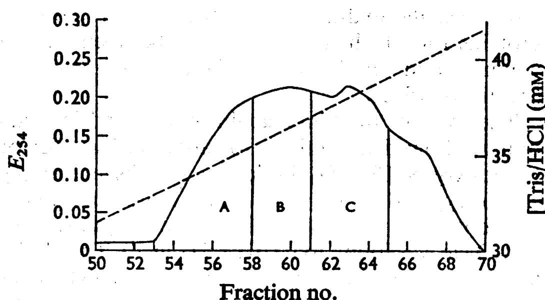

Fig. 2. *Step* 4 *of the purification procedure*

Re-fractionation of the kininogenin on DEAE-cellulose at pH 7.2. ——, E_{254}; ----, salt gradient.

Characterization of the kininogenin

Fractions A and B obtained in step 4 of the purification procedure (Table 1) were found to possess very similar enzymic activities. The fractions were potent kininogenins, being about six times more active than pure trypsin (EC 3.4.21.4). Neither of the fractions possessed any coagulant or fibrinolytic activity. Only one *N*-terminal amino acid, valine, could be detected for both fractions. The two fractions were found to be almost identical with each other on disc electrophoresis (Fig. 3), but they were heterogeneous. Heterogeneity was also seen on gel electrophoresis in the presence of sodium dodecyl sulphate and 2-mercaptoethanol; the two components had mol.wts. of 34300 and 31300. However, the fact that the frac-

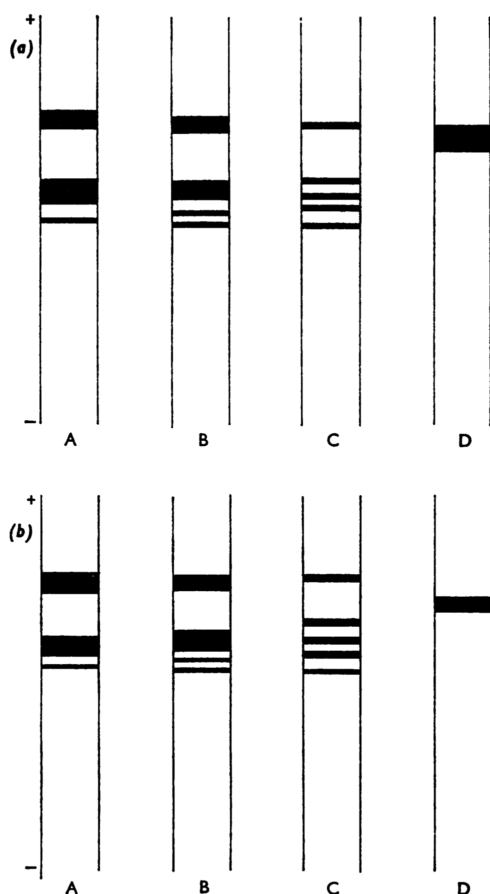

Fig. 3. *Disc electrophoresis of the fractions from step 4 of the purification procedure*

For experimental details, see the text. Electrophoresis at pH3.8 was carried out on 75μg samples for 1h in the resolving gel with a current of 4mA/gel. (*a*) Stained for protein; (*b*) stained for carbohydrate. D represents the mixture of fractions A and B after treatment with neuraminidase.

tions contained carbohydrate (Table 2) indicated that the electrophoretic heterogeneity could be due to the glycoprotein molecules containing different amounts of sialic acid residues. Therefore an attempt was made to decrease the heterogeneity by removal of sialic acid with neuraminidase. A mixture of fractions A and B (5mg of each) was incubated under sterile conditions at 37°C for 40h with neuraminidase (1.0 unit) in 5ml of sodium acetate buffer, pH5.6, containing 1% NaCl and 0.1% $CaCl_2$. After incubation the sample was thoroughly dialysed against distilled water and then freeze-dried. Disc electrophoresis of the incubated sample revealed the presence of only

Table 2. *Carbohydrate content of fractions obtained in step 4 of the purification procedure and after incubation with neuraminidase*

For experimental details see the text. D represents the mixture of fractions A and B after treatment with neuraminidase.

	Carbohydrate content (% by weight)			
Sugar Fraction ...	A	B	C	D
Fucose	0.79	0.71	0.78	0.92
Mannose	4.81	4.59	5.36	3.99
Galactose	3.83	3.83	3.44	1.24
N-Acetylglucosamine	7.62	6.23	6.43	3.48
Sialic acid	2.81	2.72	4.17	0.66
Total	19.86	18.08	20.18	10.29

one component (Fig. 3) and the mol. wt. of the reduced fraction in the presence of sodium dodecyl sulphate was found to be 29500. Also, only one component could be detected on gel electrofocusing, and the isoelectric point was estimated to be pH7.2.

Thus it appeared that the electrophoretic heterogeneity of fractions A and B was due to differences in the amount of terminal sialic acid possessed by the glycoprotein molecules. Fractions A and B were considered to represent the pure kininogenin because: (1) only one *N*-terminal amino acid residue could be detected; (2) both fractions were eluted from the calibrated Sephadex G-75 column as a single, symmetrical peak, of constant specific activity across the peak, and corresponding to a mol.wt. of 40500; (3) only a single component could be seen on electrophoresis after treatment with neuraminidase.

The difference in molecular weights found by using gel filtration and gel electrophoresis can probably be attributed to the glycoprotein nature of the molecules. The molecular weights of glycoproteins determined by gel filtration are often higher than those determined by other methods. It has been suggested that the reason for this behaviour is that glycoproteins are more heavily hydrated than the typical globular proteins for which the relationship between elution volume and molecular weight was proposed (Andrews, 1965).

The form of the kininogenin represented by fraction A showed no activity towards Ac-Tyr-OEt or Bz-DL-Arg-$NHPhNO_2$, indicating the absence of any chymotrypsin-like or trypsin-like amidase activity. The optimum activity of the enzyme against Tos-Arg-OMe was found to be pH8.5 (Fig. 4). The ability of various compounds to inhibit the esterolytic activity of fraction A was tested at pH8.5 with Bz-Arg-OEt as the substrate. Soya-bean trypsin inhibitor, 1,10-phenanthroline, EDTA and trypsin inhibitors isolated from the venom of *Vipera ammodytes ammodytes* (G. S. Bailey & R. A. Shipolini, unpublished

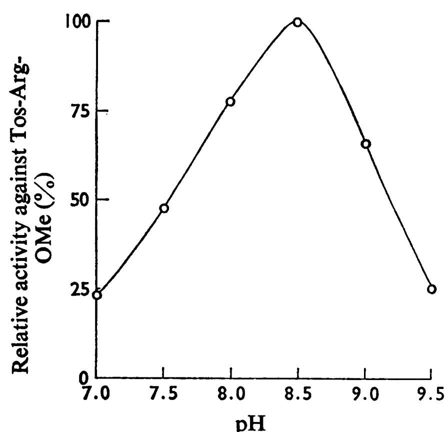

Fig. 4. *Plot of venom kininogenin activity versus pH* (*with Tos-Arg-OMe as the substrate*)

For experimental details, see the text. The 'relative activity' is the specific activity at a given pH expressed as a percentage of that at the pH optimum (100%).

Table 3. *Amino acid composition of kininogenin A*

The results are expressed as residues/molecule, assuming the mol.wt. of the polypeptide chain to be 26300. The values recorded were the averages of the results for 24h, 48h and 72h hydrolyses. However, the values for threonine and serine were extrapolated to zero time, and for valine, leucine and isoleucine the 72h values were taken.

Amino acid	Amount of amino acid (residues/molecule)
Asx	22.9
Thr	13.5
Ser	13.7
Glx	16.9
Pro	20.2
Gly	17.1
Ala	16.0
Val	12.4
½-Cys	6.2
Met	4.0
Ile	15.5
Leu	19.6
Tyr	6.8
Phe	6.3
His	9.3
Lys	11.3
Arg	11.1
Trp	3.0

work) were found to have no effect on the esterolytic activity, even at a 1:1 weight ratio. The amino acid analysis of the polypeptide chain of fraction A is given in Table 3.

Discussion

Except for trypsin, the most thoroughly studied kininogenins are the mammalian kallikreins, which are endogenous enzymes present in mammalian urine, plasma and various organs (Schacter, 1969). Nearly all of the kallikreins possess strong enzymic activity towards arginine esters and a much lower activity towards lysine esters (Webster & Prado, 1970). They have a much more restricted substrate specificity than that of trypsin, not usually attacking amides such as Bz-DL-Arg-NHPhNO$_2$ or any protein substrates apart from the natural precursors of the kinin peptides. All known kallikreins are glycoproteins and therefore show microheterogeneity on electrofocusing (Hail *et al.*, 1974). Except for the plasma enzyme, the kallikreins are not inhibited by soya-bean trypsin inhibitor (Schacter, 1969).

The kininogenin of the present study appears to be quite similar to the glandular kallikreins. It has a similar substrate specificity, and its activity is not inhibited by soya-bean trypsin inhibitor. It is a glycoprotein and is heterogeneous on electrophoresis, but the heterogeneity is removed after incubation with neuraminidase. Fritz *et al.* (1967) observed similar results with their preparation of pig pancreatic kallikrein. The venom kininogenin seems to be of a similar size to the kallikreins, having a mol.wt. of 40500 on gel filtration compared with 43600 for human urinary kallikrein (Hail *et al.*, 1974) and 33000 for pig pancreatic kallikrein (Zuber & Sache, 1974).

One of the major properties of the kallikreins is their ability to release kinins from plasma precursors. However, it is very difficult to compare kinin-releasing activities of different kallikreins. Pure kinin precursors have not yet been isolated and therefore different plasma preparations have been used as sources of the substrate. Unfortunately no standard method of assay has been evolved and in many cases specific activities have not been measured under conditions where the enzyme is saturated by the substrate. In the experiments reported in the present study great care was taken to ensure that the activity of the pure kininogenase was determined in the presence of a saturating concentration of substrate. The specific activity of the venom kininogenin was six times greater than that of pure trypsin determined under identical conditions.

The kininogenin of the present study is similar in some properties to the kininogenin isolated from *Bitis gabonica* (Gaboon viper) venom by Mebs (1970), and the kininogenin purified from the venom of *Agkistrodon halys blomhoffi* (Japanese name 'Mamushi') by Sato *et al.* (1965). The three enzymes possess low activity towards casein but are very active towards arginine esters. The *Bitis gabonica* kininogenin and the enzyme from *Agkistrodon halys blomhoffi* have optimal activity towards Bz-Arg-OEt at pH9.5 and

8.5 (Iwanaga *et al.*, 1965) respectively. But both enzymes appear to be less similar in some respects to the glandular kallikreins than is the enzyme from *Vipera ammodytes ammodytes* venom. For instance, the enzyme isolated by Mebs (1970) is inhibited to a large extent by soya-bean trypsin inhibitor and the enzyme purified by Sato *et al.* (1965) does not hydrolyse lysine esters. However, it is difficult to compare the three venom kininogenins because of insufficient reported information. For example, although the molecular weight of the *Bitis gabonica* kininogenin was reported to be 33500 on gel filtration (Mebs, 1970) the corresponding molecular weight of the enzyme from *Agkistrodon halys blomhoffi* was not recorded. Apparently the carbohydrate contents of the two enzymes were not determined.

The results reported in the present paper seem to suggest that the kininogenin of *Vipera ammodytes ammodytes* could represent the salivary kallikrein of the snake.

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