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

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A kinogenin (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 kinogenin 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 kinogenin 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, kinogenins, have not yet been well characterized. The present paper reports the isolation and characterization of a kinogenin from a viper venom which is known to possess kinin-potentiating peptides (R. A. Shipolini, unpublished work).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.

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**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 72h) were prepared by the method of Bargetti et al. (1963) and

† Abbreviations: SE, sulphonyl-; Tos-Arg-OMe, p-tosyl-L-arginine methyl ester; Bz-Arg-OEt, α-N-benzyol-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-NHPhNO₂, α-N-benzyol-DL-arginine p-nitroanilide.
analysed on a Beckman amino acid analyser. Trypto-
phan was measured by the spectrophotometric

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 (2.5 cm × 100 cm) of Sephadex G-75 in 0.05 M-
Tris/HCl buffer, pH 7.75, containing 0.1 M-NaCl. The
proteins used as standards were myoglobin (mol. wt.
17800), chymotrypsin (25700), pepsin (35000) and
bovine serum albumin (68000). Estimation of molecu-
lar 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 cyto-
chrome c (mol. wt. 11700), trypsin (23300), ovalbumin
(43000) and serum albumin (68000).

Enzyme assays
All enzyme assays were carried out at 25°C. Proteo-
lytic activity was measured by the method of Kunitz
(1947) by using a 1 % casein solution in 0.05 M-
Tris/HCl buffer, pH 7.8, containing 0.02 M-CaCl₂. The
unit of proteolytic activity was defined as the amount of
enzyme which caused an increase in E₂₈₀ 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 15 μmol of Tos-Arg-OtMe or Bz-
Arg-OEt or Ac-Lys-OtMe in 4 ml of 0.15 M-NaCl/
0.02 M-CaCl₂ solution in a pH-stat cell flushed with
N₂. The acid liberated was automatically titrated at
pH 8.5 with 0.05 M-NaOH in the pH-stat assembly of
Radiometer (Copenhagen, Denmark). The unit of
activity was defined as the amount of enzyme pro-
ducing 1 μmol 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 30 min at 25°C. Residual
enzymatic activity was then assayed by the normal
procedure. Chymotrypsin-like activity was deter-
mined by the method of Schwert & Takenaka
(1955), by using 0.93 mM Ac-Tyr-OEt in 0.05 M-
Tris/HCl buffer, pH 7.0. Trypsin-like amidase activity
was determined by the method of Erlanger et al.
(1961), by using 1 mM Bz-DL-Arg-NNH₂NO₂ in
0.05 M-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 acti-

vity was determined by using 1 % fibrinogen in 0.05 M-
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, 3 h), 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 inhibi-
tors present in the plasma. The unit of activity was
defined as the amount of enzyme which in 1 min re-
leased kinin-like peptides having an activity equivalent
to that of 1 μg of synthetic bradykinin.

Results
Purification procedure

Step 1. The crude venom (145 g) was processed in
15 g batches by gel filtration on a column
(5.4 cm × 250 cm) of Sephadex G-50 by using upward
flow at 120 ml/h, with 0.1 M-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-OtMe of 0.6 μmol/
min per mg of protein in the sample.

Step 2. Fraction 3 was fractionated in 10 g batches by
cation-exchange chromatography on a column
(2.5 cm × 45 cm) of SE-Sephadex C-25, equilibrated
with 0.05 M-Tris/0.03 M-citric acid buffer, pH 7.3, at a
flow rate of 13.5 ml/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
(2.5 cm × 45 cm) of Whatman DEAE-52 resin, equili-
ibrated with 0.005 M-Tris/HCl buffer, pH 7.2, at a
flow rate of 37 ml/h; 18.5 ml fractions were collected.
Adsorbed material was removed from the resin by
application of a linear salt gradient (0–0.30 M-NaCl in
starting buffer; total volume 925 ml). The fractions
were tested for activity against Tos-Arg-OtMe and
casein. The fraction showing the highest activity
against Tos-Arg-OtMe, fraction 1 on the elution
profile (Fig. 1), was collected.

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

Fig. 1. Step 3 of the purification procedure

Fractionation of the kininogenin on DEAE-cellulose at pH7.2. 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.

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

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-

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ions 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 (5 mg of each) was incubated under sterile conditions at 37°C for 40 h with neuraminidase (1.0 unit) in 5 ml of sodium acetate buffer, pH 5.6, containing 1 % NaCl and 0.1 % CaCl₂. 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 one component (Fig. 3) and the mol. wt. of the reduced fraction in the presence of sodium dodecyl sulphate was found to be 29 500. Also, only one component could be detected on gel electrophoresis, and the isoelectric point was estimated to be pH 7.2.

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 40 500; (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-0Et or Bz-Asp-Arg-NH-PhNO₂, indicating the absence of any chymotrypsin-like or trypsin-like amidase activity. The optimum activity of the enzyme against Tos-Arg-0Me was found to be pH 8.5 (Fig. 4). The ability of various compounds to inhibit the esterolytic activity of fraction A was tested at pH 8.5 with Bz-Arg-0Et 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

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amount of amino acid (residues/molecule)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>22.9</td>
</tr>
<tr>
<td>Thr</td>
<td>13.5</td>
</tr>
<tr>
<td>Ser</td>
<td>13.7</td>
</tr>
<tr>
<td>Glx</td>
<td>16.9</td>
</tr>
<tr>
<td>Pro</td>
<td>20.2</td>
</tr>
<tr>
<td>Gly</td>
<td>17.1</td>
</tr>
<tr>
<td>Ala</td>
<td>16.0</td>
</tr>
<tr>
<td>Val</td>
<td>12.4</td>
</tr>
<tr>
<td>3-Cys</td>
<td>6.2</td>
</tr>
<tr>
<td>Met</td>
<td>4.0</td>
</tr>
<tr>
<td>Ile</td>
<td>15.5</td>
</tr>
<tr>
<td>Leu</td>
<td>19.6</td>
</tr>
<tr>
<td>Tyr</td>
<td>6.8</td>
</tr>
<tr>
<td>Phe</td>
<td>6.3</td>
</tr>
<tr>
<td>His</td>
<td>9.3</td>
</tr>
<tr>
<td>Lys</td>
<td>11.3</td>
</tr>
<tr>
<td>Arg</td>
<td>11.1</td>
</tr>
<tr>
<td>Trp</td>
<td>3.0</td>
</tr>
</tbody>
</table>

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-NHPHO₃ or any protein substrates apart from the natural precursors of the kinin peptides. All known kallikreins are glycoproteins and therefore show microheterogeneity on electrophoresis (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 pH 9.5 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 soybean 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 33,500 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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References