ACTA CHEMICA SCANDINAVICA 21 (1967) 849–856

Isolation and Properties of Phoratoxin, a Toxic Protein from *Phoradendron serotinum* (Loranthaceae)

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A basic protein denoted as Phoratoxin has been isolated from leaves and stems of *Phoradendron serotinum* (Raf.) M. C. Johnst. The isolation method comprises the following steps: extraction of the dried plant material with 2 % acetic acid, removal of coloured impurities by passage of the extract through a column of polyamide, isolation of a high-molecular weight fraction by gel filtration on Sephadex, and chromatography on carboxymethyl cellulose and phosphate cellulose cation exchangers. Phoratoxin is chromatographically homogeneous, and shows only one band on electrophoresis in polyacrylamide gel or Sephadex. The isolelectric point is in the pH range 11.7 to 12.0. After performic acid oxidation, electrophoresis in Sephadex shows three bands. Phoratoxin contains the following amino acids: alanine, arginine, aspartic acid, cystine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, and tyrosine, with lysine as the N-terminal amino acid. No cysteine or reducing sugar is present. The yield of N-terminal lysine indicates a molecular weight of 13 000 or lower.

In connexion with studies [1–4] of a group of small toxic proteins from the European mistletoe (*Viscum album* L.), a screening was made for the occurrence of such proteins in other species within the family Loranthaceae.[5] This screening indicated the presence of such substances in three species, among them *Phoradendron serotinum* (Raf.) M. C. Johnst. This paper describes the isolation and some physical and chemical properties of a protein isolated from this plant, and denoted as *Phoratoxin*. A study of the effects of Phoratoxin on blood circulation has been published previously. Phoratoxin was found [6] to produce the same pharmacological effects as Viscotoxin from *Viscum album* L., *i.e.*, reflex bradycardia, negative inotropic effect on the heart and, in high doses, vasoconstriction of vessels in the skin and skeletal muscle. However, to produce the same effects, Phoratoxin must be administered in doses 10 times as high as Viscotoxin.

EXPERIMENTAL

Materials

Plant material. Leaves and stems from *Phoradendron serotinum* growing on *Juglans hindsii* Jepson were collected in Martinez, California, U.S.A. A reference herbarium specimen is kept at our Department. The plant parts were dried in the shade until the leaves could be easily broken off from the stems, and finally in an oven at a temperature not exceeding 45°C. For shipping, the material was packed in sealed polyethylene bags.

Polyamide powder. Two preparations of polyamide were used. One was "Ultramid Pulver für chromatographische Zwecke" from Badische Anilin- und Sodafabrik, Ludwigshafen/Rhein, Germany. The other was a powder prepared as described in the following from "Maranyl Nylon compound type A 100", a nylon 6—6 product marketed as granules by Imperial Chemical Industries Ltd., England. For preparation of polyamide powder, 500 g of nylon granules were dissolved in 1500 ml of conc. formic acid by heating on a steam bath. To this solution, 12 l of 50% ethanol (v/v) was added slowly with vigorous stirring. The powder formed was collected, washed with acetone and air dried. During drying, formation of lumps was prevented by repeated grinding of the moist powder in a mortar, eventually with addition of some acetone. The dry, coarse powder was ground in a mortar or ball mill, and sieved to give a particle size of about 0.2 mm.

Sephadex G 25, fine, in the bead form (AB Pharmacia, Uppsala, Sweden). For electrophoresis ground, block polymerized Sephadex was used.

Carboxymethyl cellulose (CMC) Whatman CM II, was washed successively with NaOH (0.1 N), water, HCl (0.1 N), NaOH (0.1 N), water, and stored at 2°C in water or buffer containing 2 % butanol.

Phosphate cellulose (PC) Whatman P 70, was washed and stored as described for CMC.

Buffers were prepared with NaOAc and HOAc in appropriate concentrations. Butanol (2 %) was added as preservative. Unless otherwise stated, all reagents used were of analytical grade.

Methods

Extraction of the plant material. 2 kg of the dried and ground plant material (leaves or stems) was extracted twice with 12 l of 2 % HOAc, by stirring for 24 h at room temperature. The combined extracts were concentrated *in vacuo* at a temperature not exceeding 30°C, and lyophilized. Yield: 680 g (34 %).

Purification with polyamide and filtration through Sephadex. 325 g of polyamide powder was stirred with 5 l of water and the suspension allowed to sediment for 2 h. Fine particles which did not sediment were removed by decantation, and the treatment repeated 4 times. The polyamide was packed as a slurry in 2 % HOAc into a column (4.8 × 94 cm) and washed with HOAc overnight. 210 g of the dry HOAc extract was dissolved in 250 ml of 2 % acetic acid. Fine insoluble particles were removed by centrifugation, after which the solution was diluted to 1200 ml and passed through the polyamide column. The column was eluted with HOAc (2 %) until the effluent was almost colourless. The eluate was concentrated *in vacuo* to 250 ml. After addition of 2 g of NaCl to facilitate detection of the low-molecular weight fraction, the solution was applied to a column (8 × 90 cm) of Sephadex G 25 in 2 % HOAc. Because of the viscosity and high density of the sample, application was best performed by Flodin's method 2,[7] in which the sample is applied in such a way that it forms a zone between the bed and the eluent above. Elution was performed with 2 % HOAc and fractions of 25 ml were collected. Fractions eluted before the appearance of Cl^- were pooled, concentrated and lyophilized. Yield: 12 g, denoted as fraction *Seph I*.

Chromatography of fraction Seph I on CMC. 8.4 g of fraction Seph I was dissolved in 80 ml of 0.01 M NaOAc buffer, pH 5.0. The solution was centrifuged to remove insoluble particles, and applied to a column (4 × 90 cm) of CMC previously equilibrated with 0.01 M NaOAc buffer, pH 5.0, which was also used for elution. Fractions of 25 ml were collected, and the UV absorption at 280 mμ was determined. When no more UV-absorbing material was eluted, the ionic strength of the eluting buffer was increased by adding NaCl to a concentration of 0.5 M. Fractions corresponding to UV-absorbing peaks were

pooled, concentrated and desalted by filtration through Sephadex G 25, using 2 % HOAc as eluent. Fraction *Seph I* was divided in this way into two fractions, denoted as fraction *Seph I, CMC A* (eluted with the low ionic strength buffer) and fraction *Seph I, CMC B* (eluted with the high ionic strength buffer). Yields: (*cf.* Fig. 1) *Seph I, CMC A:* 2.5 g, *Seph I, CMC B:* 400 mg.

Chromatography of fraction Seph I, CMC B (Fig. 1) on PC. This procedure was performed as previously described for chromatography of crude Viscotoxin on phosphate cellulose.[4] 1.5 g of fraction *Seph I, CMC B* gave the following yields: peak I: 190 mg, peak II: 260 mg, and peak III: 670 mg (*cf.* Fig. 2).

Rechromatography of fraction Seph I, CMC B peak III (Fig. 2). 295 mg of this fraction was chromatographed on a PC column (1.7 × 43 cm) with 0.05 M NaOAc buffer and a gradient of NaCl increasing linearly from 0.2 M to 1.0 M in 3.4 l, as previously described.[4] Yield: 120 mg of Phoratoxin.

Oxidation of proteins. Performic acid oxidation of proteins was performed at 0° according to Hirs.[8]

High-voltage electrophoresis. The method previously described [2] was used, with Sephadex G 25 as supporting phase. The new bead form of Sephadex was found to be less satisfactory for this purpose than the ground, block polymerized Sephadex, consisting of particles of irregular shape.

Polyacrylamide-gel electrophoresis was performed according to Shepherd and Gurley.[9]

Quantitative amino acid analysis. Proteins were hydrolyzed with constant boiling HCl, as described by Hirs *et al.*[10] and the amino acids determined with an automatic amino acid analyzer according to Spackman *et al.*[11]

Amperometric titration of sulphhydryl and disulphide groups. The method of Carter [12] was used. Titrations were performed in 8 M urea at 37°C on 1—2 mg samples of protein. In the titrations of disulphide groups, the end-point was reached at a very slow rate. It was considered that the end-point had been reached when, after addition of one drop of silver nitrate, the current did not decrease during 1 min.

Test for the presence of reducing sugar. 10 mg of protein was hydrolyzed with 0.5 ml of N H_2SO_4, according to Block *et al.*[13], p. 189 Samples of the hydrolysate corresponding to 200 and 500 μg of the protein were chromatographed on cellulose thin-layer plates, with collidine saturated with water as solvent. The dried plates were sprayed with aniline phthalate.[14]

Test for tryptophan. 5 mg of protein was hydrolyzed with 5 ml of 14 % $Ba(OH)_2$ in a sealed tube at 110°C for 26 h. Ba^{2+} was precipitated with solid CO_2 and filtrate and washings lyophilized. The residue was dissolved in 1 ml of isopropanol and 30 μl of the solution subjected to two-dimensional thin-layer chromatography on cellulose. Solvent for the first direction was butanol-water-HOAc (4:1:5) and for the second direction pyridine-water (4:1). Tryptophan was revealed by spraying with Ehrlich's reagent.[13], p. 137

Determination of N-terminal amino acids. 1. *As phenylthiohydantoins (PTH).* Coupling with phenylisothiocyanate was performed on 5 mg of protein according to Eriksson and Sjöquist.[15] Cleavage of the lyophilized PTC derivative to yield a thiazolinone derivative of the N-terminal amino acid was effected with trifluoroacetic acid, as described by Blombäck *et al.*[16] Conversion of the thiazolinone to the corresponding PTH was also performed according to these authors. PTH derivatives were identified by paper chromatography, using the systems of Sjöquist [17] and Wallén and Sjöholm.[18]

2. *As dinitrophenylderivatives (DNP).* The method of Levy [19] was used.

Toxicity tests. Substances to be tested were dissolved in 0.9 % NaCl solution, and 0.25 or 0.50 ml of the solutions administered intraperitoneally to white male mice with an average body weight of 20 g.

RESULTS AND DISCUSSION

Isolation of Phoratoxin. In preliminary experiments, separation of the acetic acid extract of the dried plant material into fractions of large and small molecules was tried by direct filtration through Sephadex G 25. Although separation was achieved, a serious drawback of the method was a strong

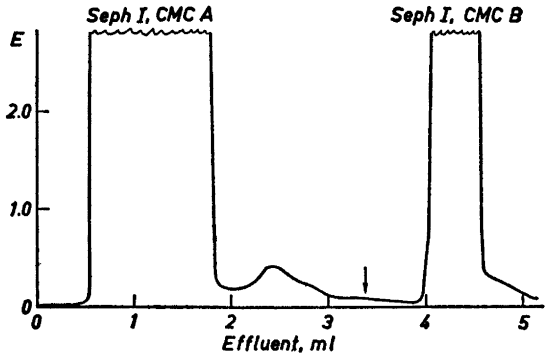

Fig. 1. Chromatography of 8.4 g of fraction *Seph I* on a column (4 × 90 cm) of carboxymethyl cellulose. E = optical density at 280 mμ. The arrow indicates change of eluting buffer.

adsorption of coloured substances to the Sephadex matrix. These coloured products were difficult to remove, and prevented repeated use of the Sephadex columns. This difficulty was overcome by passing the extract through a column of polyamide prior to gel filtration. Coloured substances were adsorbed to the polyamide. The extract had the same toxicity before and after filtration through polyamide. Gel filtration of the filtrate from the polyamide column yielded a fraction — *Seph I* — which contained the proteins and other substances with high-molecular weight. Fig. 1 shows the result of chromatography of this fraction on carboxymethyl cellulose. Two main peaks — *Seph I, CMC A* and *Seph I, CMC B* — were obtained. In toxicity tests, doses of 400 mg/kg of body weight of *Seph I, CMC A* killed the mice in 1—24 h, whereas 40 mg/kg of body weight of *Seph I, CMC B* killed the mice within 5 min. Thus, fraction *Seph I, CMC B* is most toxic and can be concluded to contain more basic

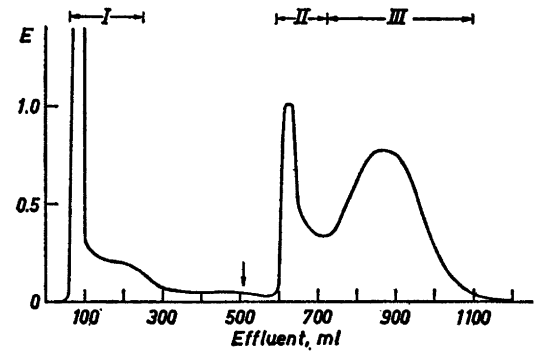

Fig. 2. Chromatography of 400 mg of fraction *Seph I, CMC B* on a column (1.7 × 43 cm) of phosphate cellulose. E = optical density at 280 mμ. The arrow indicates change of eluting buffer.

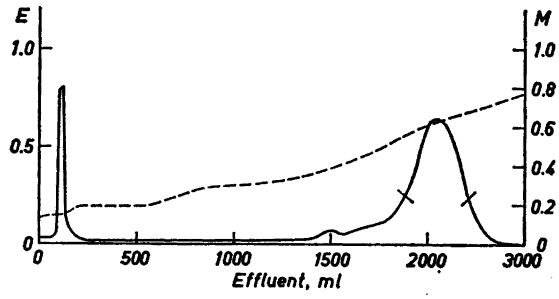

Fig. 3. Chromatography of 295 mg of material from peak III (Fig. 2) on a column (1.7 × 43 cm) of phosphate cellulose. ——— denotes optical density (E) of the eluate at 280 mμ. — — — indicates the concentration of NaCl (M) in the eluate as determined by titration with $AgNO_3$. Phoratoxin was recovered between 1880 and 2210 ml of effluent.

substances than *Seph I, CMC A*, since a higher ionic strength of the eluting buffer was necessary to release it from the cation exchanger.

Fig. 2 illustrates the result of chromatography of fraction *Seph I, CMC B* on phosphate cellulose. Three peaks were obtained. In toxicity tests, material from peak I was weakly toxic, while the substance recovered from peaks II and III had about the same toxicity; doses of 40 mg/kg of body weight killed the mice within 10 and 5 min, respectively. When chromatographed on phosphate cellulose, fraction *Seph I, CMC B* behaved very much like crude Viscotoxin from *Viscum album*.[4] The relative hold-up volume, calculated from the introduction of the high ionic strength buffer, is the same for peak III in Fig. 2 as for the peak yielding Viscotoxin A 3, obtained when crude Viscotoxin is chromatographed under the same conditions.[4] As Viscotoxin A 3 is the most extensively studied of the Viscotoxins, we considered it of greatest interest to restrict further work to the material recovered from peak III (Fig. 2) in

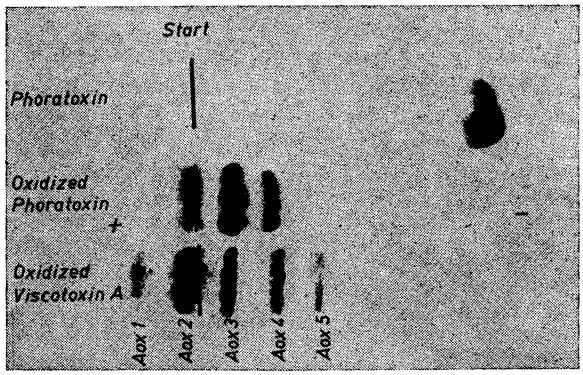

Fig. 4. Electrophoresis in Sephadex G 25 of oxidized Phoratoxin and oxidation products of Viscotoxin A. Buffer: pyridine (0.15 M)—HOAc, pH 5.6, 20 V/cm, 3.5 h. Amount of sample: 1 mg.

order to compare it with Viscotoxin A 3. This substance was therefore rechromatographed on phosphate cellulose with an increasing gradient of NaCl in the eluting buffer. The results are illustrated in Fig. 3. One small peak was eluted with the front, and one comparatively symmetrical peak was eluted at a NaCl concentration of about 0.6 M. The material from the main peak was denoted as *Phoratoxin*.

Properties of Phoratoxin. Fig. 4 shows the result of high-voltage electrophoresis in Sephadex at pH 5.6 of intact and performic acid-oxidized Phoratoxin. The intact material gave one strongly basic band, and the oxidized material three strong bands. In polyacrylamide-gel electrophoresis, Phoratoxin gave one band (Fig. 5). In both systems, Phoratoxin moved rapidly towards the cathode, indicating it to be strongly basic. In electrophoresis in Sephadex at various pH values from 5.6 to 12.0, Phoratoxin gave only one band moving towards the cathode at pH values of 11.7 and lower, and towards the anode at pH 12.0 or higher, indicating the isoelectric point to be in the pH range 11.7 to 12.0. In the systems investigated, Phoratoxin was chromatographically and electrophoretically homogeneous. The fact that oxidized Phoratoxin yielded three bands in electrophoresis in Sephadex, indicates, however, that the substance might be a mixture of closely related proteins. Further tests are therefore required to determine whether Phoratoxin is a homogeneous protein.

The amino acid composition is presented in Table 1. In addition to the amino acids in this table, tryptophan was detected in an alkaline hydrolysate. Calculated from UV data according to Goodwin and Morton,[20] the ratio of tryptophan to tyrosine was 1:5. The tryptophan content was thus low. The results of N-terminal analysis (see below) indicate a molecular weight for Phoratoxin of 13 000 or lower. This implies, if a more exact determination confirms this result, that tryptophan can hardly be a component of Phoratoxin. Amperometric determination of sulphhydryl groups showed that Phoratoxin contains no cysteine. Amperometric titration of disulphide groups, after reduction with sulphite, showed a cystine content amounting to only about

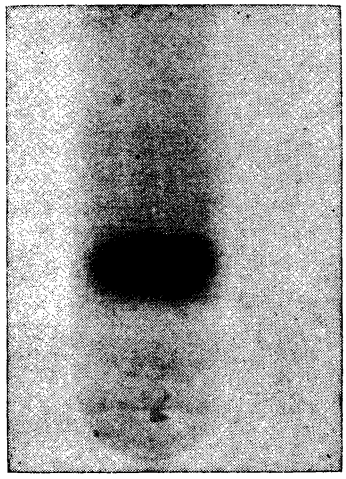

Fig. 5. Electrophoresis of Phoratoxin (20 μg) in polyacrylamide gel. Monomer concentration: 15 %. 50 V, for 30 min, thereafter 145 V, for 70 min. Stained with Amido Black 10 B.

Table 1. Amino acid composition of Phoratoxin.

Amino acid	μmole/mg *	Amino acid residues g/100 g
Lysine	0.519	6.7
Histidine	0.117	1.6
Arginine	0.410	6.4
Aspartic acid	0.566	6.5
Threonine	0.655	6.6
Serine	0.604	5.2
Proline	0.427	4.2
Glycine	0.994	5.7
Alanine	0.297	2.1
½ Cystine	0.851	13.3
Isoleucine	0.463	5.2
Leucine	0.321	3.6
Tyrosine	0.141	2.3
Phenylalanine	0.130	1.9
		71.3

* Determined after hydrolysis for 24 h. Values not corrected for ash or moisture content, or for losses due to destruction of amino acids during hydrolysis.

50 % of that calculated from the amino acid analysis. This might be due to heavy masking of the disulphide groups.

No reducing sugar was found in Phoratoxin.

In determination of N-terminal amino acids with the Edman method, lysine was the only N-terminal found. This was confirmed by dinitrophenylation, which also revealed lysine as the N-terminal amino acid. The yield of the phenylthiohydantoin corresponding to lysine was 0.076 μM/mg of Phoratoxin. Assuming only one N-terminal amino acid per molecule of protein, this indicates a molecular weight around 13 000. As the yield of the phenylthiohydantoin is probably lower than 100 %, this figure may be somewhat too high.

Comparison between the properties of Phoratoxin and Viscotoxin A 3. Phoratoxin and Viscotoxin A 3 have been shown to have similar pharmacological effects.[6] Chemically as well, Phoratoxin is similar to Viscotoxin A 3. Thus, in chromatography on phosphate cellulose, using a linearly increasing gradient of NaCl for elution, as illustrated in Fig. 3, Phoratoxin was eluted at a NaCl concentration of 0.63 M. The corresponding figure for Viscotoxin A 3, when chromatographed under the same conditions, is 0.75 M (*cf.* Fig. 3, Ref. 4). Both substances are thus strongly adsorbed to phosphate cellulose. Both substances are strongly basic, moving towards the cathode in electrophoresis up to pH values about 11.[21] In addition to the amino acids found in Viscotoxin A 3,[1,4] Phoratoxin contains histidine, phenylalanine, and tryptophan (Table 1). The significance of the tryptophan content has been discussed in the aforegoing. As regards the content of the other amino acids, no remarkable differences are present between Phoratoxin and Viscotoxin A 3. In both cases, the high concentration of cystine, threonine, serine, and proline is noteworthy.

The N-terminal amino acid of Phoratoxin is lysine. No N-terminal amino acid has been found in intact Viscotoxin,[3] but after oxidation, the N-terminal amino acid of Viscotoxin A 3 has also proved to be lysine.[3] The quantitative results of the end-group determination indicate a molecular weight for Phoratoxin of 13 000, a figure which is probably too high.

Acknowledgements. This work was made possible by grants from the *Swedish Natural Science Research Council* and from the *Swedish Medical Science Research Council* (project No. B 67-14×-2084-01). The technical assistance of Ing. Lena Seger, Mr. Ulf Andersson and Mr. Bernt Staf is gratefully acknowledged. I also wish to express my gratitude to Badische Anilin und Sodafabrik, Ludwigshafen/Rhein, Germany, and to Professor Dr. R. Hänsel, Freie Universität, Berlin, Germany, for gifts of polyamide powder.

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Received January 5, 1967.