ACTA CHEMICA SCANDINAVICA 24 (1970) 2751–2756

Separation of Viscotoxins from the European Mistletoe, *Viscum album* L. (Loranthaceae) by Chromatography on Sulfoethyl Sephadex

GUNNAR SAMUELSSON and BARBRO PETTERSSON

Department of Pharmacognosy, Faculty of Pharmacy, Lindhagensgatan 128, S-112 51 Stockholm, Sweden

Chromatography of crude Viscotoxin fractions on SE-Sephadex yields three pure Viscotoxins called Viscotoxin A2, Viscotoxin A3, and Viscotoxin B. Viscotoxins A2 and A3 correspond to substances previously isolated as oxidized material called Viscotoxin A*ox2* and A*ox3*, respectively. Viscotoxin B is a new substance with the following amino acid composition: Ala 2, Arg 4, Asp 4, Cys 6, Glu 1, Gly 5, Ile 3, Leu 2, Lys 3, Pro 3, Ser 7, Thr 4 and Tyr 2.

The European mistletoe *Viscum album* L. (Loranthaceae) contains a number of small basic proteins with high toxicity as tested by parenteral administration in mice. A mixture of these substances was first isolated by Winterfeld and Bijl [1] and given the name Viscotoxin. A different isolation method was described by Samuelsson.[2] Attempts at separation of the proteins in this mixture by counter-current distribution [2,3] or chromatography on phosphate cellulose [4] were only partly successful. Thus Viscotoxin A3 – the purest product hitherto obtained – contained small amounts of other Viscotoxins even after repeated rechromatography on phosphate cellulose.[4] However, complete separation could be obtained after performic acid oxidation of the proteins [4,5] and the amino acid sequence of oxidized Viscotoxin A3 has been reported.[6] For determination of the position of the S–S-bridges in Viscotoxin A3 access to sufficient quantities of the native protein was necessary, and further work on the separation problem proved chromatography on sulfoethyl Sephadex (SE-Sephadex®) to be much superior to chromatography on phosphate cellulose. As described in this paper the new method permits separation of Viscotoxin A3, Viscotoxin A2 (previously isolated as the oxidized product Viscotoxin A*ox2*,[5] and a new substance called Viscotoxin B.

EXPERIMENTAL

Materials and apparatus

Viscotoxin was prepared from leaves or stems of *Viscum album* as previously described.[3]

SE-Sephadex C 25, Pharmacia AB, Uppsala, Sweden.

Buffer gradients for elution of chromatographic columns were linearly increasing in pH and ionic strength and were obtained by the arrangement described by Parr.[7] The vessel connected to the column contained 1/15 M phosphate buffer of pH 5.0 — here denoted as buffer I — and the buffer in the second vessel (buffer II) was 1/15 M phosphate buffer pH 8.0 containing NaCl to a concentration of 0.125 M.

Ultracentrifuge, Spinco, model E, equipped with the Schlieren optical system.

Methods

Chromatography of crude Viscotoxin was performed on phosphate cellulose as previously described,[4] yielding five peaks numbered I-V as presented in Fig. 1.

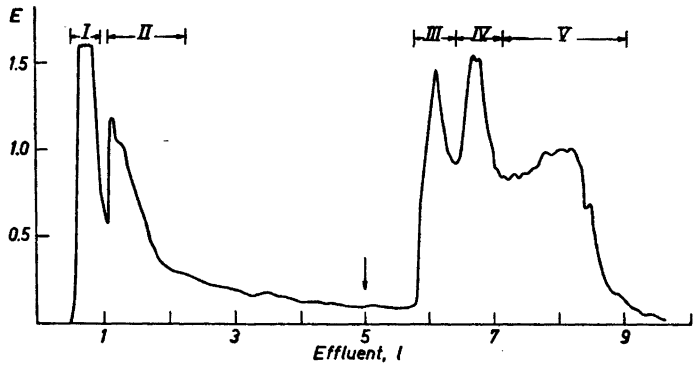

Fig. 1. Chromatography of 5 g of crude Viscotoxin on a column (3.7 × 92 cm) of phosphate cellulose (reproduced from Ref. 4).

Isolation of Viscotoxin B. 2.0 g of material from peak IV (Fig. 1) was dissolved in 7 ml 1/30 M KH_2PO_4 solution and the mixture applied to a column (2.5 × 49 cm) of SE-Sephadex previously equilibrated with buffer I (see above). Buffers I and II (6 l of each) were used for the gradient. Fractions of 25 ml were collected and their protein content was determined from the optical density at 280 mμ.

Viscotoxin B was eluted between 5.15 and 5.80 liters of effluent (Fig. 2). After desalting on a column of Sephadex G25 and freeze-drying, the yield of Viscotoxin B was 0.63 g (31 %).

Isolation of Viscotoxin A2 and A3. 0.77 g of material from peak V (Fig. 1) was fractionated and worked up as described above, using a small column (1.4 × 54 cm), 2.4 l each of buffers I and II and taking 15 ml fractions. Viscotoxin A2 (146 mg, 19 %) and Viscotoxin A3 (150 mg, 32 %) were eluted between 1.88—2.18 l and 2.40—2.85 l, respectively.

Oxidation of proteins. Performic acid oxidation of proteins was performed at 0° according to Hirs.[2]

High voltage electrophoresis. The method described by Samuelsson [9] was used with Sephadex G25 as supporting phase.

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] modified by Samuelsson.[12]

Qualitative test for tryptophan. 2 mg protein was dissolved in 1 ml $Ba(OH)_2$ solution (0.4 g/ml) and heated in a sealed tube at 105° for 20 H. After dilution with 10 ml H_2O, Ba^{2+} was precipitated with CO_2. The filtrate and washings were brought to dryness *in vacuo*, dissolved in 3 ml H_2O and filtered. 50 µl of this solution was spotted on a filter paper, dried and sprayed with Ehrlich's reagent (4 % *p*-dimethylaminobenzaldehyde in conc. HCl). One residue of tryptophan in a protein with a molecular weight of 5000 is easily detected by this test.

Test for sugars. The presence of non-nitrogenous sugars was tested by the Winzler orcinol-sulphuric acid procedure.[13] For amino sugars the Rondle and Morgan method was used.[14] The thiobarbituric acid assay [15] was used for testing the presence of sialic acids.

All these methods permit detection of one molecule of sugar in a substance with a molecular weight of 5000.

RESULTS AND DISCUSSION

As reported previously,[4] chromatography of crude Viscotoxin on phosphate cellulose yields five peaks numbered I-V (Fig. 1). Chromatography of the material from peak IV on SE-Sephadex gave the result presented in Fig. 2.

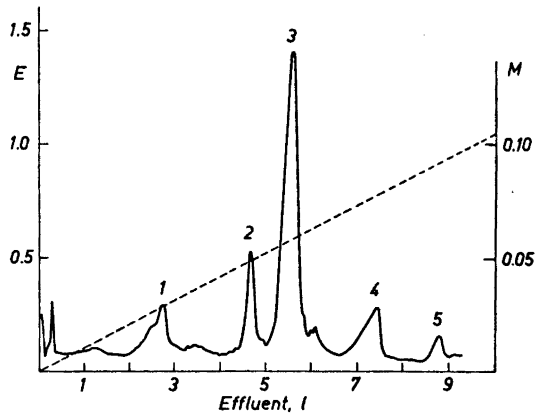

Fig. 2. Chromatography of 2.0 g of material from peak IV (Fig. 1) on a column (2.5 × 49 cm) of SE-Sephadex. For further details, see under Experimental. —— denotes optical density at 280 mµ fo the eluate (left ordinate). - - - indicates the calculated concentration of NaCl in the eluate (right ordinate). Peak 3 is Viscotoxin B.

One main peak and four small peaks were obtained. Rechromatography of the material from the main peak gave a substance which was electrophoretically homogeneous after performic acid oxidation (Fig. 4). This substance was denoted Viscotoxin B.

The result of chromatography of the material from peak V (Fig. 1) on SE-Sephadex is presented in Fig. 3. Two main peaks were obtained. Electrophoresis in Sephadex G 25 of performic-acid oxidized material from these peaks proved the substances to be electrophoretically homogeneous with the same mobilities as Viscotoxin A*ox2* and A*ox3*, respectively (Fig. 4). It is there-

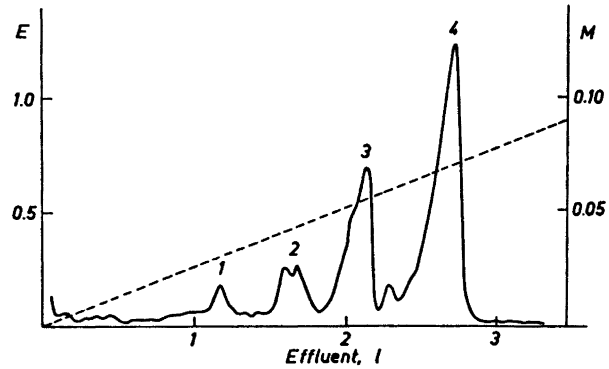

Fig. 3. Chromatography of 0.77 g of material from peak V (Fig. 1) on a column (1.4×54 cm) of SE-Sephadex. For further details, see under Experimental. —— denotes optical density at 280 mμ of the eluate (left ordinate). - - - indicates the calculated concentration of NaCl in the eluate (right ordinate). Peaks 3 and 4 are Viscotoxins A2 and A3, respectively.

fore concluded that these two peaks consist of pure native Viscotoxin A2 and Viscotoxin A3 which on performic acid oxidation give the previously studied substances Viscotoxin A*ox2* and Viscotoxin A*ox3*, respectively.

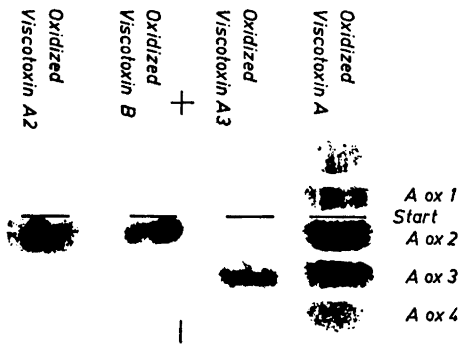

Fig. 4. Electrophoresis in Sephadex G 25 of performic acid oxidized Viscotoxins. Buffer: pyridine (0.15 M) — HOAc, pH 5.6, 20 V/cm, 3.5 h. Amount of sample 1 mg.

The amino acid compositions of Viscotoxin A2, Viscotoxin A3 and Viscotoxin B are presented in Table 1. The figures for Viscotoxins A2 and A3 are in complete agreement with the previously published amino acid compositions of Viscotoxins A*ox2* and A*ox3*.[5,6] The main differences between the three substances are that Viscotoxins B and A2 both contain glutamic acid which is absent in A3 and that valine and phenylalanine are only present in Viscotoxin A2.

Tests for tryptophan, sugars, amino sugars and sialic acids were negative in all three substances.*

* The tryptophan and sugar tests on Viscotoxin A2 were performed by Mr. Thomas Olson.

able 1. Amino acid composition of Viscotoxin A3, Viscotoxin A2, and Viscotoxin B. The results are expressed as number of residues per molecule.

Amino acid	Viscotoxin A3			Viscotoxin A2			Viscotoxin B	
	found	nearest integer	reported for Viscotoxin A*ox3*[6]	found	nearest integer	reported for Viscotoxin A*ox2*[5]	found	nearest integer
Lysine[a]	4.20	4	4	3.07	3	3	3.01	3
Arginine[a]	3.00	3	3	2.98	3	3	3.69	4
Aspartic acid[a]	4.08	4	4	4.16	4	4	4.00	4
Threonine[b]	4.62	5	5	3.90	4	4	4.07	4
Serine[b]	5.05	5	5	7.35	7	7	6.94	7
Glutamic acid[a]	—	—	—	0.97	1	1	0.96	1
Proline[a]	5.03	5	5	3.44	3	3	2.99	3
Glycine[a]	3.93	4	4	5.08	5	5	5.00	5
Alanine[a]	2.98	3	3	2.16	2	2	1.93	2
½ Cystine	5.91[b]	6	6[c]	5.65[b]	6	6[c]	6.16[c]	6
Valine[b]	—	—	—	0.93	1	1	—	—
Isoleucine[b]	2.87	3	3	2.93	3	3	2.94	3
Leucine[b]	1.93	2	2	1.03	1	1	2.08	2
Tyrosine[b]	1.62	2	2	1.97	2	2	1.95	2
Phenylalanine[a]	—	—	—	0.98	1	1	—	—

[a] Mean value of 2—5 determinations.
[b] Value extrapolated to zero time or maximum values as calculated from analysis of samples ydrolyzed for 24 h and 72 h.
[c] Determined as cysteic acid.

Table 2. Determination of molecular weights of Viscotoxin A2 and Viscotoxin B according to Archibald.

Min	$\frac{M \cdot (1-\overline{V}\varrho)}{RT} \times 10^{-8}$	
	Viscotoxin A2	Viscotoxin B
24	8.4	—
30	8.6	—
38	8.2	—
46	8.1	7.1
54	8.6	7.1
62	8.6	6.9
70	8.8	7.1
78	8.6	7.0
86	8.8	7.5
94	—	7.5
Mean value (graphically determined)	8.5	7.2

The molecular weights of Viscotoxin A2 and Viscotoxin B were determined in the ultracentrifuge by the Archibald method [16] as modified by Ehrenberg.[17] The results are presented in Table 2. The values for $M\cdot(1-\bar{V}\varrho)/RT$ do not change with time, indicating the sedimenting substances to be homogeneous. Calculation of the partial specific volumes [18] from the amino acid compositions (Table 1) of Viscotoxin A2 and Viscotoxin B gives the value 0.706 for both substances. Inserting this value in the mean value of $M\cdot(1-\bar{V}\varrho)/RT$ from Table 2 gives the molecular weights 7040 for Viscotoxin A2 and 5960 for Viscotoxin B. The minimum molecular weights for the two substances as calculated from the amino acid compositions are 4833 (Viscotoxin A2) and 4856 (Viscotoxin B). The molecular weight of Viscotoxin A*ox3* is 5123 [6] while determination by the Archibald method gave the value 9600.[4] This indicates that the Archibald method tends to give too high values for the molecular weights of the Viscotoxins, probably due to association phenomena. The molecular weights calculated from the amino acid compositions of Viscotoxin A2 and Viscotoxin B are in agreement with the number of peptides isolated from the tryptic hydrolysates of the performic acid oxidized proteins (details will be reported elsewhere).

Acknowledgements. This work was supported by grant No. B69-13x-2084-03 from the *Swedish Medical Science Research Council.* We also wish to express our gratitude to miss Puck Brodin and miss Eva Weckell for skilful technical assistance. The ultracentrifugation experiments were performed by Ing. Håkan Pertoft at the Department of Medical Chemistry, University of Uppsala, through the courtesy of Prof. Thorvald Laurent. This valuable help is hereby gratefully acknowledged.

REFERENCES

1. Winterfeld, K. and Bijl, L. M. *Ann.* **561** (1948) 107.
2. Samuelsson, G. *Svensk Farm. Tidskr.* **65** (1961) 481.
3. Samuelsson, G. *Svensk Farm. Tidskr.* **65** (1961) 209.
4. Samuelsson, G. *Acta Chem. Scand.* **20** (1966) 1546.
5. Olson, T. and Samuelsson, G. *Acta Chem. Scand.* **24** (1970) 720.
6. Samuelsson, G., Seger, L. and Olson, T. *Acta Chem. Scand.* **22** (1968) 2624.
7. Parr, C. W. *Proc. Biochem. Soc. 324th meeting XXVII.*
8. Hirs, C. H. W. *J. Biol. Chem.* **219** (1956) 611.
9. Samuelsson, G. *Svensk Farm. Tidskr.* **66** (1962) 201.
10. Hirs, C. H. W., Stein, W. H. and Moore, S. *J. Biol. Chem.* **211** (1954) 941.
11. Spackman, D. H., Stein, W. H. and Moore, S. *Anal. Chem.* **30** (1958) 1190.
12. Samuelsson, G. *Svensk Kem. Tidskr.* **80** (1967) 98.
13. François, C., Marshall, D. and Neuberger, A. *Biochem. J.* **83** (1962) 335.
14. Rondle, C. J. M. and Morgan, W. T. J. *Biochem. J.* **61** (1955) 586.
15. Warren, L. *J. Biol. Chem.* **234** (1959) 1971.
16. Archibald, W. *J. Phys. and Colloid Chem.* **51** (1947) 1204.
17. Ehrenberg, A. *Acta Chem. Scand.* **11** (1957) 1257.
18. Mc Meekin, T. L. and Marshall, K. *Science* **116** (1952) 142.

Received February 6, 1970.