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The Disulfide Bonds of Viscotoxin A3 from the European Mistletoe (*Viscum album* L., Loranthaceae)

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Viscotoxin A3 has been digested with pepsin and trypsin, and the resulting peptides have been isolated. Two peptides are key fragments for deduction of the position of the disulfide bonds. The amino acid composition of the two peptides, and partial amino acid sequences of fragments isolated after performic acid oxidation permit the following conclusions. One disulfide bond links residue 16 (Cys) with residue 26 (Cys) in the amino acid sequence of Viscotoxin A3. Two other disulfide bonds link residues 3 (Cys) and 4 (Cys) with residues 32 (Cys) and 40 (Cys), but it has not been possible to show if residue 3 (Cys) is linked to residue 32 (Cys) or to residue 40 (Cys), and *vice versa* for residue 4 (Cys). This arrangement of the disulfide bonds gives Viscotoxin A3 a very compact structure, which is also reflected in the remarkable stability of the substance, *e.g.* against heat.

Viscotoxin A3 is one of the pharmacologically active proteins, first isolated from the European mistletoe, *Viscum album* L., as a mixture called Viscotoxin.[1,2] Following performic acid oxidation of Viscotoxin, the oxidation product of Viscotoxin A3 was isolated[3] and the amino acid sequence determined.[4] A method for isolation of pure native Viscotoxin A3 was later developed.[5] Viscotoxin A3 contains 6 cysteine residues, forming 3 disulfide bonds. The availability of pure native Viscotoxin A3 has now permitted determination of the position of these disulfide bonds, as reported in this paper.

EXPERIMENTAL

Material and apparatus

Viscotoxin A3 was isolated from crude Viscotoxin [2] by chromatography on cellulose phosphate and SE-Sephadex®, as described previously.[3,5]

Trypsin, Worthington, 2 × crystallized, salt free. Batch 6118. The enzyme was treated with L-(1-tosylamido-2-phenyl) ethyl-chloromethyl ketone to inhibit chymotryptic activity, as described by Kostka and Carpenter.[6]

Pepsin, Bovine, 3 × crystallized. Serva Entwicklungslabor., Heidelberg, DBR.

Cation exchanger Dowex 50 W-X2. Purified and subjected to hydraulic separation of particle sizes as described previously.[4]

SE-Sephadex®. AB Pharmacia, Uppsala, Sweden.

Sephadex® G-10, G-15, and G-25. AB Pharmacia, Uppsala, Sweden.

pH-stat. Radiometer titrator TTTlc with titrigraph SBR2c. Radiometer, Copenhagen, Denmark.

Methods

Digestion of Viscotoxin A3 with pepsin and trypsin. 200 mg Viscotoxin A3 was dissolved in 20 ml of water, and the pH of the solution adjusted to 2.0 by addition of 6 N HCl. 8 mg pepsin was added, and the solution was kept at 37°C overnight. The pH was then adjusted to 6.5, and 3 ml 0.15 M $CaCl_2$ solution was added. After adjustment of the pH to 6.5, 2 ml of an aqueous trypsin solution (2 mg/ml) was added, and the alkali uptake during digestion at 37°C recorded. Another 2 ml of trypsin solution was added 3.5 h later (final enzyme : protein ratio 1 : 25), and the digestion continued overnight. The mixture was concentrated *in vacuo*, diluted with 2 % acetic acid, desalted by gel filtration in 2 % HOAc on Sephadex G – 25, and lyophilized.

Separation of peptides from the pepsin-trypsin digestion. The desalted, dry mixture of peptides was dissolved in 2 ml 1/30 M KH_2PO_4, and the solution applied to a column (10 × 300 mm) of SE-Sephadex, previously equilibrated with 1/15 M PO_4^{3-} buffer of pH 5.0. Elution was performed with a straight line buffer gradient, starting with 1/15 M PO_4^{3-} buffer of pH 5.0, and ending with 1/15 M PO_4^{3-} buffer, pH 8.0, containing NaCl to a concentration of 0.125 M. The gradient was obtained by the arrangement described by Parr,[7] and the total gradient volume was 1200 ml. Fractions of 5 ml were collected, and their optical density at 232 mμ determined. Fractions corresponding to UV-absorbing peaks were pooled, concentrated *in vacuo*, and desalted on Sephadex G-15. Isolated peptides were dissolved in 2 % HOAc, and the solutions stored in the refrigerator.

Amino acid analysis. Hydrolysis of peptides for amino acid analysis was performed as described by Hirs *et al.*,[8] with modifications according to Crestfield *et al.*[9] The amino acids were determined with an automatic amino acid analyzer, according to Spackman *et al.*,[10] as modified by Samuelsson.[11]

Oxidation of peptides. Oxidation was performed with performic acid at 0°C, according to Hirs.[12]

Separation of oxidized peptides. The procedure of Schroeder *et al.*[13] was used. The column of Dowex 50W-X2 had the dimensions 9 × 1200 mm, and the total volume of the buffer gradient was 1935 ml. Fractions of 5 ml were collected, and aliquots of 0.20 or 0.40 ml were taken from every second fraction for hydrolysis and analysis by the ninhydrin method, as described previously.[4] Fractions corresponding to ninhydrin positive peaks were pooled, desalted on Sephadex G-10, concentrated to dryness *in vacuo*, and stored in the refrigerator as solutions in 2 % HOAc, until further investigated.

Determination of amino acid sequences. Subtractive Edman degradation was used, as previously described.[4] Amino terminal amino acids were also identified by treatment of the peptides with dansyl chloride,* hydrolysis of the reaction product, and identification of the liberated dansyl amino acids by thin layer chromatography, as described by Stehelin and Duranton.[15]

RESULTS AND DISCUSSION

The conditions used for the enzymatic degradation of native Viscotoxin A3 are essentially the same as those used by Spackman *et al.* in the studies of the disulfide bonds of ribonuclease.[14] However, digestion with chymotrypsin was omitted, as better results were obtained by digestion with pepsin and trypsin only.

Separation of the digestion products by chromatography on SE-Sephadex is illustrated in Fig. 1. Eight peaks were obtained, numbered 1 – 8 in the

* 1-Dimethylamino-naphthalene-5-sulfonyl chloride.

figure. Amino acid analysis showed peaks 3, 4, 6, and 8 to contain cystine (Table 1). Except for the analysis of the material from peak 8, the figures of Table 1 have not been corrected for the destruction of serine, threonine,

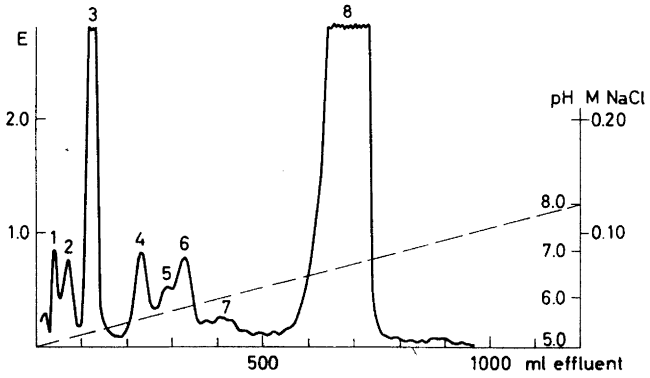

Fig. 1. Separation of peptides from Viscotoxin A3 after digestion with pepsin and trypsin. Column: SE-Sephadex (10 × 300 mm). Gradient elution with PO_4^{3-} buffer. —, optical density at 232 mμ (left ordinate). – – –, calculated pH and NaCl concentration of gradient (right ordinate). For details: see text.

Table 1. Amino acid analysis of cystine-containing peptides, isolated from a pepsin-trypsin hydrolysate of Viscotoxin A3 (*cf.* Fig. 1).

Amino acid	Peak 3[a]	Peak 4[a]	Peak 6[a]	Peak 8[b]
Lysine	2.1	1.2	2.6	4.1
Arginine	0.9	1.0	0.9	2.9
Aspartic acid	1.9	1.0	2.3	4.0
Threonine	2.6	0.8	3.1	4.9[c]
Serine	3.7	0.4	5.2	4.9[c]
Proline	2.8	1.0	3.4	5.0
Glycine	2.6	0.3	3.0	3.9
Alanine	–	1.6	–	2.9
½ Cystine	3.5	1.9	3.7	6.0[d]
Isoleucine	0.6	0.2	0.9	2.7[e]
Leucine	0.8	0.1	1.0	1.9[e]
Tyrosine	0.7	0.2	0.9	1.8

[a] Single analysis. 24 h hydrolysis.
[b] Mean values of 3 analyses.
[c] Corrected for destruction during hydrolysis.
[d] Determined as cysteic acid.
[e] After 72 h hydrolysis.

or tyrosine. The values for isoleucine are probably also too low, as the –Ile–Ile-bond (residues 34 and 35 in Viscotoxin A3) is very resistant to acid hydrolysis. When these facts are taken into account, the results of the amino acid analyses indicate that peak 3 represents a peptide containing residues

2 – 10 and 29 – 46 of Viscotoxin A3, with two disulfide bonds (*cf.* Fig. 4). Peak 6 could be the same peptide as peak 3, but with the amino terminal lysine (residue 1) intact. Peak 4 corresponds to a peptide formed from residues 14 – 17 and 24 – 28, with one disulfide bond (*cf.* Fig. 4). Peak 8 represents undigested Viscotoxin A3, as seen from the amino acid composition. The peptides obtained from peaks 3, 4, and 6 are in the following denoted as peptides PT3, PT4, and PT6.

Peptide PT4. This peptide was oxidized with performic acid, and the fragments separated on Dowex 50W-X2, as illustrated in Fig. 2. Two peptides

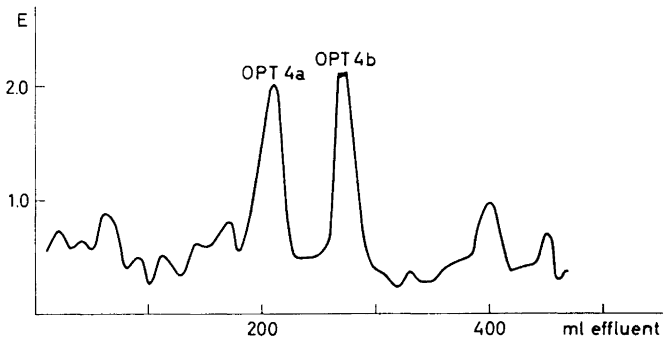

Fig. 2. Separation of oxidation products of peptide *PT4* on Dowex 50W-X2 (9 × 1200 mm). Gradient elution with pyridine-HOAc buffer, as described in the text.

were obtained, denoted *OPT4a*, and *OPT4b*. Edman degradation of these peptides, as presented in Table 2, gave the partial amino acid sequence *Pro-Thr-Cys*(SO_3H)-(*Ala*, *Lys*) for *OPT4a*, and *Asx-Ala*-(*Cys*(SO_3H), *Arg*)* for *OPT4b*, thereby demonstrating a disulfide bond between residues 16 and 26 in the amino acid sequence of Viscotoxin A3 (*cf.* Fig. 4).

Table 2. Amino acid composition and Edman degradation of peptides *OPT4a* and *OPT4b*.

Amino acid	*OPT4a* Step				*OPT4b* Step		
	0	1	2	3	0	1	2
Lysine	1.0	[a]	[a]	[a]	–	–	–
Arginine	–	–	–	–	1.0	[a]	[a]
Cysteic acid	0.9	1.1	1.1	*0.6*	1.2	1.2	1.0
Aspartic acid	–	–	–	–	1.0	*0.2*	0.1
Threonine	0.7	0.8	*0.5*	0.4	–	–	–
Proline	0.9	*0*	0	0	–	–	–
Alanine	1.0	1.0	1.0	1.0	1.0	1.0	*0.2*

[a] Not determined.

* Asx = Aspartic acid or asparagine.

Peptide PT6. Chromatographic separation on Dowex 50W-X2 of the oxidation products of peptide *PT6* yielded 4 peptides, denoted *OPT6a*, *OPT6b*, *OPT6c*, and *OPT6d* (Fig. 3). Table 3 shows the amino acid composition of these peptides (step 0). The isoleucine values of peptides *OPT6a* and *OPT6b* are too low, as they were obtained from a 24 h hydrolysate only.

Dansylation of the peptides showed that the amino terminal amino acids of the peptides were the following: *OPT6a* and *OPT6b*: isoleucine; *OPT6c*: lysine; and *OPT6d*: leucine. Three steps of Edman degradation of peptides

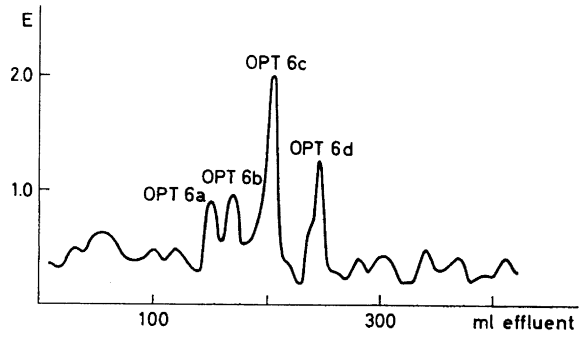

Fig. 3. Separation of oxidation products of peptide *PT6* on Dowex 50W-X2 (9 × 1200 mm). Gradient elution with pyridine-HOAc buffers, as described in the text.

Table 3. Amino acid composition and Edman degradation of peptides *OPT6a*–*OPT6d*.

Amino acid	*OPT6a* Step				*OPT6b* Step				*OPT6c* Step			*OPT6d* Step		
	0	1	2	3	0	1	2	3	0	1	2	0	1	2
Lysine	0.9	×	×	×	1.1	×	×	×	0.9	*0*	×	0.8	×	×
Arginine	—	—	—	—	—	—	—	—	0.7	0.6	×	—	—	—
Cysteic acid	1.2	1.0	1.0	1.0	1.0	1.0	1.1	0.7	2.1	2.0	1.7	0.8	1.0	1.0
Aspartic acid	1.0	1.0	1.0	1.2	1.0	*a*	1.1	1.0	1.0	1.0	1.0	—	—	—
Threonine	0.9	0.8	0.8	1.0	1.0	1.0	1.0	0.9	1.8	1.7	1.6	—	—	—
Serine	3.0[b]	2.8[b]	2.9[b]	*2.2*[b]	2.9[b]	3.1[b]	2.7[b]	*2.1*[b]	1.3[b]	1.3[b]	*0.4*[b]	1.2[b]	1.1[b]	*0.2*[b]
Proline	2.1	1.7	2.0	1.9	1.9	2.0	2.0	1.9	1.0	1.1	1.0	—	—	—
Glycine	1.3	1.1	1.0	1.0	1.4	1.0	1.2	1.3	1.2	1.0	1.0	1.0	1.1	0.9
Isoleucine	1.2[c] (2)	*0.9*[d]	*0.2*[d]	0	0.6[c] (2)	*0.8*	*0.1*	0	—	—	—	—	—	—
Leucine	—	—	—	—	—	—	—	—	—	—	—	0.6	*0*	0
Tyrosine	0.7[c]	0.4[d]	0.5[d]	+	0.2	—	—	—	—	—	—	—	—	—
3-Chlorotyrosine	—	—	—	—	+	+	+	+	—	—	—	—	—	—

× Not determined.
[a] Not determined, due to technical error.
[b] Values corrected for destruction during hydrolysis, assuming 10 % loss in 24 h and 30 % in 72 h.
[c] 24 h hydrolysis.
[d] 72 h hydrolysis.
\+ Present, but not determined.

OPT6a and *OPT6b*, and two degradation steps of peptides *OPT6c* and *OPT6d* are presented in Table 3. These results give the following partial amino acid sequences for the four peptides: *OPT6a*: *Ile-Ile-Ser-(Gly,2Ser,Thr,Cys(SO_3H), 2Pro,Tyr,Asx,Lys)*. *OPT6b*: *Ile-Ile-Ser-(Gly,2Ser,Thr,Cys(SO_3H),2Pro,TyrCl, Asx,Lys)*. *OPT6c*: *Lys-Ser-(2Cys(SO_3H),Pro,Asx,2Thr,Gly,Arg)*. *OPT6d*: *Leu-Ser-(Gly,Cys(SO_3H),Lys)*.

These results can be interpreted as follows: *OPT6a* consists of residues 34 – 46 of Viscotoxin A3. *OPT6b* is an artefact, formed by chlorination of the tyrosine residue in *OPT6a*. *OPT6c* and *OPT6d* correspond to peptides containing residues 1 – 10 and 29 – 33, respectively (*cf.* Fig. 4). Thus, peptide *PT6* contains residues 1 – 10 and 29 – 46 of the Viscotoxin A3 sequence, and two disulfide bonds connect residues 3 (Cys) and 4 (Cys) with residues 32 (Cys) and 40 (Cys).

Peptide PT3. The amino acid composition of this peptide (Table 1) indicates that it has been formed from peptide *PT6* by tryptic cleavage of the amino terminal lysine residue. This peptide was therefore subjected to partial acid hydrolysis with the aim of breaking the peptide bond between the two cysteine residues Nos. 3 and 4, and isolating peptide fragments containing the disulfide bridges and a sufficient number of connected amino acids, to give an answer to the question if residue 3 is linked to residue 32 or to residue 40, and *vice versa* for residue 4. However, despite several attempts, these experiments were unsuccessful, and this question must therefore remain open until better methods are developed.

The arrangement of the disulfide bridges in Viscotoxin A3. The results of this investigation are summarized in Fig. 4. One disulfide bond links residue 16 (Cys) to residue 26 (Cys), forming a loop of 9 amino acids in the chain. The amino terminal and carboxyl terminal ends of the molecule are brought close to each other by two disulfide bridges, connecting residues 3 (Cys) and

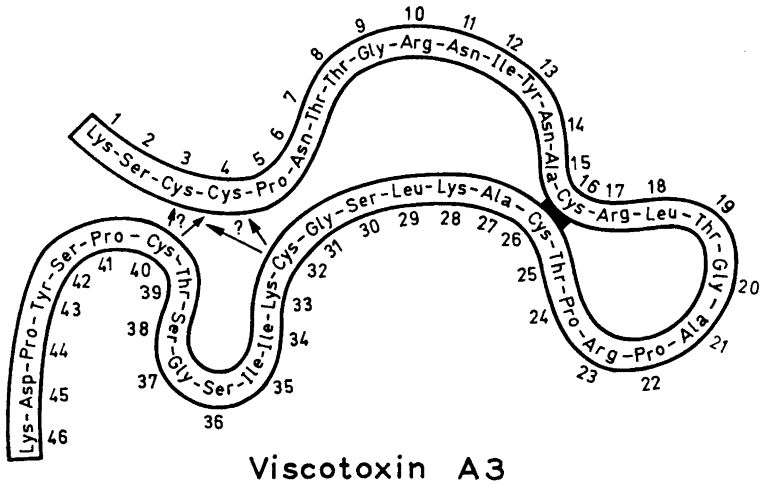

Fig. 4. The arrangement of the disulfide bonds in Viscotoxin A3.

4 (Cys) with residues 32 (Cys) and 40 (Cys) also forming a second loop of 7 amino acids (residues 33 – 39). This arrangement of the disulfide bonds gives the molecule a very compact structure, which explains the unusual stability of Viscotoxin A3. Thus heating an aqueous solution to 100°C for 30 min has no influence on the toxicity of the substance, and aqueous solutions have also been kept in the refrigator for several years with no loss of biological activity.

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