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The Amino Acid Sequence of Oxidized Viscotoxin A3 from the European Mistletoe (*Viscum album* L, Loranthaceae)

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Oxidized Viscotoxin A3 has been digested with trypsin and chymotrypsin. The resulting peptides have been separated by ion exchange chromatography and subjected to amino acid sequence determination by Edman degradation. 7 tryptic and 5 chymotryptic peptides are primary digestion products, and permit deduction of the following amino acid sequence for the 46 amino acids of oxidized Viscotoxin A:

Lys—Ser—Cys(SO_3H)—Cys(SO_3H)—Pro—Asn—Thr—Thr—Gly—
—Arg—Asn—Ile—Tyr—Asn—Ala—Cys(SO_3H)—Arg—Leu—Thr—
—Gly—Ala—Pro—Arg—Pro—Thr—Cys(SO_3H)—Ala—Lys—Leu—
—Ser—Gly—Cys(SO_3H)—Lys—Ile—Ile—Ser—Gly—Ser—Thr—
—Cys(SO_3H)—Pro—Ser—Try—Pro—Asp—Lys.

The actions of trypsin and chymotrypsin are normal except for a Thr—Cys(SO_3H) bond (residues 25 and 26) which is broken by chymotrypsin. The molecular weight of oxidized Viscotoxin A3, as calculated from this sequence, is 5123.

Viscotoxin A3 is one of the main components of Viscotoxin, a mixture of pharmacologically active, small, basic proteins isolated from the European mistletoe, *Viscum album* L. Viscotoxin A3 and its oxidation product, Viscotoxin A*ox3*, are obtained by chromatography on phosphate cellulose.[1] This paper describes the determination of the amino acid sequence of Viscotoxin A*ox3*.

EXPERIMENTAL

Materials and apparatus

*Viscotoxin A*ox3 was obtained by previously described methods.[1]

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 according to Kostka and Carpenter.[2]

α-*Chymotrypsin*, Worthington CDI 6114—5.

Leucine aminopeptidase, Sigma type III from hog kidney. Lot 16B—0201.

Carboxypeptidase A, Worthington, 2× crystallized, Batch No. COA 726, 52 U/mg protein. 1 ml of the suspension was mixed with 5 μl diisopropyl fluorophosphate (DFP),

and 3 ml water. The crystals were collected by centrifugation and washed once with 3 ml water containing 5 μl DFP. They were then mixed with 5 ml 2 M NH_4HCO_3, 5 μl DFP and dissolved by addition of conc. ammonia to pH 10.3—10.5 (pH meter). When everything was dissolved the pH was lowered to 8.0 by addition of conc. acetic acid. Aliquots of the solution (0.3 ml) were dispensed into ampoules which were sealed and kept frozen until use.

Carboxypeptidase B, Worthington. Batch COBDFP7GA. 120 U/mg protein.

Cation exchanger Dowex 50 W—X2. Before use, the ion-exchanger was purified and subjected to hydraulic separation of particle sizes as described by Moore *et al.*[3] with the exception of the treatment with NaOH which was performed at room temperature instead of on the steam bath. The fraction collected at a water flow of 517—650 ml/min was used for packing the column.

H-Stat. Radiometer titrator TTTlc with titrigraph SBR2c.

Methods

Digestion with trypsin. 200 mg of Viscotoxin A*ox3* was digested at pH 8.5 and a temperature of 37°C for 21 h. The procedure was essentially the same as described by Samuelsson and Li [4] for tryptic digestion of lactogenic hormone. Hence the protein was dissolved in 38 ml of water and the solution titrated in the pH-stat with 0.25 N NaOH to pH 8.5. A N_2 barrier prevented uptake of CO_2 from the air. After the addition of 1.9 ml 0.15 M $CaCl_2$, 1 ml of a trypsin solution (2 mg/ml) was added and the alkali uptake recorded. Another 1 ml of trypsin solution was added after 40 min (final enzyme: protein ratio 1:50). After the completion of the reaction, HCl was added to bring the pH to 6.0. 2.9 ml of a pyridine—3 N formic acid buffer of pH 3.0 was added, followed by a few drops of 6 N HCl to bring the pH to 2.5. The mixture was finally diluted with water to 50 ml.

Digestion with chymotrypsin. 560 mg of Viscotoxin A*ox3* was dissolved in 275 ml water in the reaction vessel of the pH-stat and the pH brought to 8.5 by addition of 0.1 N NaOH. 1 ml of a solution of chymotrypsin (18 mg/ml) was added and the alkali uptake recorded. After 15 h the pH was lowered to 3.3. A solution of 5 mg L(1-tosylamido-2-phenyl)ethyl-chloromethyl ketone was added, and the solution was lyophilized.

Chromatography of peptides on Dowex 50W—X2. For separation of the peptides from the tryptic and chymotryptic digestions, the procedure of Schroeder *et al.*[5] was followed. For analysis aliquots of 0.50 ml or 0.20 ml were taken from every second fraction. 1 ml 2.5 N NaOH was added to each sample and the mixture heated in a boiling water bath for 2.5 h. 0.5 ml of water was added after 1.5 h. After completed hydrolysis 1.0 ml of 30 % acetic acid was added followed by 1.00 ml of ninhydrin reagent.[6] The tubes were covered with glass balls and heated in a boiling water bath for 15 min. After addition of 4.0 ml of aqueous ethanol (1:1 V/V) and shaking, the optical density at 570 mμ was determined. Tubes, corresponding to the peaks obtained, were pooled, concentrated *in vacuo* in a rotary evaporator and lyophilized. The peptide content of the fractions was checked by peptide mapping.

Chromatography of peptides on Dowex 1—X2. The procedure of Schroeder *et al.*[5] was followed in complete detail. Analysis of the fractions were performed on 0.20 ml aliquots as described above. Fractions corresponding to ninhydrin positive peaks were pooled, concentrated, lyophilized and examined by peptide mapping. Pure peptides were desalted on Sephadex G15, lyophilized and dissolved in 5.0 ml of 2 % aqueous butanol containing 2 % HOAc and stored in the deep freeze until further investigated. The concentrations of the peptide solutions were calculated from the results of the amino acid analyses.

Separation of peptides by paper chromatography. Peptides which did not separate in ion-exchange chromatography were subjected to preparative descending paper chromatography on Whatman 3M filter paper with propanol-conc. ammonia-water (60:6:34) as solvent. The sample was applied as a streak in an amount of about 0.5 μmole of peptide per cm. After the development of the chromatogram, 0.5 cm wide guidestrips were cut on both sides and in the middle. The guidestrips were treated with ninhydrin and strips corresponding to ninhydrin positive bands on the guide strips were cut from the chromatogram and eluted with 2 % acetic acid.

Peptide maps. The purity of the isolated peptides was checked by peptide mapping using the thin-layer techniques of Ritschard.[7] Plates 20×40 cm were coated with Silica Gel G (0.25 mm) and 10 µl of the sample corresponding to about 0.1 µmole was applied to a spot 20 cm from the short edge and 4 cm from the long edge of the plate. Chromatography was performed in the short direction of the plate using propanol-conc. ammonia-water (60:6:34) as solvent. When the front had reached 11 cm from the starting point, the plates were dried for 5 min at 105°C. For electrophoresis, the plate was sprayed with buffer (4 ml pyridine, 40 ml glacial acetic acid and water to 2 l, pH 3.5) in direction from the short edges towards the middle. The plate was placed on a water cooled aluminium block and connected to the electrode vessels *via* paperwicks and cellophane membranes to prevent buffer flow, as described for electrophoresis in Sephadex gel.[8] The plate was covered with a glass plate, separated from the silica gel layer by strips of filter paper placed along the long edges of the thin-layer plate. Electrophoresis was performed for 2.5 h at 2000 V, whereupon the plates were dried at 105°C for 20 min and then sprayed with ninhydrin reagent (0.05 g ninhydrin, 50 ml ethanol, 2 ml collidine and 15 ml conc. acetic acid) and heated for 5 min at 105°C. To detect peptides giving none or only a faint reaction with ninhydrin, the chlorotolidine test of Reindel and Hoppe [9] was used.

Preparative thin-layer chromatography and electrophoresis. The technique for peptide mapping was also used for preparative purposes. The mixture to be separated was applied as a streak on a 1 mm thick layer of Silica Gel H (20×40 cm) in an amount of about 0.15 µmole/cm. Depending on the results of previous peptide mapping, the chromatographic or the electrophoretic step was chosen for the preparative separation. The separated bands were located by spraying the edges of the plate with ninhydrin. Zones containing peptides were scraped off, transferred to a chromatographic tube and eluted with 50 ml 10 % HOAc.

Quantitative amino acid analysis. Peptides 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]

Amino acid sequence analysis. The phenylisothiocyanate (PITC) method of Sjöquist *et al.*[12] and of Blombäck *et al.*[12a] was used with the following modifications. N_2 was bubbled through all reagents before use and in all reactions the air was removed from the reaction vessel by flushing with N_2. For coupling with PITC 1—5 µmole of peptide was dissolved in 0.10 ml buffer (2.40 ml newly distilled triethylamine, 4 ml 2 N HOAc, water to 25 ml) and a solution of 23 µmole of PITC in 0.20 ml pyridine was added. After completion of the reaction, the mixture was shaken two times with 0.30 ml of benzene. The combined benzene phases were shaken with 0.2 ml water which was added to the aqueous phase which was then lyophilized over-night. The remaining PITC was removed from the residue by sublimation *in vacuo* at 50°C. After cleavage with trifluoroacetic acid, the peptide residue was precipitated by the rapid addition of a mixture of dichloroethane and peroxide free ether (0.75+0.90 ml). The precipitate was collected by centrifugation and washed twice with 0.4 ml of the dichloroethane-ether mixture. The combined organic phases were washed twice with 0.2 ml of water and the water phases added to the peptide and lyophilized. The organic phases were rapidly evaporated at 40°C in a jet of nitrogen and the thiazolinone converted to the thiohydantoin by the addition of 0.20 ml 1 N HCl and heating at 80°C for 30 min. In cases where little or no precipitate was formed, on addition of the dichloroethane-ether mixture, water (0.2 ml) was added directly [13] and the mixture shaken and centrifuged. The aqueous phase was washed twice with 0.4 ml of the dichloroethane-ether mixture and the phases separated by centrifugation and treated as described above.

The thiohydantoins were identified by paper chromatography as described by Sjöquist.[14] PTH-lysine was separated from PTH-glycine and PTH-alanine in the solvent system of Wallén and Sjöholm.[15] In later stages of the work the TLC system of Jeppson and Sjöquist [16] was also used.

Before subjecting the peptide residue to the next cycle, an aliquot was taken for hydrolysis and quantitative amino acid analysis.

Digestion with carboxypeptidase A. 0.3 µmole peptide was dissolved in 0.6 ml water and 10 µl carboxypeptidase A solution (see above) added. The digestion was performed at room temperature and aliquots of 200 µl taken at 5, 15, and 60 min after start of the experiment. The aliquots were immediately mixed with 0.1 ml conc. HOAc. 1 ml citrate buffer pH 2.2 was added and the mixture applied to the amino acid analyzer.

When a more extensive digestion was required, the enzyme concentration and digestion time were increased.

Digestion with carboxypeptidase B. 0.2 μmole peptide was dissolved in 0.1 ml 0.02 M phosphate buffer of pH 8.0. 70 μl carboxypeptidase B solution (as delivered by the manufacturer) was added and the mixture kept at 37°C over-night followed by determination of liberated amino acids.

Digestion with LAP. 0.2 μmole peptide was dissolved in 0.4 ml water. 50 μl 0.025 M $MgCl_2$, 50 μl 0.5 M TRIS buffer pH 8.5, and 10 μl LAP (as delivered by the manufacturer) were added and the mixture kept at 37°C over-night. Liberated amino acids were determined using the automatic amino acid analyzer.

Partial acid hydrolysis of peptide OT2. 6 μmole peptide was hydrolyzed with 3 ml 0.1 N HCl at 110°C for 24 h. The hydrolyzate was desalted on Sephadex G 10 and lyophilized. The residue was separated into 7 fractions by preparative thin-layer electrophoresis on Silica Gel H as described above.

RESULTS AND DISCUSSION

Separation of tryptic peptides

Chromatography of the tryptic digestion mixture of Viscotoxin A*ox3* on Dowex 50W—X2 is illustrated in Fig. 1. Eight peaks were obtained, numbered 1—8 in the figure. Peptide mapping showed peaks 1, 3, 5, 6, and 8 to contain pure peptides. They were designated as peptides *OT1*, *OT2*, *OT3*, *OT4*, and *OT5*, respectively. The other peaks represented mixtures of peptides, and were separated by chromatography on Dowex 1—X2.

Peptides from peak 2 (Fig. 1). Material from this peak gave one faint and one heavy spot in the peptide map. The result of chromatography on Dowex 1—X2 is presented in Fig. 2. One very high peak was obtained together with some small peaks. As all samples were hydrolyzed prior to the ninhydrin reaction, it is not likely that these small peaks can be attributed to main peptides in the tryptic digest of Viscotoxin A*ox3*. Peptide mapping showed that the high peak B corresponded to the heavy spot in the peptide map of

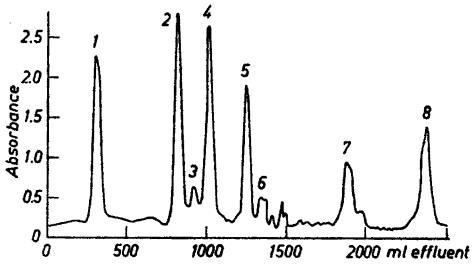

Fig. 1. Chromatography of a tryptic hydrolysate of 200 mg of Viscotoxin A*ox3* on Dowex 50W—X2. Gradient elution with pyridine-acetic buffer as described in the text. The figure shows the chromatogram only for the first 2500 ml of eluate. No more peaks were obtained at higher buffer concentrations or with 2 N NaOH.

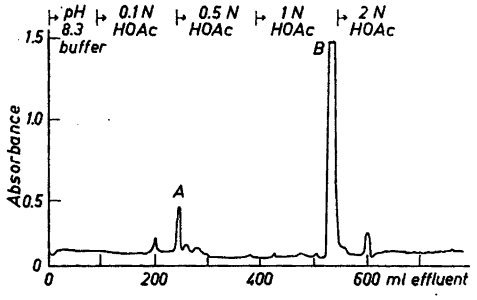

Fig. 2. Chromatography of peptides from peak 2 (Fig. 1) on Dowex 1—X2. The composition of the liquid flowing into the mixing chamber is indicated in the upper part of the figure.

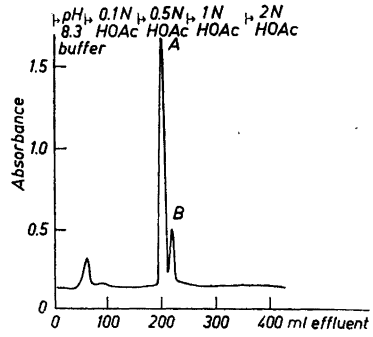

Fig. 3. Chromatography of peptides from peak 4 (Fig. 1) on Dowex 1—X2. The composition of the liquid flowing into the mixing chamber is indicated in the upper part of the figure.

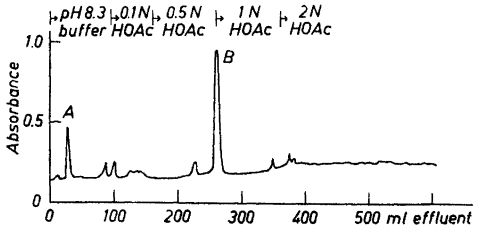

Fig. 4. Chromatography of peptides from peak 7 (Fig. 1) on Dowex 1—X2. The composition of the liquid flowing into the mixing chamber is indicated in the upper part of the figure.

the material from peak 2 in the Dowex 50 chromatogram. This material was designated as peptide *OT6*. Peak A in the Dowex 1 chromatogram corresponded to the faint spot in the peptide map of peak 2 (Fig. 1).

Peptides from peak 4 (Fig. 1). Peptide mapping of material from this peak gave 3 spots. Fig. 3 shows the result of chromatography on Dowex 1—X2. Peptide mapping showed peak A to be a mixture, while peak B represented small amounts (< 1 μmole) of a peptide which contained lysine, cysteic acid, threonine, and alanine. Material from peak A was separated by preparative paper chromatography yielding 3 fractions. One of these proved to consist of a pure peptide designated as *OT7* while the two others were still mixtures. On chromatography on Dowex 1—X2 in another buffer system, one of the remaining 2 fractions from the separation on paper yielded another pure peptide called *OT8*. The third fraction contained only small amounts of peptides which were not investigated further.

Peptides from peak 7 (Fig. 1). Peptide maps showed three heavy spots as well as some faint spots. The result of chromatography on Dowex 1—X2 is presented in Fig. 4. Two main peaks were obtained. Peptide maps proved the first eluted peak (A) to be a pure substance designated as *OT9*, while the second peak (B) still represented a mixture. Preparative paper chromatography of this mixture gave two pure peptides *OT10* and *OT11* together with very small amounts of a still unresolved mixture. Peptide mapping showed that none of the other peaks of Fig. 4 represented peptides.

The amino acid composition of the peptides from the tryptic digest of Viscotoxin A*ox3* is presented in Table 1. The yield of the various peptides as indicated in the table have been calculated from the amino acid analyses of the purified substances and have not been corrected for losses in the various separation steps and from the taking of samples for identification. In the steps involving preparative paper chromatography, these losses can be estimated to be heavy.

Table 1. Amino acid composition of tryptic peptides from Viscotoxin A*ox3*.

Amino acid	OT1	OT2	OT3	OT4	OT5	OT6	OT7	OT8	OT9	OT10	OT11
Lysine		1.0	0.8			1.0	0.9	0.7	1.0		
Arginine	1.0			1.0	0.8		0.8			1.0	
Cysteic acid	2.0	1.1	1.0	1.2		1.1	2.3	1.1		1.0	
Aspartic acid	1.0	0.9		1.0		1.2	1.0			2.0	1.0
Threonine	2.0	1.1			1.0	1.0	1.8	0.9			
Serine	1.0	2.4	0.9			2.5	1.1				
Proline	1.0	1.9			1.0	2.0	1.1	1.0			
Glycine	1.0	0.7	1.0		1.0	1.0	1.1				
Alanine				1.0	1.0			1.0		1.0	
Isoleucine		1.5				1.8				1.0	1.0
Leucine			1.0		1.0						
Tyrosine						0.9				0.6	
3-Chlorotyrosine		×									×
Yield, μmoles from 32 μmoles Viscotoxin A*ox3*	18	4	17	7	12	12	4.1	2.5	11	8	2

Amino acid sequences of tryptic peptides

The determination of amino acid sequences of the peptides was founded on the results of Edman degradation of the peptides, followed by amino acid analysis of an aliquot of the remaining peptide fragment after each degradation step. The results were in most cases confirmed by paper chromatographic identification of the phenyl-thiohydantoins formed from the liberated N-terminal amino acids. Asparagine was distinguished from aspartic acid by TLC chromatography of the PTH derivative or from results of digestion with carboxypeptidase or LAP.

Peptide OT1. The results of Edman degradation are presented in Table 2. The degradation was carried through 8 steps (7 presented in the table). After

Table 2. Edman degradation of peptide *OT1*.

Amino acid	Residues after step No.							
	0	1	2	3	4	5	6	7
Serine	1.0	*0.3*	0.1	0.1	0.1	0.1	0.2	0.2
Cysteic acid	2.0	2.0	*1.3*	*0.7*	0.6	0.7	0.7	0.7
Proline	1.0	1.1	1.1	1.0	*0.3*	0	0	0
Aspartic acid	1.0	1.0	1.0	1.0	1.0	*0.4*	0.4	0.4
Threonine	2.0	1.8	1.8	1.8	1.8	1.8	*1.5*	*1.1*
Glycine	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Arginine	1.0	*	*	*	*	*	*	*

* Not determined.

Table 3. Edman degradation of peptide *OT2*.

Amino acid	Residues after step No.									
	0	1	2	3	4	5	6	7	8	9
Isoleucine	(1.1) 1.5 (75h)	*0.8*	*0.1*	0	0	0	0	0	0	0
Serine	2.4	2.7	2.4	*1.7*	1.4	*1.0*	1.0	1.0	1.0	*0.8*
Glycine	0.7	0.8	0.7	0.7	*0.3*	0.2	0.2	0.2	0.3	0.3
Threonine	1.1	1.0	0.8	0.8	0.7	0.7	*0.3*	0.3	0.3	0.3
Cysteic acid	1.1	1.0	1.1	1.1	0.9	1.0	1.1	*0.7*	0.6	0.7
Proline	1.9	1.7	1.8	1.8	1.8	1.8	2.1	1.9	*1.5*	1.6
3-Chlorotyrosine	×	×	×	×	×	×	×	×	×	×
Aspartic acid	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Lysine	1.0	*	*	*	*	*	*	*	*	*

* No determined.

step 8, the presence of free arginine was demonstrated. Carboxypeptidase A+B at 37° for 24 h liberated glycine, threonine, and asparagine in the ratio: 1:1.6:0.6. (Arginine not determined.) The amino acid sequence of peptide *OT1* is thus: *Ser—Cys(SO_3H)—Cys(SO_3H)—Pro—Asn—Thr—Thr—Gly—Arg.*

Peptide OT2. This peptide contains 13 amino acids. The chromatograms from the amino acid analyses show one peak appearing much later than phenylalanine on the long column and just before lysine on the short column. The position of this peak corresponds exactly with that of 3-chlorotyrosine which is known to be formed on performic acid oxidation of proteins containing tyrosine in the presence of traces of Cl^- ion. In an ordinary 24 h hydrolysate of the peptide only 1 residue of isoleucine seemed to be present. After hydrolysis for 75 h this figure increased to 1.5 indicating the presence of 2 residues of isoleucine. This was confirmed by Edman degradation (Table 3) which was carried through 9 steps indicating the following sequence for the first 9 N-terminal amino acids:

Ile—	Ile—	Ser—	Gly—	Ser—	Thr—	Cys(SO_3H)—	Pro—	Ser—
1	2	3	4	5	6	7	8	9

Various attempts at further degradation of peptide *OT2* with other proteolytic enzymes such as pepsin, papain, and subtilisin gave no fragments which could be used for determination of the rest of the sequence. As a rule only the first two or three amino acids were split off and the remaining fragment was still too big for a complete Edman degradation. Digestion with carboxypeptidase B liberated lysine in good yield indicating this amino acid to be C-terminal. Digestion with carboxypeptidase A+B liberated lysine and aspartic acid. Hydrolysis of peptide *OT2* with 0.01 N HCl at 110° for 24 h liberated only aspartic acid and lysine in equimolecular yield. Since aspartic acid is known to be the amino acid which is most easily split off by acid hydrolysis, this result confirms the C-terminal sequence Asp—Lys. Partial acid

Table 4. Edman degradation of a peptide liberated from peptide *OT2* by partial acid hydrolysis.

Amino acid	Residues after step No.			
	0	1	2	3
Threonine	1.0	*0.6*	0.5	0.5
Cysteic acid	1.2	1.0	*0.8*	0.7
Proline	1.0	0.9	0.9	*0.7*
Serine	0.9	1.1	1.0	1.0

hydrolysis with 0.1 N HCl yielded several fragments. One of these was isolated and subjected to Edman degradation. The results are presented in Table 4. Amino acid analysis of the non-hydrolyzed aqueous phase from the third step showed the presence of free serine. Thus the amino acid sequence of this peptide is Thr—Cys(SO_3H)—Pro—Ser. This confirms the sequence of amino acids 6—9 of *OT2* found on Edman degradation of the whole peptide. This leaves only the amino acids 3-chlorotyrosine and proline to be placed in sequence. As will be shown later, peptide *OT2* is the same peptide as *OT6* with tyrosine chlorinated. For peptide *OT6* the corresponding sequence is most probably Tyr—Pro. From this follows that the amino acid sequence of peptide *OT2* is *Ile—Ile—Ser—Gly—Ser—Thr—Cys(SO_3H)—Pro—Ser—TyrCl —Pro—Asp—Lys.*

Peptide OT3. Results of the Edman degradation of this peptide are presented in Table 5. The degradation was carried through four steps. Assuming normal tryptic action, the sequence of this peptide is *Leu—Ser—Gly—Cys(SO_3H)—Lys.*

Peptide OT4. Table 6 shows the results of the Edman degradation of this peptide. The phenylthiohydantoin of cysteic acid was found in the third degradation step (identified by paper chromatography in Sjöquist's solvent IV [14]). Amino acid analysis of the non-hydrolyzed aqueous phase from the

Table 5. Edman degradation of peptide *OT3.*

Amino acid	Residues after step No.				
	0	1	2	3	4
Leucine	1.0	*0.2*	0.1	0	0
Serine	0.9	0.8	*0.3*	0.3	0.3
Glycine	1.0	0.9	1.0	*0.7*	0.7
Cysteic acid	1.0	1.1	1.1	1.0	*0.1*
Lysine	0.8	*	*	*	*

* Not determined.

Table 6. Edman degradation of peptide *OT4*.

Amino acid	Residues after step No.		
	0	1	2
Aspartic acid	1.0	*0.5*	0.2
Alanine	1.0	1.0	*0.5*
Cysteic acid	1.2	1.0	1.0
Arginine	1.0	*	*

* Not determined.

third step showed the presence of free arginine. The amino acid sequence of peptide *OT4* is as follows: *Asp(n)—Ala—Cys(SO_3H)—Arg*.

Peptide OT5. For results of the Edman degradation refer to Table 7. After completion of step 6 the presence of free arginine was demonstrated. The amino acid sequence of the peptide is thus: *Leu—Thr—Gly—Ala—Pro—Arg*.

Peptide OT6. Edman degradation of this peptide was carried through 7 steps, whereupon no conclusive results were obtained. The peptide contains 13 amino acid residues. The results of the Edman degradation (Table 8) gives the following partial amino acid sequence: Ile—Ile—Ser—(Gly,Ser)—Thr——Cys(SO_3H)—(2Pro,Ser,Tyr,Asp,Lys). Carboxypeptidase B liberated lysine in good yield indicating this amino acid to be C-terminal. Carboxypeptidase A+B liberated lysine and aspartic acid. These results show that peptides *OT6* and *OT2* are identical, the only difference being that the tyrosine of *OT2* is chlorinated. The yields of peptides *OT2* and *OT6* were very different when two different batches of Viscotoxin A*ox3* were digested with trypsin. In the experiment illustrated in Fig. 1, *OT6* was the main peptide while in another experiment, *OT2* was obtained in a 10 times higher yield than *OT6*. Peptide *OT6* is not affected by digestion with chymotrypsin. This indicates a

Table 7. Edman degradation of peptide *OT5*.

Amino acid	Residues after step No.				
	0	1	2	3	4
Leucine	1.0	*0.1*	0.1	0	0
Threonine	1.0	0.8	*0.4*	0.3	0.3
Glycine	1.0	1.0	1.0	*0.5*	0.5
Alanine	1.0	1.0	1.0	1.0	*0.5*
Proline	1.0	0.9	1.0	0 9	1.0
Arginine	0.8	*	*	*	*

* Not determined.

Table 8. Edman degradation of peptide *OT6*.

Amino acid	Residues after step No.							
	0	1	2	3	4	5	6	7
Isoleucine	1.8	*1.0*	*0*	0	[a]	0	0	0
Serine	2.5	2.7	2.5	*1.8*	2.0	*1.6*	1.6	1.5
Glycine	1.0	1.0	1.0	1.0	[a]	*0.4*	0.4	0.5
Threonine	1.0	0.9	0.9	0.9	0.9	1.0	*0.6*	0.6
Cysteic acid	1.1	1.0	1.0	1.1	1.0	1.0	1.1	*0.7*
Proline	2.0	2.0	1.9	1.8	1.9	2.2	2.0	1.9
Tyrosine	0.9	1.0	0.9	0.8	[a]	0.7	0.8	0.8
Aspartic acid	1.2	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lysine	1.0	[b]	[b]	[b]	[b]	[b]	[b]	[b]

[a] Not determined due to technical error.
[b] Not determined.

sequence —Tyr—Pro— in the peptide which sequence is known not to be split by chymotrypsin. From these data the following amino acid sequence of peptide *OT6* is deduced: *Ile—Ile—Ser—Gly—Ser—Thr—Cys(SO_3H)—Pro——Ser—Tyr—Pro—Asp—Lys*.

Peptide OT7. The results of Edman degradation are presented in Table 9. These results and the specificity of trypsin permit deduction of the following amino acid sequence. Lys—Ser—Cys(SO_3H)—Cys(SO_3H)—Pro—Asp(n)—Thr——Thr—Gly—Arg. With the exception of the N-terminal lysine, the amino acid sequence is the same as for peptide *OT1*. Peptide *OT7* is thus resulting from incomplete tryptic splitting of the Lys—Ser bond. As has been shown for peptide *OT1*, the aspartic acid is present as the amide. Thus, the amino acid sequence of peptide *OT7* is: *Lys—Ser—Cys(SO_3H)—Cys(SO_3H)—Pro—Asn——Thr—Thr—Gly—Arg*.

Table 9. Edman degradation of peptide *OT7*.

Amino acid	Residues after step No.								
	0	1	2	3	4	5	6	7	8
Lysine	0.9	*0.04*	*	*	*	*	*	*	*
Serine	1.1	1.2	*0.4*	0.3	0.3	0.3	0.3	0.2	0.3
Cysteic acid	2.3	2.3	2.4	*1.9*	*1.2*	1.1	1.0	1.0	0.8
Proline	1.1	1.1	1.1	1.0	1.1	*0.6*	0.4	0.5	0.5
Aspartic acid	1.0	1.0	1.0	1.0	1.0	1.0	*0.5*	0.5	0.5
Threonine	1.8	1.9	1.7	1.7	1.7	1.7	1.7	*1.4*	*1.0*
Glycine	1.1	1.2	1.2	1.1	1.1	1.0	1.1	1.1	1.1
Arginine	0.8	1.0	*	*	*	*	*	*	*

* Not determined.

Table 10. Edman degradation of peptide *OT8*.

Amino acid	Residues after step No.			
	0	1	2	3
Proline	1.0	*0*	0	0
Threonine	0.9	0.8	*0.5*	0.4
Cysteic acid	1.1	1.1	1.1	*0.8*
Alanine	1.0	1.0	1.0	1.0
Lysine	1.0	*	*	*

* Not determined.

Peptide OT8. The Edman degradation was carried through three steps, the result of which is presented in Table 10. Assuming normal tryptic action, the sequence of peptide *OT9* is: *Pro—Thr—Cys(SO_3H)—Ala—Lys.*

Peptide OT9. This substance was found to be free lysine, presumably representing the N-terminal amino acid of peptide *OT7*.

Peptide OT10. The result of Edman degradation (5 steps) is illustrated in Table 11. Digestion with LAP showed both aspartic acid residues to be present as asparagine. The phenylthiohydantoin of cysteic acid and free arginine were demonstrated to be present after completion of the 6th degradation step. Thus peptide *OT10* has the following amino acid sequence: *Asn—Ile—Tyr—Asn—Ala—Cys(SO_3H)—Arg.*

Peptide OT11. This peptide which was obtained in low yield contained aspartic acid, isoleucine, and 3-chlorotyrosine. Edman degradation showed the sequence to be *Asp—Ile—TyrCl.*

Table 11. Edman degradation of peptide *OT10*.

Amino acid	Residues after step No.					
	0	1	2	3	4	5
Aspartic acid	2.0	*1.2*	1.1	1.0	*0.6*	0.4
Isoleucine	1.0	1.0	*0.2*	0.1	0	0
Tyrosine	0.6	0.7	0.8	*0.2*	0	0
Alanine	1.0	1.0	1.0	1.0	1.0	*0.6*
Cysteic acid	1.0	1.1	1.0	1.1	1.1	1.0
Arginine	1.0	*	*	*	*	*

* Not determined.

Number of tryptic peptides and the molecular weight of Viscotoxin *Aox3*

The amino acid analysis of Viscotoxin A*ox3* indicated 88 amino acid residues for a molecular weight of 9736.[1] The total number of amino acid residues contained in peptides *OT1—OT11* is 71. Peptide *OT2* is an artefact formed by chlorination of the tyrosine in peptide *OT6*. Peptides *OT4* and *OT11* are probably fragments of peptide *OT10* resulting from chymotryptic activity of the trypsine. Finally peptide *OT7* is the result of incomplete tryptic release of the N-terminal lysine which normally should give free lysine (=*OT9*) and peptide *OT1*. No more peptides were found in amounts sufficiently high to be of structural importance (< 1 μmole). This result was confirmed in a second experiment. In addition to spots corresponding to peptides *OT1—OT11*, peptide maps of the total hydrolysate of Viscotoxin A*ox3* showed only a few very weak spots (as revealed by ninhydrine and *o*-tolidine reagents). These spots were not reproducible in different hydrolysates.

Thus only peptides *OT1*, *OT3*, *OT5*, *OT6*, *OT8*, *OT9* and *OT10* can be considered to be primary peptides from the tryptic digestion of Viscotoxin A*ox3*. Table 12 shows the summarized amino acid composition of these peptides compared with the results of the previous amino acid analysis of Viscotoxin A*ox3*[1] calculated for half the previously assumed molecular weight. The agreement is very good indicating that the molecular weight 9600 found on ultracentrifugation[1] is too high, which could be due to the formation of a dimer.

Table 12. Summarized amino acid composition of peptides *OT1*, *OT3*, *OT5*, *OT6*, *OT8*, *OT9*, and *OT10*.

Amino acid	Sum of residues from peptides	Residues per mole of Viscotoxin if one residue = 0.183 μmole/mg (from Table 1, Ref. 1)	No. of residues to nearest integer
Lysine	4	3.6	4
Arginine	3	2.8	3
Cysteic acid	6	6.2	6
Aspartic acid	4	4.0	4
Threonine	5	5.0	5
Serine	5	4.5	5
Proline	5	5.0	5
Glycine	4	4.1	4
Alanine	3	3.0	3
Isoleucine	3	2.5	3
Leucine	2	2.0	2
Tyrosine	2	1.5	2
	46		46

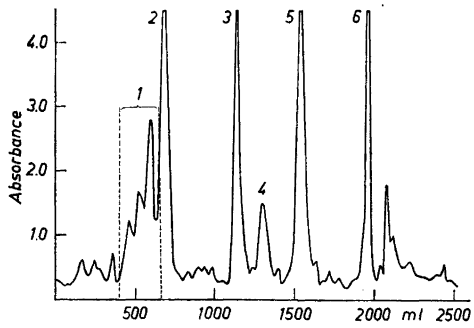

Fig. 5. Chromatography of a chymotryptic hydrolysate of 560 mg of Viscotoxin A*ox3* on Dowex 50W—X2. Gradient elution with pyridine-acetic acid buffer. The figure shows the chromatogram only for the first 2500 ml of eluate. No more peaks were obtained at higher buffer concentrations or with 2 N NaOH.

Separation of chymotryptic peptides

Chromatography on Dowex 50W—X2 of the mixture of peptides from digestion of Viscotoxin A*ox3* with chymotrypsin is presented in Fig. 5. 6 main peaks were obtained numbered 1—6 in the figure. Peptide mapping showed that peaks 2, 3, 4, and 6 contained pure peptides. These peptides were designated as peptides *OC2*, *OC3*, *OC4* and *OC7*.

Peaks 2 and 5 contained mixtures of peptides and were further separated by chromatography on Dowex 1—X2. Substance was also recovered from the smaller peaks in Fig. 5. Amino acid analysis of this material showed that they represented only small amounts of peptides (<10 % of the amount of the main peptides) which were not investigated further.

Peptides from peak 1 (*Fig. 5*). The result of chromatography on Dowex 1—X2 of the material from this peak is illustrated in Fig. 6. Two peaks were obtained both of which by peptide mapping were found to represent pure peptides. Amino acid analysis and position on the peptide map showed the second peak to be identical with peptide *OC2*. The peptide from the first peak was designated as peptide *OC1*.

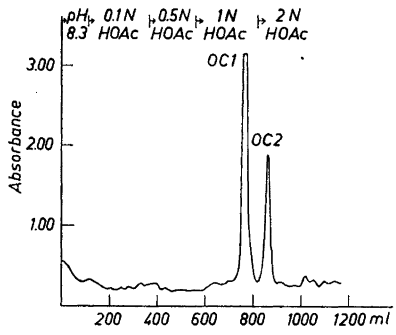

Fig. 6. Separation of peptides *OC1* and *OC2* by chromatography on Dowex 1—X2. The composition of the liquid flowing into the mixing chamber is indicated in the upper part of the figure.

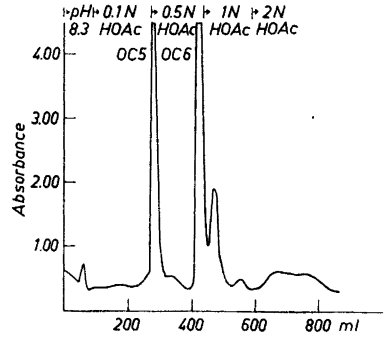

Fig. 7. Separation of peptides *OC5* and *OC6* by chromatography on Dowex 1—X2. The composition of the liquid flowing into the mixing chamber is shown in the upper part of the figure.

Peptides from peak 5 (Fig. 5). Chromatography of this material on Dowex 1—X2 gave 3 peaks (Fig. 7). Peptide mapping showed all three to be pure peptides but from amino acid analysis, the third peak was found to represent only small amounts of peptide. The peptides from the first two peaks were designated as peptides *OC5* and *OC6*, respectively. Yields and amino acid composition of peptides *OC1—OC6* are presented in Table 13.

Table 13. Amino acid composition of chymotryptic peptides from Viscotoxin A*ox3*.

Amino acid	OC1	OC2	OC3	OC4	OC5	OC6	OC7
Lysine	2.2	1.6	1.0	1.0		1.0	
Arginine				1.0	0.9	0.9	1.0
Cysteic acid	2.5	2.2	1.1	2.0	1.1	2.1	
Aspartic acid	1.0	1.1		1.9	1.0	2.2	
Threonine	0.9	1.0		1.8		1.9	1.6
Serine	3.4	3.5		1.1		0.8	
Proline	2.0	2.0		1.0		1.0	2.1
Glycine	1.8	1.8		1.0		1.0	1.1
Alanine			1.0		0.9		1.0
	(1.0)	(1.0)					
Isoleucine	1.7	1.9		0.9		0.9	
	(75h)	(75h)					
Leucine			1.0		1.0		
Tyrosine	0.8			0.6			
3-Chlorotyrosine		×				×	
Yield, μmoles from 75 μmoles Viscotoxin A*ox3*	16	40	54	31	44	24	51

Table 14. Edman degradation of peptide *OC1*.

Amino acid	Residues after step No.					
	0	1	2	3	4	5
Serine	3.4	*2.8*	2.9	2.8	2.8	2.9
Glycine	1.8	1.7	*1.2*	0.9	1.0	0.9
Cysteic acid	2.5	2.0	2.0	*1.6*	1.5	1.4
Lysine	2.2	1.4	1.7	1.5	*1.2*	1.1
Isoleucine	(1.0) 1.7	1.2	1.2	1.2	1.1	*0.9*
Threonine	0.9	1.1	1.0	0.8	0.8	0.7
Proline	2.0	2.0	2.0	2.0	2.0	2.0
Tyrosine	0.8	0.6	*	0.6	0.8	0.6
Aspartic acid	1.0	0.9	1.0	0.8	0.9	0.9

* Not determined.

Amino acid sequence of chymotryptic peptides

Amino acid sequences of the chymotryptic peptides were determined as described for the tryptic peptides.

Peptide OC1. The results of Edman degradation are presented in Table 14. These results and demonstrations of the formation of corresponding phenylthiohydantoins permitted deduction of the following partial amino acid sequence: *Ser—Gly—Cys(SO_3H)—Lys—Ile(3Ser,Gly,Cys(SO_3H),Lys,Ile,Thr, 2Pro,Tyr)*. Carboxypeptidase B liberated lysine indicating this amino acid to be C-terminal. The partial amino acid sequence and the amino acid composition shows that peptide *OC1*-forms the overlap between the tryptic peptides *OT3* and *OT6*.

Peptide OC2. Results of Edman degradation of this peptide are presented in Table 15. Carboxypeptidase B liberated lysine. These results and the amino acid composition show that peptides *OC1* and *OC2* are identical except for the tyrosine residue, which is chlorinated in peptide *OC2*.

Peptide OC3. 3 steps of Edman degradation of this peptide are presented in Table 16. Free leucine was demonstrated in the residue after step 3. From these results the amino acid sequence of peptide *OC3* is: *Cys(SO_3H)—Ala——Lys—Leu*.

Table 15. Edman degradation of peptide *OC2*.

Amino acid	Residues after step No.					
	0	1	2	3	4	5
Serine	3.5	*2.6*	2.5	2.5	2.5	2.3
Glycine	1.8	1.7	*1.0*	1.1	1.0	0.8
Cysteic acid	2.2	2.3	2.1	*1.4*	1.4	1.0
Lysine	1.6	1.4	1.3	1.5	*1.0*	0.3
Isoleucine	(1.0) 1.9	1.2	1.1	1.3	1.0	*0.8*
Threonine	1.0	1.0	0.9	1.0	1.0	0.8
Proline	2.0	2.0	2.0	2.0	2.0	2.0
3-Chlorotyrosine	×	×	×	×	×	×
Aspartic acid	1.1	1.0	0.9	1.0	1.0	1.0

Table 16. Edman degradation of peptide *OC3*.

Amino acid	Residues after step No.			
	0	1	2	3
Cysteic acid	1.1	*0.4*	0.4	0.3
Alanine	1.0	1.0	*0.4*	0.3
Lysine	1.0	0.9	0.9	*0.6*
Leucine	1.0	1.0	1.0	1.0

Table 17. Edman degradation of peptide *OC4*.

Amino acid	Residues after step No.				
	0	1	2	3	4
Lysine	1.0	*0.2*	0	0	0
Serine	1.1	1.0	*0.2*	0	0
Cysteic acid	2.0	2.3	2.2	*1.5*	*0.9*
Proline	1.0	1.1	1.1	1.1	1.4
Aspartic acid	1.9	1.9	1.8	1.8	1.8
Threonine	1.8	1.8	1.8	1.6	1.7
Glycine	1.0	1.0	1.0	0.9	1.0
Arginine	1.0	1.0	1.0	0.8	0.9
Isoleucine	0.9	0.9	0.9	0.9	0.9
Tyrosine	0.6	0.9	0.7	0.6	0.8

Peptide OC4. Table 17 shows the results of 4 steps of Edman degradation of this peptide, establishing the sequence of the first 4 amino acids as Lys—Ser—Cys(SO_3H)—Cys(SO_3H)—. Digestion with carboxypeptidase A for 5 min liberated 1 residue of tyrosine and 0.2 residues each of isoleucine and asparagine. After 60 min digestion with carboxypeptidase A, 1 residue each of tyrosine, isoleucine, and asparagine had been liberated. After tryptic digestion of peptide *OC4*, a peptide map showed 3 spots, two of which were in the same positions as the tryptic peptides *OT1* and *OT7*, respectively. The peptide corresponding to the third spot was isolated by preparative thin layer chromatography. Amino acid analysis and Edman degradation showed the amino acid sequence of the peptide to be Asn—Ile—Tyr. These results permitted deduction of the following partial amino acid sequence for peptide *OC4*. *Lys—Ser—Cys(SO_3H)—Cys(SO_3H)—(Pro,Asp,2Thr,Gly,Arg)—Asp—Ile—Tyr*. From this follows that peptide *OC4* forms the overlap between the tryptic peptides *OT1*, *OT10* and the amino terminal lysine (*OT9*).

Peptide OC5. The results of Edman degradation of this peptide are presented in Table 18. Free leucine was present in the residue after step No. 4. Digestion with LAP showed the aspartic acid residue to be present as asparagine. From

Table 18. Edman degradation of peptide *OC5*.

Amino acid	Residues after step No.				
	0	1	2	3	4
Aspartic acid	1.0	*0.2*	0.2	0	0
Alanine	0.9	0.9	*0.4*	0	0
Cysteic acid	1.1	0.8	1.1	*0.4*	0
Arginine	0.9	0.9	1.0	1.0	*0.1*
Leucine	1.0	1.0	1.0	0.9	1.0

Table 19. Edman degradation of peptide *OC6*.

Amino acid	Residues after step No.					
	0	1	2	3	4	5
Lysine	1.0	*0*	0	0	0	0
Serine	0.8	0.8	*0*	0	0	0
Cysteic acid	2.1	2.1	2.2	*1.1*	*0.5*	0.5
Proline	1.0	1.0	1.0	1.1	1.0	*0.6*
Aspartic acid	2.2	1.8	1.8	2.0	1.8	1.8
Threonine	1.9	1.8	1.8	1.9	1.6	1.7
Glycine	1.0	0.9	1.0	0.9	1.0	1.0
Arginine	0.9	1.0	0.9	0.9	0.9	0.8
Isoleucine	0.9	0.9	1.0	1.0	1.0	0.9
3-Chlorotyrosine	×	×	×	×	×	×

these results it follows that the amino acid sequence of peptide *OC5* is: *Asn—Ala—Cys(SO_3H)—Arg—Leu*.

Peptide OC6. Edman degradation of this peptide was carried through 5 steps, the results of which are presented in Table 19. A peptide map of a tryptic hydrolysate showed 3 spots, two of which occupied the positions of tryptic peptides *OT1* and *OT7*. The peptide corresponding to the third spot was isolated by preparative thin-layer chromatography and shown to contain asparagine (from digestion with LAP), isoleucine, and 3-chlorotyrosine, with asparagine as the N-terminal amino acid. These results show that peptide *OC6* is identical with peptide *OC4* but with the tyrosine residue chlorinated.

Peptide OC7. The results from Edman degradation of this peptide are shown in Table 20. The results give the following amino acid sequence for peptide *OC7*: *Thr—Gly—Ala—Pro—Arg—Pro—Thr*. This sequence forms the overlap between tryptic peptides *OT5* and *OT8*.

Table 20. Edman degradation of peptide *OC7*.

Amino acid	Residues after step No.						
	0	1	2	3	4	5	6
Threonine	1.6	*1.2*	1.1	0.9	1.0	1.0	1.0
Glycine	1.1	1.0	*0.4*	0.3	0.3	0.3	0.3
Alanine	1.0	1.0	1.0	*0.2*	0.3	0.3	0.2
Proline	2.1	2.2	2.2	1.9	*1.3*	1.2	*0.8*
Arginine	1.0	*	1.0	1.0	1.0	*0.2*	0.2

* Not determined.

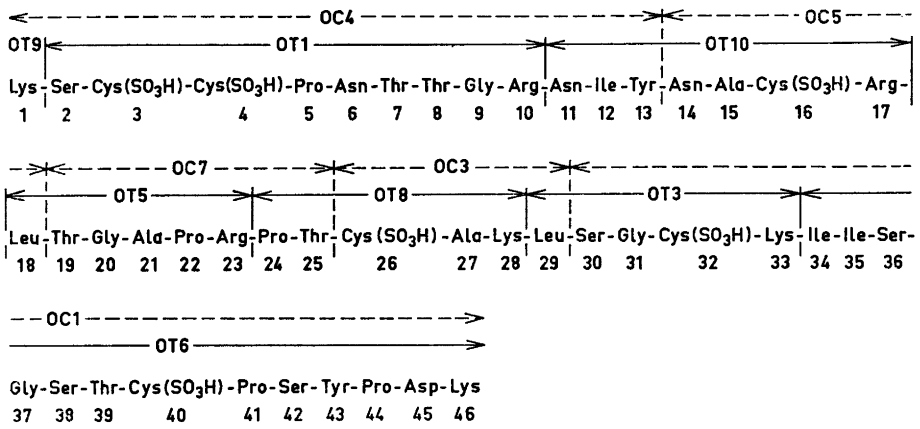

Fig. 8. The amino acid sequence of oxidized Viscotoxin A3.

The complete amino acid sequence of oxidized Viscotoxin A3

The results of determination of amino acid sequences of peptides formed by digestion of oxidized Viscotoxin A3 with chymotrypsin shows that peptides *OC1*, *OC3*, *OC4*, *OC5*, and *OC7* are true chymotryptic peptides, while *OC2* and *OC6* are artefacts formed by chlorination of a tyrosine residue during oxidation of the protein. Peptides *OC1—5* and *OC7* account for all the amino acids of the primary tryptic peptides (Table 12). As has been previously shown [17] the sequence of the first two amino acids of Viscotoxin A*ox3* is Lys—Ser—. Overlapping sequences in the tryptic and chymotryptic series of peptides permit the establishment of the complete amino acid sequence of oxidized Viscotoxin A3 as presented in Fig. 8.

The sites of attack by trypsin and chymotrypsin have been indicated in the figure. They are all normal except for the cleavage by chymotrypsin of a Thr—Cys(SO_3H) bond between residues 25 and 26. The failure of chymotrypsin to attack a Tyr—Pro bond (residues 43—44) has been reported several times, *e.g.* in corticotropin and ribonuclease.

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