Chromatography of Viscotoxin and Oxidized Viscotoxin on Phosphate Cellulose

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Purification of Viscotoxin by chromatography on phosphate cellulose has been found to be a simple and more effective method than purification by countercurrent distribution. Following performic acid oxidation, of the purest substance obtained by the new method, electrophoresis in Sephadex G 25 reveals the presence of Viscotoxin Aox 3, and small amounts of Viscotoxin Aox 2. Oxidation products of Viscotoxin are readily separated by chromatography on phosphate cellulose. Viscotoxin Aox 3 has the following amino acid composition: Ala 6, Arg 6, Asp 8, Cysteic acid 12, Gly 8, Glu 5, Leu 4, Lys 7, Pro 10, Ser 9, Thr 10, Tyr 3, and Amide NH₂ 6. The molecular weight calculated from these data is 9736. Viscotoxin Aox 3 is homogeneous in a sedimentation experiment in the ultracentrifuge and the molecular weight determined according to Archibald is 9600.

Viscotoxin is a mixture of small basic proteins with high toxicity as tested by parenteral administration in mice. Viscotoxin was first isolated by Winterfeld and Bijl,¹ and further studied by Winterfeld and Rink,² Winterfeld and Leiner,³ and Winterfeld and Heuken.⁴ A different isolation method for Viscotoxin was described by Samuelsson.⁵ This method involved adsorption of the basic proteins on a weak cation exchanger and gel filtration of the eluted material giving a crude Viscotoxin. Further purification was obtained by countercurrent distribution yielding a substance called Viscotoxin A.

Although Viscotoxin A and the purest product isolated by Winterfeld et al. — Viscotoxin S4 — were similar in many respects, some differences existed. Thus Viscotoxin S4 contained arabinose, glucose, and the amino acids histidine, methionine, and phenylalanine. None of these substances was found in Viscotoxin A. These discrepancies have now been clarified since Winterfeld and Kirschbaum⁶ on reinvestigation of Viscotoxin S4 found it to be impure. Through a modified technique involving gel filtration through Sephadex G 25 and G 50 and purification by continuous paper electrophoresis they isolated two proteins which have amino acid compositions very similar to that of Viscotoxin A.

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Although Viscotoxin A behaved as a homogeneous substance in countercurrent distribution experiments, there was evidence for it being a mixture of closely related substances. Thus, performic acid oxidation gave a product which showed five bands in paper electrophoresis. The amino acid composition of the corresponding substances indicated that they had not been formed by rupture of SS-bonds in a big protein molecule. Small amounts of the oxidation products were isolated by electrophoresis in Sephadex and one of them was more extensively studied.

Countercurrent distribution thus gave only partial separation of the Viscotoxins and the electrophoretic technique permitted isolation of the oxidation products of Viscotoxin A only on a semimicro scale. This paper describes methods for preparative separation of Viscotoxins and their oxidation products by chromatography on phosphate cellulose, and some properties of one of the oxidized proteins: Viscotoxin Ace 3.

EXPERIMENTAL

Materials and apparatus

Viscotoxin was prepared from leaves or stems of Viscum album as described previously. The peptide fraction obtained after gelfiltration through Sephadex G 25 is in the following called crude Viscotoxin.

Phosphate cellulose. Whatman P 70, was successively washed with NaOH (0.1 N), water, HCl (0.1 N), water, NaOH (0.1 N), water, and stored at 2°C in water or buffer containing 2% butanol.

Sephadex G 25, fine in the bead form. AB. Pharmacia, Uppsala, Sweden.

Buffers were prepared with sodium acetate (reagent grade) and acetic acid (reagent grade) in appropriate concentrations. Butanol (2%) was added as a preservative.

Uvicord spectrophotometer, LKB-Produkter AB, Stockholm 12, was used for continuous recording of the optical density from chromatographic columns at 254 μm. Peaks recorded with the instrument were also checked by determining the optical density at 280 μm.

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

Methods

Chromatography of crude Viscotoxin. 5 g of crude Viscotoxin was dissolved in 25 ml of 0.05 M NaOAc buffer, pH 5.0, containing 0.2 M NaCl. The solution was applied to a column (3.7 x 92 cm) of phosphate cellulose previously equilibrated with this buffer. The same buffer was also used for elution. The eluate was collected in 25 ml fractions. When 5 liters of the effluent had passed the column, the NaCl concentration of the eluant was raised to 0.7 M and another 4.5 liters collected. Further elution with 1 N NaOH gave only small amounts of UV-absorbing material. The fractions corresponding to the different peaks were pooled and concentrated to 40 ml in vacuo in a rotary evaporator at a temperature not exceeding 40°C. Precipitated NaCl was filtered off and washed with a small amount of water, which was added to the filtrate. NaCl was removed from the solution by filtration through a column of Sephadex G 25 (3.7 x 64 cm) using 2% HOAc as eluant. The salt-free proteins were recovered by freeze-drying. Yields: (cf. Fig. 1) peak I: 0.31 g, peak II: 0.20 g, peak III: 0.64 g, peak IV: 0.87 g, peak V: 1.44 g.

Rechromatography of material from peak V in Fig. 1. 2.9 g of material from peak V was dissolved in 40 ml of 0.05 M NaOAc buffer pH 5.0 containing 0.2 M NaCl and the solution applied to a column (4.2 x 82 cm) of phosphate cellulose previously equilibrated with the same buffer. Elution was performed with 0.05 M NaOAc buffer pH 5.0 and a gradient of NaCl, linearly increasing from 0.2 M to 1.0 M in 18 liters. The gradient was obtained by the arrangement described by Parr. Fractions of 25 ml were collected,
pooled, concentrated, desalted and freeze-dried as described above. Yields: (cf. Fig. 3) section a: 0.41 g, section b: 0.96 g, section c: 1.00 g.

**Chromatography of oxidized Viscotoxins.** 1.4 g performic acid oxidized material from peak V (cf. chromatography of crude Viscotoxin) was dissolved in 15 ml of 0.01 M NaOAc buffer pH 4.0 and the solution applied to a column (1.7 × 32 cm) of phosphate cellulose, previously equilibrated with this buffer. Elution was started with the buffer used for dissolving the sample. When 600 ml had passed the column, the eluate was changed to a straight line NaOAC-NaCl gradient from 0.01 M NaOAc to 0.05 M NaOAc + 0.05 M NaCl in 5.6 liters. Fractions of 10 ml were collected until a total volume of 1500 ml had passed the column, when the fraction volume was changed to 15 ml. When 2600 ml had been collected the eluant was changed to 0.5 M NaOAc + 0.5 M NaCl and another 675 ml collected. Fractions corresponding to the peaks were pooled, desalted and freeze-dried as described above for chromatography of crude Viscotoxin. Yields: (cf. Fig. 5): peaks 1 + 2: 0.41 g, peak 3: (Viscotoxin Aox 3) 0.46 g.

**Oxidation of proteins.** Performic acid oxidation of proteins was performed at 0°C according to Hirs.10

**High voltage electrophoresis.** The method described by Samuelsson7 was used with Sephadex G 25 as supporting phase.

**Quantitative amino acid analysis.** Proteins were hydrolyzed with constant boiling HCl as described by Hirs et al.11 and the amino acids determined with an automatic amino acid analyzer according to Spackman et al.12

**Ultracentrifugation of Viscotoxin Aox 3.** The substance was dissolved in 0.1 M NaCl to a concentration of 10 mg/ml. Sedimentation experiments were performed at 20°C and maximum speed (59 780 rpm). The molecular weight was determined by the method of Archibald 15 using the same solution as for the sedimentation experiment. The centrifuge was run at 59 780 rpm at 20°C.

**RESULTS AND DISCUSSION**

Fig. 1 shows the result of chromatography of crude Viscotoxin on phosphate cellulose. Five peaks, I—V, were obtained. Peak I and II were eluted with a buffer of low ionic strength and peaks III—V after increasing the ionic strength of the buffer. Fig. 2 shows the result of high voltage electrophoresis in Sephadex G 25 of performic acid oxidized material from peaks I—V. The compositions

![Fig. 1. Chromatography of 5 g of crude Viscotoxin on a column (3.7 × 92 cm) of phosphate cellulose. For further details see text under "Experimental". E, optical density at 280 mμ. The arrow indicates change of eluting buffer.](image)

of the materials from peaks I and II are distinctively different from that of Viscotoxin A isolated by previously described methods. Peak III contains material which after oxidation gives four bands with mobilities corresponding to Viscotoxin Aox 1, Aox 2, Aox 3, and Aox 4. In addition there are materials giving undistinct bands with high mobilities towards the cathode. Fig. 2 also shows that materials recovered from peaks IV and V are similar in composition to Viscotoxin A, the main difference between them being, that, as judged from the intensity of the bands, the content of Viscotoxin Aox 1 and Viscotoxin Aox 2 is relatively higher in oxidized substance from peak IV than from peak V.

In toxicity tests by intraperitoneal injection in mice, material from peaks I and II were weakly toxic, while material from peaks III, IV, and V had about the same high toxicity as Viscotoxin A.

As shown in Fig. 2 electrophoresis in Sephadex G 25 of oxidized material from peak V revealed a comparatively strong band corresponding to Viscotoxin Aox 3. Viscotoxin Aox 3 is the main component of oxidized Viscotoxin A and has previously been subjected to endgroup studies. Attempts were therefore made to isolate the protein (Viscotoxin A 3) which on oxidation gives Viscotoxin Aox 3, by rechromatography of material recovered from peak V.

The results of an experiment where elution was performed at constant pH with a straight line gradient of NaCl is illustrated in Fig. 3. The elution curve was divided in three parts numbered a, b, and c as indicated in the figure, and the material from the corresponding fractions recovered. Fig. 4 shows the results of high voltage electrophoresis of oxidized material from these fractions. It is remarkable that the first eluted fraction a contains material with the same mobility as Viscotoxin Aox 4 and Aox 5 while fraction b is essentially free from bands with higher mobility. Such bands appear again in fraction c. It is thus possible that the fast moving bands of fraction a represent other substances than the corresponding bands of fraction c. In this experiment fraction b was comparatively pure containing mainly Viscotoxin A 3 (the protein which on oxidation gives Viscotoxin Aox 3). In a series of chromato-
Fig. 3. Chromatography of 2.9 g of material from peak V (Fig. 1) on a column (4.2 × 82 cm) of phosphate cellulose. For further details see text under "Experimental". — denotes optical density at 280 mμ of the eluate. - - - indicates the concentration of NaCl in the eluate. As the first 8 liters of effluent contained no UV-absorbing material only the last part of the elution curve is illustrated.

Fig. 4. Electrophoresis in Sephadex G 25 of oxidized material from sections a, b, and c (Fig. 3) and oxidation products of Viscotoxin A. Conditions: see Fig. 2. Amount of sample: 2 mg.

Fig. 5. Chromatography of 1.4 g of oxidized material from peak V (Fig. 1) on a column (1.7 × 32 cm) of phosphate cellulose. For further details see text under "Experimental". — denotes optical density at 280 mμ of the eluate. - - - indicates concentration of Na+ in the eluate.

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Table 1. Amino acid composition of Viscotoxin Aε 3.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Average or extrapolated value μmole/mg</th>
<th>Amino acid residues g/100 g</th>
<th>Nitrogen g/100 g</th>
<th>Calculated No. of residues for mol. wt. 9700</th>
<th>No. of residues to nearest integer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.664&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.51</td>
<td>1.86</td>
<td>7.3</td>
<td>7</td>
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<tr>
<td>Arginine</td>
<td>0.518&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.09</td>
<td>2.90</td>
<td>5.7</td>
<td>6</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>1.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.21</td>
<td>1.60</td>
<td>12.4</td>
<td>12</td>
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<tr>
<td>Aspartic acid</td>
<td>0.732&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.43</td>
<td>1.03</td>
<td>8.0</td>
<td>8</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.921&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.31</td>
<td>1.29</td>
<td>10.0</td>
<td>10</td>
</tr>
<tr>
<td>Serine</td>
<td>0.816&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.11</td>
<td>1.14</td>
<td>8.9</td>
<td>9</td>
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<tr>
<td>Proline</td>
<td>0.911&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.85</td>
<td>1.28</td>
<td>10.0</td>
<td>10</td>
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<tr>
<td>Glycine</td>
<td>0.753&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.30</td>
<td>1.05</td>
<td>8.2</td>
<td>8</td>
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<tr>
<td>Alanine</td>
<td>0.550&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.91</td>
<td>0.77</td>
<td>6.0</td>
<td>6</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.455&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.15</td>
<td>0.64</td>
<td>5.0</td>
<td>5</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.366&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.14</td>
<td>0.51</td>
<td>4.0</td>
<td>4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.277&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.52</td>
<td>0.39</td>
<td>3.0</td>
<td>3</td>
</tr>
<tr>
<td>Amide NH₃</td>
<td>0.506&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>0.71</td>
<td>5.7</td>
<td>(6)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

89.53                  15.18                   88

<sup>a</sup> Average value of analysis of two samples hydrolyzed for 24 h.

<sup>b</sup> Value extrapolated to zero time or maximum value as calculated from analysis of two samples hydrolyzed for 24 h and 2 samples hydrolyzed for 95 h.

<sup>c</sup> Corr. for NH₃ cont. of reagents and NH₃ formed by destruction of threonine and serine.

<sup>d</sup> The amide groups are not included in the summation of the amino acid residues.
This might indicate that some kind of protein-protein interaction exists between the Viscotoxins.

As rechromatography of material from peak V (Fig. 1) did not give a completely pure Viscotoxin A3, the oxidation product — Viscotoxin Aox 3 — was more conveniently obtained by performic acid oxidation of the whole material from peak V and chromatographic separation of the oxidation products.

Chromatography of performic acid oxidized material from peak V is illustrated in Fig. 5. High voltage electrophoresis (Fig. 6) of the substances recovered from the different peaks showed that peak 1 and 2 contain Viscotoxin Aox 1 and Aox 2 while pure Viscotoxin Aox 3 was obtained from peak 3. Peak 4 which is eluted with a high Na+ concentration contains Viscotoxin Aox 4 and Aox 5. This chromatographic system thus allows preparative isolation of pure Viscotoxin Aox 3.

The amino acid composition of Viscotoxin Aox 3 is illustrated in Table 1. The values are corrected for the content of moisture (8.6 %, as determined by drying in vacuo at 100°C*) and ash (1.7 %*). The amino acid composition is well in agreement with preliminary data for Viscotoxin Aox 3 published previously. The amino acids amount to 89.5 % of the weight of the dry and ash free material. The nitrogen content calculated from the amino acid analysis is 15.2 % and elementary analysis showed a nitrogen content of 15.53 %. The amino acids thus account for 98 % of the nitrogen content of the dry and ashfree protein. The sulfur content calculated from the amount of cysteic

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Fig. 6. Electrophoresis in Sephadex G 25 of material recovered from peaks 1 + 2, 3, and 4 (Fig. 5). Conditions: see Fig. 2. Amount of sample: 1 mg.

Fig. 7. The sedimentation pattern of a solution (10 mg/ml) of Viscotoxin Aox 3 in 0.1 N NaCl. Photographs from left to right were taken at 44, 108, 204, and 268 min from the start of the experiment. Diaphragm angle: 60° at 44, 108, and 204 min; 50° at 268 min. Speed of rotor: 59 780 rpm.

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acid as determined by the amino acid analysis is 3.6 % and as determined by elementary analysis 3.27 %.*

The molecular weight calculated on the basis of the integral numbers of residues is 9736 including one terminal molecule of water and the amide group.⁸

A sedimentation experiment in the ultracentrifuge indicated Viscotoxin Aox 3 to be homogeneous (Fig. 7). The partial specific volume for Viscotoxin Aox 3 as calculated from the amino acid composition according to Mc Meckin and Marshall,¹⁴ using data for the specific volume of the amino acid residues given by Schachman,¹⁵ was 0.76 g/ml. From this value and the sedimentation data the sedimentation coefficient (S₉₀₀₀₀) was calculated to be 0.82.

The results of determination of the molecular weight according to Archibald¹³ in the modification by Ehrenberg¹⁰ is presented in Table 2. Using the calculated value 0.76 g/ml for the partial specific volume, the molecular weight of Viscotoxin Aox 3 was 9600 which is in good agreement with the value 9736 calculated from the amino acid analysis.

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**REFERENCES**


* Determinations performed by A. Bernhardt, Mikroanalytisches Laboratorium im Max Planck Institut für Kohlenforschung, Mülheim (Ruhr), Germany.

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