THE DISULPHIDE BRIDGES OF APAMIN

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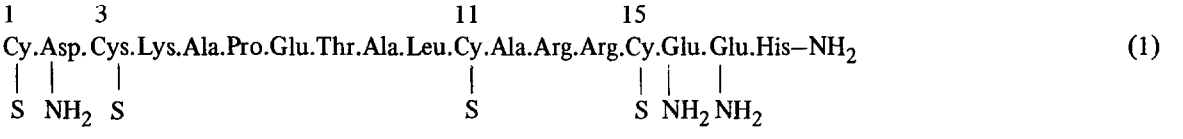
It has been known for some years that bee venom contains an easily dialysable substance which has central excitatory properties [1]. The substance, which has been given the name apamin, was first isolated by Habermann and his colleagues, and shown to be a peptide containing eighteen residues and two disulphide bridges [2a,b]. Recently [3], we found the aminoacid sequence of apamin is:



(1)

The German workers [4] have also described the primary sequence but their structure did not include the amidation of the C-terminal carboxyl group. We now report the presence of a disulphide bridge linking the half-cystine residues at positions 3 and 15 and, since there are no free sulphydryl groups, there must be another disulphide bridge linking positions 1 and 11.

Tryptic hydrolysis of apamin in pyridine-acetic acid buffer (pH 6.5) gave only one peptide and free arginine. The arginine arises from cleavage of the bonds between residues 13 and 14, and 14 and 15. The bond between residues 4 and 5 was also, since only alanine was detected on N-terminal analysis of the peptide. Consequently the disulphide bridges in apamin do not connect positions 1 and 3 and positions 11 and 15.

Distinction between the remaining two possibilities was made by an investigation of some of the peptides arising from partial acid hydrolysis of apamin, using conditions similar to those described by Ryle and Sanger [5]. Apamin (25 mg) was dissolved in a mixture (2.5 ml) of sulphuric acid, acetic acid and water (5 : 3 : 2 v/v) containing thioglycollic acid (0.05%) and heated for 45 minutes at 100°. The hydrolysate was freed from sulphuric acid by passage through a column of Permutit De-Acidite G anion exchange resin equilibrated with a solution containing 20% acetic acid. The larger peptide fragments were separated from smaller peptides and free aminoacids by passage through a column of Sephadex G-10 equilibrated with a solution containing 5% acetic acid. The fraction containing the larger peptides was concentrated to a small volume by rotary evaporation and freeze-dried.

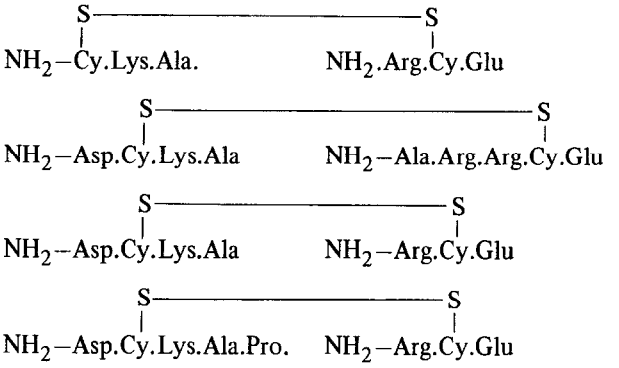
Analysis of the peptide mixture was made by electrophoresis on cellulose thin layer plates (5 × 20 cm, Marck A.G.) as follows. The sample (100 μg peptide dissolved in 5 μl of buffer) was applied to the plate as a band of length 1 cm. The plate was then lightly sprayed with pyridine-acetic acid buffer (pH 5.2) and mounted horizontally over two buffer tanks fitted with platinum electrodes. Electrical contact between the cellulose and the buf-

fer tanks was made by two strips of filter paper lightly damped with buffer. The apparatus was immersed in ligroin as coolant and a potential of 75 V cm^{-1} was applied for 12 min. The plate was dried and the positions of the peptides located by spraying with cadmium ninhydrin reagent.

Peptides containing disulphide bridges were identified by application of the "diagonal technique" introduced by Brown and Hartley [6] who used paper. We have found that results are more rapidly and conveniently obtained by using cellulose thin layer plates (20 X 20 cm). The sample (100 μg) was applied to the centre of the plate as a band of length 0.5 cm. Electrophoresis was carried out as described above. The plate was dried in a stream of warm air and exposed to performic acid vapour (2 hr) to oxidise the disulphide bonds. Ater removal of performic acid vapour electrophoresis was again carried out but in a direction perpendicular to the first. The plate was dried and sprayed with cadmium ninhydrin reagent. Peptides originally containing disulphide bridges were moved off the diagonal.

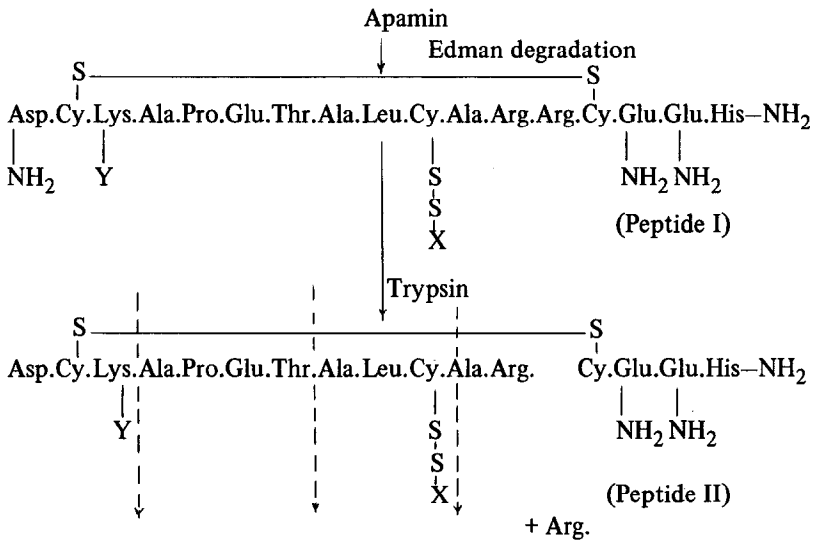
Preparative electrophoresis was carried out using larger amounts of peptide mixture (ca. 1.5 mg) applied to a cellulose thin layer plate (20 X 20 cm) as a band of length 15 cm. Peptides were located by spraying 1 cm guide strips at the edges of the band and those containing disulphide bridges were identified by comparison with the "diagonal" plate and were marked out with a sharp blade. They were concentrated by chromatographic development with acetic acid (5%) solution in a direction perpendicular to that used for the electrophoresis. The thin layer material containing the peptides was collected and the peptides were eluted from it with acetic acid (5%) solution. Residual layer material was removed by centrifugation and the solution obtained was evaporated to dryness in vacuo. Performic acid (20 μl) was added and oxidation allowed to proceed for 30 minutes. The solution was then evaporated to dryness in vacuo. The oxidized peptides were applied to thin layer plates as a band of length 5 cm. Electrophoresis, identification and elution were carried out as described above.

Appropriate peptides were hydrolysed by heating with 6N HCl (100 μl) at 105°. The resulting amino acids were identified as "Dansyl" derivatives by thin layer chromatography on silica gel G using the solvent systems: – methyl acetate: isopropanol: concentrated ammonia solution (45 : 35 : 20 v/v) and chloroform: methanol: acetic acid (75 : 20 : 5 v/v) [7]. The relative amounts of the amino acid derivatives were determined by visual inspection of the chromatograms. The results obtained, together with the known primary structure of apamin, showed that four peptides containing disulphide bridges and with the structures given below had been isolated.



This establishes that apamin contains a disulphide bridge linking residues 3 and 15.

This result was confirmed by a study of the peptides obtained on tryptic digest of apamin, which had been degraded by the Edman procedure. Apamin (12 mg) was subjected to one complete Edman degradation cycle (8a, b) thus breaking the bond between half-cystine-1 and asparagine-2. In the following experiments *N*-terminal analysis was carried out by the "Dansyl" method [9] and electrophoresis at pH 6.5 on strips of Whatman 3 mm paper held between cooled pressed plates 50 cm in length (Locarte Co. London) [10]. The product from the Edman degradation had a lower electrophoretic mobility than native apamin and its *N*-terminal amino acid was found to be aspartic acid (from asparagine-2; the amide group being lost during hydrolysis). The peptide (10 mg) was then hydrolysed with trypsin (0.5 mg) for 16 hr at pH 6.5. Electrophoresis showed that only two products were obtained, free arginine and a peptide. This result can only arise if a disulphide bridge links the half-cystine residues 3 and 15, e.g.,



(2)

where X denotes the phenyl thiohydantoin derivatives of half-cystine or some degradation product derived from it and Y (which presents trypsin cleavage between lysine-4 and alanine-5) is the ϵ-phenyl thioureido derivative of lysine.

Peptide II, which gave an *N*-terminal analysis aspartic acid (arising from asparagine-2) and cysteic acid (arising as an artefact from half-cystine-15), was separated from free arginine by gel filtration on Sephadex G-15. It was then hydrolysed with chymotrypsin (0.3 mg for 6 mg peptide) at pH 8.0 for 4 hr. *N*-terminal analysis of the hydrolysate showed aspartic acid, cysteic acid and alanine. Hence cleavage had occurred on the amino side of an alanine residue: the possible points of attack are shown in the structure of peptide II given above by dotted arrows. Electrophoresis of the hydrolysate shows the presence of two peptides, one with *N*-terminal alanine and the other with *N*-terminal aspartic acid and cysteic acid. The second peptide on treatment with performic acid generated two peptides. It must, therefore, have contained a disulphide bridge. The whole sequence is uniquely consistent with a disulphide bridge linking residues 3 and 15.

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References

[1] G.Hahn and M.E.Fernholz, Ber. 72 (1939) 1281.
[2] E.Habermann, Recent Advances in the Pharmacology of Toxins (Pergamon Press, London, 1963) p. 53.
E.Habermann and K.G.Reiz, Biochem. Z. 343 (1965) 192.
[3] R.Shipolini, A.F.Bradbury, G.L.Callewaert and C.A. Vernon, Chem. Commun. (1967) 679.
[4] P.Von Haux, H.Sowerthal and E.Habermann, Z. Physiol. Chem. 348 (1967) 737.
[5] A.P.Ryle and F.Sanger, Biochem. J. 60 (1955) 535.
[6] J.R.Brown and B.S.Hartley, Biochem. J. 101 (1966) 214.
[7] N.Seiler and J.Wiechmann, Experientia 20 (1964) 559.
[8] P.Edman, Proc. Roy. Australian Chem. Inst. (1957) 434;
Ann. N.Y. Acad. Sci. 88 (1960) 602.
[9] W.R.Gray and B.S.Hartley, Biochem. J. 89 (1963) 59P.
[10] D.Gross, J. Chromatog. 5 (1961) 194.