

## The Disulphide Bridges of Phospholipase A<sub>2</sub> from Bee Venom

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The protein part of phospholipase A<sub>2</sub> from the venom of the common European honey-bee consists of a single chain of 128 amino acid residues [1], eight of which are half-cystine. The absence of free sulphhydryl groups indicates that the enzyme contains four disulphide bridges. Digestion of the native enzyme with pepsin resulted in formation of a fragment containing all four disulphide bridges. Further digestion of this fragment with trypsin allowed the isolation of two singly cross-linked peptides, one containing half-cystine residues 37 and 107, and the other containing residues 59 and 99. A fragment containing the remaining two disulphide bridges was digested with dilute sulphuric acid and a peptide was isolated from the digest in which half-cystine residues 9 and 30 were linked. The remaining disulphide bridge is between residues 31 and 89.

In the accompanying publication [1] is presented the amino acid sequence of the phospholipase A<sub>2</sub> from bee venom. The enzyme consists of a single chain of 128 amino acid residues and has a carbohydrate moiety attached at position 13. Eight of the amino acid residues are half-cystine (positions 9, 30, 31, 37, 59, 89, 99 and 107). It has previously been reported [2] that treatment of the enzyme, with *p*-chloromercuribenzoate both in the presence and absence of 8 M urea resulted in no detectable spectrophotometric change. These results indicate either that the enzyme does not contain free sulphhydryl groups or that any such groups are deeply buried in the interior of the molecule. The results reported here show that the former possibility is correct and have led to an unambiguous assignment of the positions of the four disulphide bridges.

### MATERIALS AND METHODS

#### *Materials*

Pepsin (twice recrystallised) was obtained from Sigma (London) Chemical Co. (London SW6, U. K.). The sources of all other materials were as described in the preceding publication [1].

*Abbreviation.* Dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

*Enzymes.* Phospholipase A<sub>2</sub> (EC 3.1.1.4); trypsin (EC 3.4.21.4); pepsin (EC 3.4.23.1); chymotrypsin (EC 3.4.21.1).

#### *Methods*

The general methods of peptide purification, amino acid analysis and amino acid sequence determination were as previously described [1] except that all purification procedures were carried out at acid pH to avoid the possibility of disulphide interchange [3]. Specific methods used were as follows.

*Digestion of the Native Enzyme with Pepsin.* Native phospholipase A<sub>2</sub> (120 mg) was dissolved in formic acid solution (6 ml, 5% w/v) and pepsin (2.4 mg) was added. The mixture was incubated under nitrogen for 6 h at 37 °C after which more pepsin (2.4 mg) was added and incubation continued for a further 18 h. The resulting mixture of soluble peptides was lyophilized.

*Digestion of Fraction P-1 with Trypsin.* Fraction P-1 from the peptic digest (40 mg) was dissolved in pyridine–acetic acid buffer (4 ml, pH 6.5) and trypsin (4 mg) was added. The solution was incubated under nitrogen for 24 h at 37 °C after which the peptide mixture was separated by high-voltage paper electrophoresis at pH 6.5 (60 V/cm, 60 min).

*Digestion of Peptide PT-3-C with Dilute Sulphuric Acid.* The method used was a modification of that of Ryle and Sanger [4]. The peptide was dissolved in a mixture (0.5 ml) of sulphuric acid (10 M)–acetic acid–water (6:18:6 by vol.) containing 1 part in 3000 (by vol.) of thioglycolic acid. The tube containing the sample was sealed under vacuum and incubated at 100 °C for 60 min. The hydrolysate was applied

to a column of Permutit Deacidite G anion-exchange resin equilibrated with acetic acid (20% v/v) and the peptides were eluted with the same solvent. The mixture was then lyophilised and redissolved in dilute (5% v/v) acetic acid.

**Detection of Peptides Containing Disulphide Bridges.** Peptides produced by digestion of the native enzyme with pepsin were examined for the presence of sulphur-containing amino acids using the chloroplatinic acid reagent described by Toennies and Kolb [5]. In subsequent procedures disulphide-containing peptides were identified by the technique of diagonal electrophoresis [6].

**Oxidation of Purified Disulphide Peptides.** The purified peptides were oxidised with performic acid essentially as described by Hirs [7]. Oxidised fragments were purified by high-voltage paper electrophoresis at pH 3.5 or 6.5.

### Peptide Nomenclature

The letter P denotes a peptide produced by digestion of the native enzyme with pepsin, and PT is used for peptides resulting from digestion of peptic peptides with trypsin. Further digestion of peptide PT-3 with chymotrypsin produced a set of peptides labelled PT-3-C one of which was further digested with sulphuric acid (PT-C-3-A). The prefix O (as in O-PT *etc.*) indicates fragments of disulphide peptides obtained after oxidation.

## RESULTS

### Digestion of Native Phospholipase A<sub>2</sub> with Pepsin

The lyophilized digest was dissolved in acetic acid (5% v/v, 2 ml) and fractionated by passage through Sephadex G-25 (superfine). The elution profile is shown in Fig. 1. Eight pooled fractions were taken as shown in Fig. 1 and examined by analytical high-voltage paper electrophoresis at pH 3.5 and 6.5. The electropherograms were stained for the presence of sulphur-containing peptides by spraying with chloroplatinic acid reagent [5]. Only fraction P-1 (yield 55 mg) gave a positive reaction to this stain. Fraction P-1 contained a single peptide as judged by analytical electrophoresis and gel filtration through Sephadex G-50 and G-100 (superfine grades). It appeared, therefore, that cleavage with pepsin had produced a fragment which contained all four disulphide bridges.

The components of fractions P-2 to P-8 were purified by the usual techniques and were found to consist of free amino acids and peptides containing all the residues from positions 17–21, 42–57, 63–84,

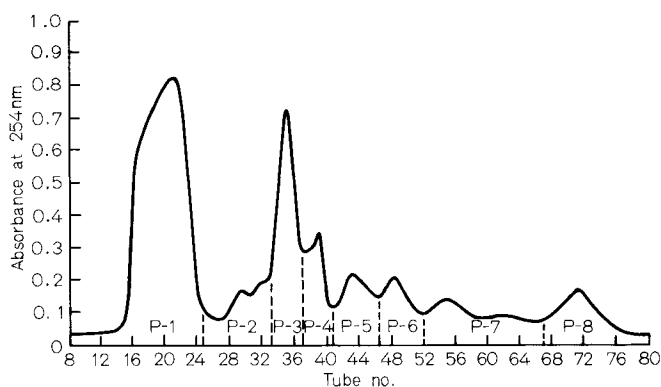


Fig. 1. Elution profile of the peptic digest of native phospholipase A<sub>2</sub> after passage through Sephadex G-25. The column (1.5 × 180 cm) was equilibrated with acetic acid (5% v/v). The fractions which were pooled for further examination are indicated by vertical bars

93–97 and 111–128 of the known structure of phospholipase A<sub>2</sub> [1] (see Fig. 6). In addition, small amounts of the peptides Ile-Ile-Tyr-Pro-Gly-Thr-Leu (positions 1–7) and Gly-Arg-Phe (positions 22–24) were found.

These results suggested that fragment P-1 contained the amino acid residues shown in Fig. 2 but that the population of molecules was heterogeneous in that partial cleavage had occurred at the two positions marked. Consistently N-terminal analysis by the dansylation method [8] showed isoleucine (residues 1 and 85), glycine (residues 22 and 98) and serine (residue 58); there was also a strong spot corresponding to the dansyl derivative of the dipeptide Ile-Ile, as would be expected on the basis of the known resistance of this dipeptide to cleavage with acid, and a weak spot of bisdansyl lysine arising from those molecules which lacked the residues 22 to 24. Amino acid analysis of fragment P-1 gave the results listed in Table 1. The analysis is generally consistent with the structure given in Fig. 2 except that the values for threonine, proline, glycine, isoleucine, leucine, tyrosine, phenylalanine and arginine are somewhat low owing to partial cleavage and loss of residues 1–7 and 22–24.

### Digestion of Fragment P-1 with Trypsin

Fragment P-1 was digested with trypsin and the resulting peptide mixture separated by high-voltage paper electrophoresis at pH 6.5 (see Methods); a guide strip stained after electrophoresis showed seven ninhydrin-positive bands. A strip of the preparative paper 2.5 cm wide was cut off and the peptides oxidised by exposure to performic acid vapour. The strip was sewn to a new sheet of paper and electrophoresis

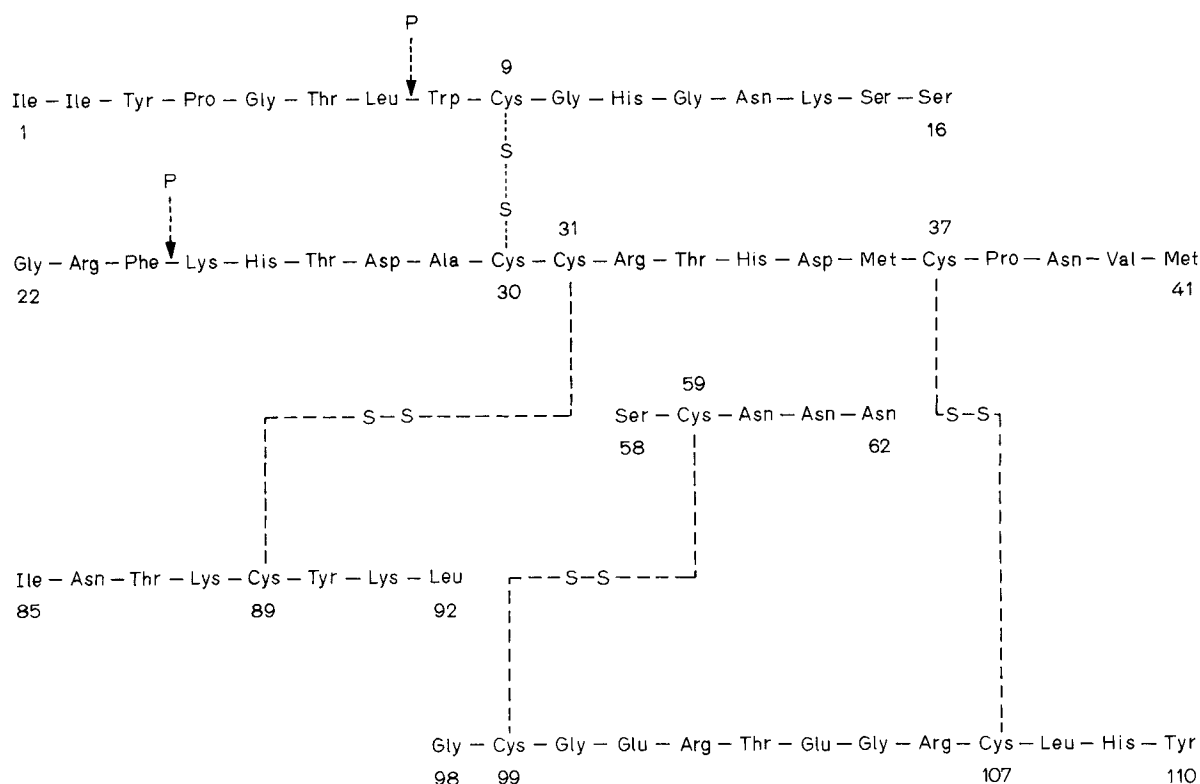


Fig. 2. Proposed structure for fragment P-1 produced by the action of pepsin on native phospholipase  $A_2$ . Evidence that the fragment contains the five polypeptide chains shown cross-linked into a single unit is discussed in the text. The arrange-

ment of disulphide bridges shown is not the only one possible but is that derived from subsequent experiments. The dashed arrows indicate points of partial cleavage by pepsin

(pH 6.5) carried out at right-angles to the original direction; the resulting "diagonal map" is shown in Fig. 3. Only three of the original components of the mixture produced peptides with altered mobilities after oxidation, and all of these altered peptides stained positively with chloroplatinic acid. The original peptides were designated PT-1, PT-2 and PT-3 and the oxidation products labelled as shown in Fig. 3. The strips from the preparative electrophoresis carrying peptides PT-1, PT-2 and PT-3 were eluted with acetic acid (5% v/v) and the peptides lyophilised. The amino acid compositions of the peptides are given in Table 1.

**Peptide PT-1.** This peptide had N-terminal glycine and serine. This information, and the amino acid analysis data suggested that it contained residues 58-62 and 98-102. The peptide was oxidised with performic acid and the two product peptides (O-PT-1-1 and O-PT-1-2) were separated by high-voltage electrophoresis at pH 6.5. The amino acid compositions of these peptides are shown in Table 1 and the amino acid sequences, determined by the dansyl-Edman method [9], are shown in Fig. 4. These results clearly establish the link between half-cystine residues 59 and 99 (Fig. 5).

**Peptide PT-2.** The amino acid composition of peptide PT-2 (Table 1) was consistent with it containing residues 33-41 and 107-110, although this could not be considered conclusive, since the diagonal electrophoresis (Fig. 3) suggested the presence of a non-sulphur-containing contaminant. Peptide PT-2 was oxidised and the product peptides separated by high-voltage electrophoresis at pH 6.5. Two peptides containing cysteic acid were obtained (O-PT-2-1 and O-PT-2-2) the amino acid compositions of which are given in Table 1 and the amino acid sequences in Fig. 4. The data show that peptide PT-2 contains a disulphide bridge between residues 37 and 107 (Fig. 5).

**Peptide PT-3.** This component gave rise to four sulphur-containing peptides on oxidation (Fig. 3) and amino acid analysis showed (Table 1) that it contained four half-cystine residues (presumably 9, 30, 31 and 89). Previous studies of fragment P-1 (see before) had indicated that partial cleavage had occurred between Leu<sup>7</sup> and Trp<sup>8</sup>. In order to avoid this possible source of confusion, peptide PT-3 was digested with chymotrypsin (pyridine-acetic acid buffer, pH 6.5) to remove the N-terminal fragment completely. After



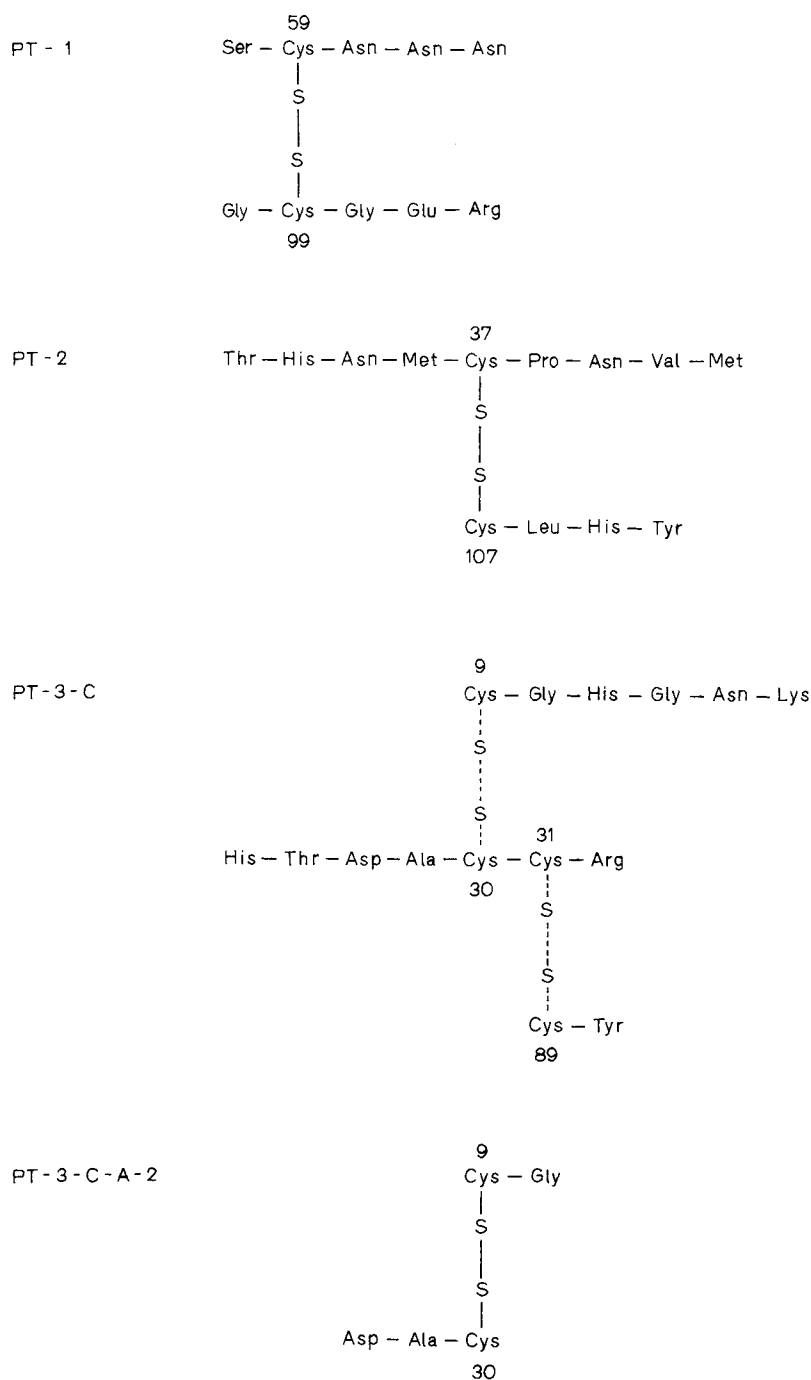


Fig. 5. *Proposed structures for the major disulphide-bridge-containing peptides.* The structure of peptide PT-3-C was unambiguously assigned on the basis of the structure of

PT-3-C-A-2 obtained from PT-3-C by hydrolysis with dilute acid. The details are given in the text

analysis showed only histidine. The amino acid composition is given in Table 1. These data are consistent with peptide PT-3-C being composed of residues 9–14, 26–32 and 89–90. This was confirmed by studies of the three fragments (O-PT-3-C-1, O-PT-3-C-2 and O-PT-3-C-3) produced by oxidation of a

sample of peptide PT-3-C. The amino acid compositions and sequences of these oxidised fragments are given in Table 1 and Fig. 4 respectively. These results show that peptide PT-3-C contains half-cystine<sup>9</sup> linked to either residue 30 or to 31 and half-cystine<sup>89</sup> linked to the other one of the pair. The structure shown

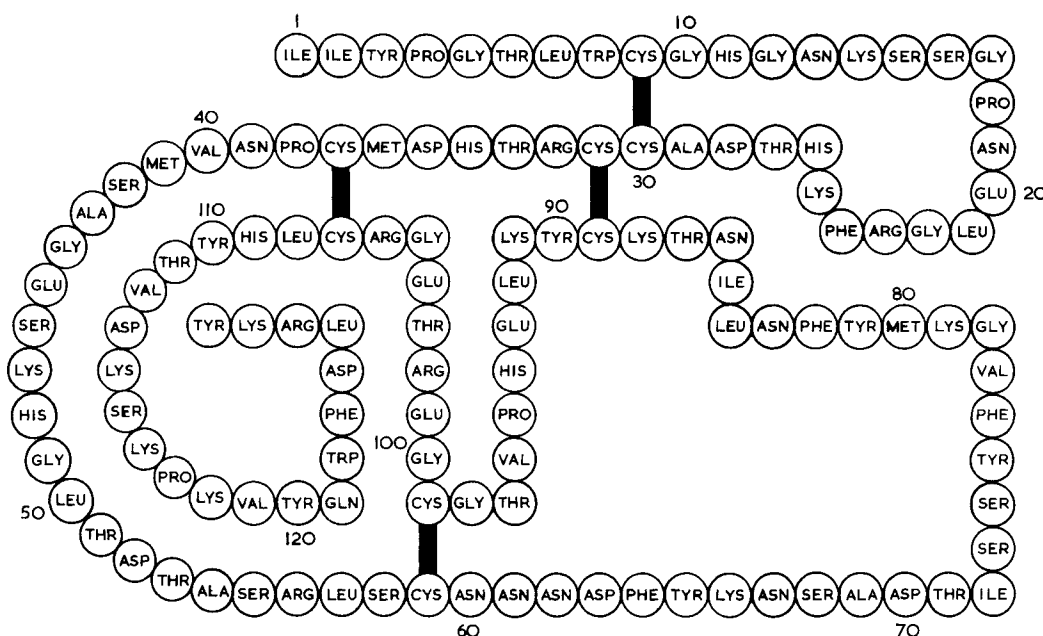


Fig. 6. The amino-acid sequence of phospholipase A<sub>2</sub> showing the positions of the four disulphide bridges

in Fig. 5 is the correct one, which is based on the observations described below.

#### *Digestion of Peptide PT-3-C with Sulphuric Acid*

The remainder of the sample of peptide PT-3-C was digested with dilute sulphuric acid, as described in the Methods section. After removal of sulphuric acid the peptide mixture was fractionated by passage through Sephadex G-25 (superfine) equilibrated in acetic acid (5% v/v). The first two fractions to emerge from the column were further purified by high-voltage paper electrophoresis at pH 6.5. Only one peptide (PT-3-C-A-2) was found which contained an intact disulphide bridge. Amino acid analysis (Table 1) indicated that the peptide contained residues 9–10 and 28–30. The peptide was oxidised with performic acid and the two pieces separated by high-voltage electrophoresis at pH 6.5. Sequence analysis by the dansyl-Edman method gave the sequences as CysSO<sub>3</sub>H-Gly (9–10) and Asp-Ala-CysSO<sub>3</sub>H (28–30). Thus it was established unambiguously that the third disulphide bridge is between residues 9 and 30 and hence, by elimination, the remaining bridge links residues 31 and 89.

#### DISCUSSION

The complete amino acid sequence of phospholipase A<sub>2</sub>, including the positions of the four disulphide

bridges, is shown in Fig. 6. It can be seen that the molecule contains a heavily cross-linked 'core' region, and this is reflected in the action of pepsin on the native enzyme. Digestion with pepsin produced a large fragment (Fig. 2) containing all four disulphide bridges. Characterization of this fragment yielded useful information in that it restricted the number of possible ways in which the disulphide bridges could be arranged. It is clear from Fig. 2 that for the five polypeptide fragments to be linked in a single unit, Cys<sup>9</sup> cannot be linked to either Cys<sup>59</sup> or Cys<sup>89</sup>, residues 59 and 89 cannot be joined and, moreover, a bridge cannot exist between any pair of the three residues 30, 31 and 37 or between residues 99 and 107. In fact the number of allowed sets of disulphide bridges is reduced from 105 to 36.

The information available concerning the structure of P-1 indicated that cleavage with trypsin should be the next step, in that hydrolysis at Arg<sup>32</sup> and Arg<sup>106</sup> would produce either one or two singly cross-linked peptides, depending on the actual arrangement of the bridges. In fact, two such peptides were obtained (Fig. 5), which were used to establish the links between residues 37 and 107 and residues 59 and 99.

The remaining two disulphide bridges presented a more severe problem owing to two of the half-cystine residues being adjacent (positions 31 and 32). This problem was first encountered by Ryle and Sanger [4] who used hydrolysis with sulphuric acid to cleave between adjacent half-cystine residues. An initial unsuccessful attempt was made to cleave

peptide PT-3-C under conditions identical to those used by Ryle and Sanger. The digest was darkly coloured and on examination by diagonal electrophoresis failed to show any disulphide-containing peptides. This failure was attributed to the presence of the carbohydrate side-chain known to be attached at position 13 in phospholipase A<sub>2</sub> [1]; this residue forms a part of peptide PT-3-C. It seemed likely that degradation products from the polysaccharide chain caused oxidation of the disulphide bridges during the treatment with sulphuric acid. In an attempt to remove the carbohydrate moiety before further digestion, a sample of peptide PT-3-C was subjected to hydrolysis under less-acid conditions, as described in the Methods section. Subsequent examination of the hydrolysate showed that it contained a singly cross-linked disulphide peptide, isolation and characterization of which established the link between residues 9 and 30. It is possible that the modified conditions of hydrolysis may be generally useful for obtaining disulphide bridge peptides from glycoproteins and glycopeptides.

It remains to consider the structural relationships between the phospholipase A<sub>2</sub> of bee venom and those from other sources. Little work has so far been published on the structures of the enzymes from snake venoms. Partial sequences of the enzyme from *Agistrodon halys blomhoffi* have been reported [10], but the small amount of data precludes comparison with bee-venom enzyme. The complete structure, including positions of the six disulphide bridges, has been reported for the enzyme from porcine pancreas [11, 12]. The enzyme is very similar in size to that from bee venom, the zymogen containing 130 amino acid residues and the active enzyme 123. Both enzymes show a preponderance of basic amino acid side-chains in the C-terminal region. There do not, however, appear to be any obviously homologous sequences between the two proteins. It is possible to align the two amino acid sequences such that, with a small number of gaps in one or the other, 20 residues occupy identical positions. In no case, however, are there more than two adjacent identical residues (e.g. the sequence Leu-Trp at positions 7–8 and 9–10 in the bee-venom enzyme and the pancreatic zymogen, respectively). The most striking similarity is at the C-terminal regions, where the bee-venom enzyme has the sequence -Arg-Lys-Tyr and the pancreatic enzyme has -Lys-Lys-Tyr-Cys. It is, however, interesting to compare the positions in the molecules of disulphide bridges. For example the two adjacent half-

cystine residues in the bee-venom enzyme at positions 30 and 31 are linked to residues 9 and 89, respectively, whereas the two most-nearly equivalent half-cystine residues in the pancreatic enzyme (positions 34 and 36) are both linked to residues further towards the C-terminal end of the molecule (positions 130 and 51, respectively). In general, the positions of the disulphide bridges in the two proteins do not appear to show any similarities. Thus, although comparison of the amino acid sequences might suggest a distant structural relationship between the two enzymes, this similarity does not seem to be reflected in the dispositions of the disulphide bridges. A more firm decision on the evolutionary relationship, if any, between the bee-venom and porcine pancreatic enzymes would require a detailed analysis of possible alignments of the two sequences using the relatedness-odds calculation methods which have been developed by Dayhoff and her coworkers [13].

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