The disulphide bridges in a cellobiohydrolase and an endoglucanase from *Trichoderma reesei*

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The positions of the disulphide bridges of the 1,4- β -glucan cellobiohydrolase (CBH I) of the fungus *Trichoderma reesei* have been investigated. The results can be summarized as follows. (1) The enzyme contains 12 disulphide bridges and no free cysteine residues. (2) The location of six disulphide bridges have been determined experimentally. (3) The bonding patterns of the two disulphide bridges in the C-terminal region is suggested on the basis of internal homology. (4) The remaining four disulphide bridges are put into two groups, each containing four half-cysteine residues where two are adjacent. (5) A repeating bonding pattern is observed along the peptide chain and a non-local disulphide bond with an unusually long separation distance links the N-terminal and the C-terminal region. (6) The disulphide-bonded CNBr peptides of a 1,4- β -glucan glucanohydrolase (endoglucanase II) from *T. reesei* have been isolated and a disulphide bonding pattern is suggested on the basis of the sequence homology between the two enzymes.

Cellulases function as catalysts in one of the most important degradation processes in Nature, and because cellulose is a renewable resource, its conversion into food, fuel and chemicals by the use of cellulolytic enzymes is being actively researched. The enzymology of cellulose degradation is complicated, and a number of enzymes are involved. Therefore still more knowledge is needed before a practical industrial process can be designed.

In order to understand the function and mechanism of cellulases we need to know their three-dimensional structures. The study of the primary structure of these enzymes is currently a subject of intensive investigation. Thus the amino acid sequence of a 1,4- β -glucan cellobiohydrolase (CBH I) (EC 3.2.1.91) from *T. reesei* QM 9414 has been determined in our laboratory (Fägerstam *et al.*, 1984) and the nucleotide sequence of the corresponding gene from another strain L27 has been reported by Shoemaker *et al.* (1983). Recently the partial amino acid sequence of a 1,4- β -glucan

glucanohydrolase (EC 3.2.1.4) (endo II) has also been reported from our group (Bhikhabhai & Pettersson, 1984). In the present paper we have investigated the positions of the disulphide bridges in CBH I and have compared them to the disulphide pairs deduced from the amino acid compositions of the CNBr peptides of endo II.

Materials and methods

The availability of the culture filtrate of *T. reesei* QM 9414, purification of the enzymes, chemicals, proteolytic enzymes, CNBr cleavage (Gross & Witkop *et al.*, 1961, 1962), enzymic digestion, amino acid analysis, carbohydrate analysis by orcinol method (Vasseur, 1948), manual Edman degradation (Edman & Henschen, 1975), identification of PTH derivatives of amino acids are described by Fägerstam *et al.* (1984) and Bhikhabhai & Pettersson (1984).

Reversed-phase h.p.l.c.

Peptides were chromatographed using an Altex 110 chromatographic system with a 4.5 mm $\times$ 250 mm Lichrosorb RP-8 column equilibrated with 0.05 M-ammonium acetate, pH 6.4. A linear gradient of a suitable organic solvent was used to elute the peptides. The organic solvent was

Abbreviations used: CBH I, 1,4- β -glucan cellobiohydrolase; endo II, 1,4- β -glucan glucanohydrolase; RCM, reduced and carboxymethylated; PTH, phenylthiohydantoin; h.p.l.c., high-performance liquid chromatography.

either 70% ethanol or 70% propanol in 0.05M-ammonium acetate or acetonitrile. Analytical separations were made with linear gradients (0–70%, v/v) of organic solvent. Preparative separations were designed on the basis of the analytical results. After pooling the appropriate fractions, spectra were recorded and two portions of each sample were taken for amino acid analysis. One portion was analysed normally, whereas the other was oxidized with performic acid before hydrolysis to afford accurate determination of half-cystine as cysteic acid.

SDS/polyacrylamide-gel electrophoresis

Discontinuous SDS/polyacrylamide-gel electrophoresis was performed with 18% (w/v) acrylamide in the separation gel. The experimental conditions were essentially those described by Maizel (1969).

Analytical peptide mapping h.p.l.c. for tracing cystine peptides

A portion of a proteolytic digest of a peptide was chromatographed by using a 0–70% linear gradient of organic solvent for 1 h. Another portion was oxidized by performic acid (Hirs, 1956), freeze-dried and chromatographed under the same conditions. Preparative runs were performed on the unoxidized digest. Those peptides that changed their elution positions on oxidation were collected and examined for half-cystine content by amino acid analysis after oxidation with performic acid.

Reaction with 2-thiopyridyl disulphide

5mg of CBH I was incubated with 1ml of a 4mM solution of 2-thiopyridyl disulphide in the presence of 8M-urea and 50mM- Na_2EDTA at pH4.0. The reaction was allowed to proceed for 3 h at room temperature. The extent of reaction was determined from the absorbance of the released 2-thiopyridone at 343nm, assuming $\epsilon_{343} = 8.11 \cdot 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Rydén & Norder, 1981).

Nomenclature

Peptide nomenclature. The disulphide-bonded peptides resulting from CNBr cleavage of CBH I are denoted A, B and C in order of elution. These peptides were further digested with various proteolytic enzymes. T, Ch and Th denote trypsin, chymotrypsin and thermolysin respectively. Chromatographic subfractions are denoted by Roman or Arabic numerals or by small letters in order of elution. For example, B-Ch : 3 corresponds to the third gel-filtration peak of the chymotryptic digest of CNBr fragment B. The CNBr peptides derived from native endo II were denoted A, B, C, D and E. The CNBr peptides derived from the RCM derivatives of both enzymes are denoted by

Roman numerals from I to VIII (see Fig. 2 below). **Nomenclature of half-cystine residues.** Each half-cystine (Cys) residue is given a number (1–24) that describes its position related to the other cysteine residues in the complete sequence. [To avoid confusion with the position occupied in the sequence as a whole, a space, not a hyphen is used (e.g. Cys 1).] The residue number of half-cystine residues in relation to the other amino acids in the sequence is illustrated in Fig. 1.

Results

Fig. 1 shows the complete sequence of CBH I (497 residues). Fig. 2 shows the connectivity diagrams of CBH I and endo II, where the disulphide bridges elucidated directly from the experiments or deduced from homology are indicated. To facilitate the discussion below, the points of cleavage with CNBr are also indicated.

Detection of free thiol groups

Treatment of the enzyme with 2-thiopyridyl disulphide revealed no free thiol group. All 24 cystine residues are therefore linked in pairs by disulphide bonds.

CNBr cleavage. The CBH I enzyme was treated with CNBr and gel-chromatographed. Three disulphide-bonded peptides (A, B and C) were isolated (Fig. 3). These peptides were characterized further as follows.

Peptides B and C. The peptides B and C behaved in gel filtration and on SDS polyacrylamide-gel electrophoresis as single chains after the cleavage of S–S bonds by reduction and alkylation. The amino acid composition of B and C corresponded to CNBr peptides 1–111 (Cys 1–8) and 150–213 (Cys 10–13) respectively (Table 1). The *N*-terminal amino acid of peptide B was blocked, as is the *N*-terminus of the CBH I protein, whereas peptide C had the *N*-terminal sequence Asp-Ala. Peptide C (150–213), which contains four half-cystine residues, breaks the enzyme into two parts each containing an odd number of half-cystine residues, indicating a disulphide bond between *N*-terminal and *C*-terminal regions.

Peptide fraction A. *N*-Terminal analysis of the peptide fraction A gave the PTH derivatives of aspartic acid, alanine and leucine, as the first residues, indicating the presence of three disulphide-bonded peptides. When peptide fraction A was reduced and subjected to gel electrophoresis in SDS, it separated into three bands, one of which corresponded to an M_r of 3000 and the other two corresponded to M_r values of about 15000. The amino acid composition of the peptide fraction A corresponded to a mixture of the following CNBr peptides: 112–149 (Cys 9), 214–360 (Cys 14–19)

Fig. 1. Complete amino acid sequence of CBH I

The numbers above the sequence indicate the positions of the residues in the alignment, whereas the number below each half-cystine residue (C) is the residue number of its partner half-cystine residue. As there was an excellent agreement between the published nucleotide and amino acid sequences, amino acid sequence data have been used except for a few short regions (underlined) which were not determined by direct protein sequencing (Fägerstam *et al.*, 1984). These short regions are filled in by using the nucleotide sequence (Shoemaker *et al.*, 1983). The numbers 1–24 in the circles indicate the relative position of cysteine residues as they occur along the sequence.

Fig. 2. Connectivity diagram illustrating the disulphide bridges and points of cleavage with CNBr in CBH I and endo II. Cysteine numbers 1 to 24 refer to the half-cystine residues of CBH I. The thick solid horizontal lines represent the determined sequences whereas the thinner solid horizontal lines correspond to the known amino acid composition. Positions of methionine (M) and half-cystine residues (S) are indicated. The CNBr peptides of endo II are aligned with the CBH I sequence on the basis of homology as described in Bhikhabhai & Pettersson (1984). The shaded region in endo II represents an unknown sequence or a deletion. The disulphide bridges determined experimentally are joined by solid lines, whereas those joined by broken lines are deduced as explained in the text. The Roman numerals describe the CNBr peptides of endo II also mentioned in Fig. 5.

and 375–497 (Cys 20–24) (Table 1). Furthermore, the orcinol test for carbohydrate provided evidence that fraction A contained the most heavily glycosylated CNBr peptide (375–497) of the molecule. From these results we deduced that the CNBr peptide (112–149) containing Cys 9 is linked with one of the half-cysteine residues of the last two CNBr peptides of the C-terminal region of the enzyme.

Fig. 3. Separation of disulphide-bonded CNBr peptides of CBH I

The digest was chromatographed on a 1.6 mm × 100 cm column of Sephadex G 75 (super-fine grade) in 0.05 M-NH₄HCO₃. Fractions (2.7 ml) were collected at a flow rate of 8 ml/h and A₂₈₀ read (●). The orcinol test to detect carbohydrate was done on 100 μl of each fraction (×). All peaks were further purified, oxidized by performic acid and examined for cysteic acid from the amino acid analysis. Peaks A, B and C contained cysteic acid.

Each of these peptides (A, B and C) was further treated with thermolysin and the subpeptides obtained were mapped and purified by h.p.l.c. Even though amino acid analysis of the oxidized peptides gave poor recoveries of several amino acids, it was nevertheless possible to identify the cysteine-containing peptides. To confirm the tentative data, further digestions of CNBr peptides A, B and C were designed. The experimental procedure is summarized in Fig. 4 and the results are described below. All peptides were purified by h.p.l.c before amino acid analysis. The amino acid composition data allowed identification of the cysteine peptides by comparison with known sequence data.

Pairing of Cys 1–8

Tryptic digestion of B. One of the largest peptides in the tryptic digest of B (B-T : II) corresponded in amino acid composition to the sum of sequences 1–18 and 40–78 (Table 2). This indicated that Cys 1 and Cys 4 up to and including Cys 8 are interconnected with each other in three S=S bridges. Chymotryptic digestion of B-T : II yielded a peptide (B-T : II-Ch : 2) corresponding to the two sequences 1–16 and 43–56, indicating a disulphide bridge between Cys 1 and Cys 4. Since Cys 7 and Cys 8 are adjacent residues and therefore very unlikely to form a disulphide bond, they must be paired with Cys 5 and Cys 6 in one way or another.

Chymotryptic digestion of B. This yielded a peptide (B-Ch : 2) with an amino acid composition similar to that of B-T : II-Ch : 2 and another peptide (B-Ch : 1) corresponding to residues 17–38,

Table 1. Amino acid composition of three S=S linked peptides (A, B and C) obtained from the CNBr digest of CBH I. Values in parentheses indicate the sum of amino acid compositions of three constituent RCM CNBr peptides (112–149, 214–360 and 375–497).

	A	Sequence 112–149	Sequence 214–360	Sequence 375–497	B	Sequence 1–111	C	Sequence 150–213
Cys	12.5 (12)	1	6	5	7.9	8	4.4	4
Asp	35.0 (31)	5	18	8	11.2	12	9.5	10
Thr	35.0 (37)	3	16	18	14.8	16	4.2	4
Ser	35.0 (36)	4	14	18	14.6	14	6.3	6
Glu	28.4 (27)	4	15	8	9.4	9	5.3	5
Pro	19.5 (22)	1	6	15	3.5	3	3.1	3
Gly	42.5 (42)	3	20	19	9.5	9	11.3	11
Ala	16.4 (14)	2	7	5	7.9	9	4.9	5
Val	11.5 (14)	3	5	6	5.0	5	2.2	2
Iso	6.3 (8)	0	5	3	1.8	2	2.1	2
Leu	15.7 (17)	5	8	4	6.9	7	1.2	1
Tyr	15.1 (15)	2	8	5	3.8	4	3.0	3
Phe	12.0 (13)	4	7	2	1.4	1	0.9	1
His	2.2 (2)	0	1	1	2.0	2	1.2	1
Lys	7.2 (7)	0	5	2	2.8	3	3.0	3
Trp	4.5 (3)	0	2	1	5.1	4	1.2	1
Arg	6.0 (6)	0	3	3	1.9	2	1.0	1
Hse	2.3 (2)	1	1	0	1.3	1	1.3	1

Fig. 4. Summary of the methods used for obtaining cystine peptides of CBH I. The disulphide-bonded peptides resulting from CNBr cleavage of CBH I are denoted A, B and C in order of elution. These peptides were further digested with various proteolytic enzymes. The resulting peptides from the digest were fractionated by gel chromatography or h.p.l.c. T, Ch, Th and Asp denote trypsin, chymotrypsin, thermolysin and aspartic acid cleavage respectively. Chromatographic subfractions are denoted by Roman or Arabic numerals or by small letters in order of elution. For example, B-Ch :3 corresponds to the third gel filtration peak of the chymotryptic digest of CNBr fragment B. Tables 1-5 show the amino acid compositions of the corresponding peptides.

Table 2. Amino acid composition of the S-S-linked tryptic and chymotryptic peptides of CNBr peptide B of CBH I. Values in parentheses indicate the sum of the amino acid compositions of two cystine peptides.

	B-T:II	Sequence		B-T:II -Ch:2	Sequence		B-Ch:1	Sequence
		1-18	40-78		1-16	43-56		17-38
Cys	4.6 (6)	1	5	2.0 (2)	1	1	1.5	2
Asp	7.5 (8)	0	8	4.5 (4)	0	4	2.5	2
Thr	8.6 (9)	3	6	4.5 (6)	3	3	2.9	3
Ser	6.8 (6)	2	4	3.5 (4)	2	2	3.0	3
Glu	5.0 (5)	4	1	2.4 (3)	3	0	3.0	3
Pro	2.8 (3)	2	1	1.3 (2)	2	0	0	0
Gly	3.0 (2)	0	2	1.7 (1)	0	1	3.2	3
Ala	5.0 (5)	1	4	2.7 (2)	1	1	1.2	1
Val	0	0	0	0	0	0	1.3	2
Iso	0	0	0	0	0	0	1.0	1
Leu	4.0 (4)	2	2	2.0 (2)	2	0	0.5	0
Tyr	1.7 (2)	0	2	1.0 (1)	0	1	0	0
Phe	0	0	0	0	0	0	0	0
His	1.8 (2)	1	1	1.0 (1)	1	0	0	0
Lys	1.5 (2)	1	1	0	0	0	0.9	1
Trp	3	1	2	2.3 (2)	1	1	1.0	1
Arg	0	0	0	0	0	0	0	0

indicating a disulphide bond between Cys 2 and Cys 3 (Table 2).

Pairing of Cys 10-13

Tryptic digestion of C. A peptide (C-T:1), containing four half-cystine residues (Cys 10-13), was isolated from the tryptic digest of peptide C. Since Cys 12 and Cys 13 are adjacent, a bond

between them is very unlikely. Therefore Cys 10 and Cys 11 must be paired with Cys 12 and Cys 13 in one way or another (Table 3).

Pairing of Cys 21-24

Thermolytic and chymotryptic digest of A. One of the largest thermolytic peptides of A corresponded to residues 426-497, which is the most heavily

Table 3. *Amino acid composition of the S-S-linked tryptic peptide of CNBr peptide C of CBH I*

Values in parentheses show the sum of the two cystine peptides.

	C-T:I	Sequence 167-178	Sequence 182-213
Cys	3.4 (4)	2	2
Asp	6.0 (6)	1	5
Thr	2.1 (2)	1	1
Ser	4.9 (5)	1	4
Glu	5.2 (5)	1	4
Pro	2.2 (2)	1	1
Gly	7.8 (8)	2	6
Ala	2.3 (2)	0	2
Val	1.0 (1)	0	1
Iso	2.0 (2)	0	2
Tyr	2.1 (2)	2	0
Phe	0.9 (1)	0	1
His	0.9 (0)	0	1
Lys	0	0	0
Trp	1.6 (1)	0	1
Arg	0.9 (1)	1	0
Hse	0 (1)	0	1

Table 4. *Amino acid composition of the S-S-linked peptide obtained from the chymotryptic digest of peptide P from the tryptic digest of CBH I*

Values in parentheses show the sum of amino acid composition of the three cystine peptides.

		Sequence		
	P-Ch:1	217-247	253-266	322-338
Cys	5.7 (6)	3	2	1
Asp	9.2 (10)	2	4	4
Thr	5.7 (6)	4	1	1
Ser	3.0 (3)	2	0	1
Glu	8.5 (9)	5	0	4
Pro	4.4 (4)	2	2	0
Gly	8.5 (9)	5	3	1
Ala	4.0 (4)	2	0	2
Val	1.0 (1)	1	0	0
Iso	2.0 (2)	2	0	0
Leu	2.0 (2)	1	0	1
Tyr	3.7 (3)	1	1	1
Phe	1.0 (1)	0	0	1
His	1.0 (1)	1	0	0
Trp	1.2 (1)	0	1	0

glycosylated region of the enzyme. Although the known sequence derived from the RCM derivative (Fägerstam *et al.*, 1984) contains two or three sites at which thermolysin should cleave, the disulphide-containing fragment 426-497 was not fragmented by this enzyme or by chymotrypsin, indicating that Cys 21-24 must be internally connected. On the basis of studies of internal homology, the pairing Cys 21-Cys 24 and Cys 22-Cys 23 is proposed (see the Discussion section).

Fig. 5. *Grouping of disulphide pairs in CNBr peptides of endo II*

The disulphide-bonded CNBr peptides obtained by gel filtration of a CNBr digest of native endo II are denoted A, B, C, D and E. Roman numerals refer to the CNBr peptide of the RCM protein as aligned in Fig. 2. Arabic numerals denote the total number of cysteine residues from amino acid analysis (Bhikhabhai & Pettersson, 1984).

Pairing of Cys 14-19

Tryptic digest of CBH I. The gel filtration of the tryptic digest of CBH I gave five peaks. The largest fraction, P, was digested with chymotrypsin and gave two disulphide-bonded peptides (P-Ch:1 and P-Ch:2). The amino acid composition of P-Ch:1 indicated that it consisted of three S-S-linked peptides indicating links between Cys 14 and Cys 19 (Table 4). Thermolytic digestion of P-Ch:1 yielded two disulphide-bonded peptides. One of the peptides indicated a disulphide bond between Cys 15 and Cys 16, whereas the other contained three S-S-linked peptides, indicating two cystine bridges between Cys 14 and Cys 17, and between Cys 18 and Cys 19 (Table 5).

To locate these two S-S bridges, the peptide was cleaved at aspartic acid with dilute acid hydrolysis (Inglis, 1983). The resulting peptides were separated by h.p.l.c. The amino acid composition of one of the peptides made it evident that Cys 18 is paired with Cys 19, and hence Cys 14 must be paired with Cys 17.

Pairing of Cys 9 and 20

By subtractive deduction one can pair Cys 9 and Cys 20. A disulphide bond between the CNBr peptide (112-149) containing Cys 9 and one of the last two CNBr peptides of the C-terminal region was also indicated from the gel electrophoresis and N-terminal determination of peptide fraction A.

Disulphide pairing in endo II

The partial sequences of the CNBr peptides of endo II and their alignment on the basis of

Table 5. Amino acid composition of the S-S-linked thermolytic peptides of P-Ch:1
Values in parentheses show the sum of amino acid composition of the three cystine peptides.

	P-Ch:1 -Th:a	Sequence			P-Ch:1 -Th:b	Sequence 233-247
		225-232	253-266	322-338		
Cys	4.0 (4)	1	2	1	2.0	2
Asp	7.0 (8)	0	4	4	1.2	1
Thr	4.8 (5)	3	1	1	1.1	1
Ser	1.0 (1)	0	0	1	0	0
Glu	3.8 (4)	0	0	4	3.2	3
Pro	3.8 (4)	2	2	0	0	0
Gly	4.0 (4)	0	3	1	5.3	5
Ala	2.0 (2)	0	0	2	0	0
Val	0	0	0	0	1.0	1
Iso	0	0	0	0	0.9	1
Leu	1.8 (2)	1	0	1	0	0
Tyr	2.6 (2)	0	1	1	1.0	1
Phe	0.9 (1)	0	0	1	0	0
His	1.0 (1)	1	0	0	0	0
Trp	(1)	0	1	0	0	0

Fig. 6. Separation of the disulphide-bonded CNBr peptides of endo II

The same column and conditions were used as in Fig. 3. The absorbance of fractions was measured at 276 nm. The presence of cysteine was deduced from amino acid analysis. Cysteine acid was observed in fractions A, B, C and D.

homology with CBH I have been reported. Gel filtration of a CNBr digest of native endo II resulted in five main peaks A, B, C, D and E (Fig. 6). Each peak was further purified and subjected to amino acid analysis. The compositions were compared with the amino acid compositions of the individual CNBr peptides from the RCM protein. Fig. 5 summarizes the results and Fig. 2 shows the connectivity diagram illustrating the disulphide bonds.

Discussion

The connectivity diagram in Fig. 2 shows a similar bonding pattern within the peptide chain

of CBH I and between the two proteins. The disulphide bridges between Cys 1 and Cys 4 and between Cys 14 and Cys 19 have been demonstrated experimentally. The C-terminal region (465-497) containing Cys 21-24 is homologous with the (N-terminal) region containing Cys 1-4. The sequence between Cys 22 and Cys 23 (CASGTTC) is highly similar to that between the interconnected Cys 2 and Cys 3 (CSSGGTC), and hence the following pattern is proposed: Cys 21-Cys 24 and Cys 22-Cys 23. The repeating bonding pattern reflects the presence of a domain structure such as has been observed in proteins like serum albumin (17 S-S pairs), wheat-germ agglutinin (16 S-S pairs) and prothrombin (12 S-S pairs) (Thornton, 1981). Cys 5-8 and Cys 10-13 form local disulphide patterns. Since adjacent residues are almost certainly not connected by disulphide bridges due to sterical hindrance the following semi-resolved pattern is proposed: Cys (5-6)-Cys (7-8) and Cys (10-11)-Cys (12-13). Complete resolution would require cleavage between the adjacent half-cystine residues or crystallographic analysis.

Two non-local disulphide bridges have been observed. Cys 18 and Cys 19 are separated by 60 residues. Cys 9 and Cys 20 are separated by 260 residues. The non-local disulphide bonds are usually between 45 and 125 residues long. The Cys 9-Cys 20 bond is the next longest found so far. In Asian-influenza-virus neuraminidase (Ward *et al.*, 1983), a disulphide bond separated by 350 residues has been observed. In many cases a non-local disulphide bond joins the N-terminal and C-terminal of a protein. A similar bond is also suggested to be present in endo II.

The partial amino acid sequences of the CNBr peptides of endo II indicate that the following

disulphide bridges are conserved in both enzymes: Cys 2–Cys 3, Cys 9–Cys 20 and Cys 22–Cys 23. The disulphide pairs analogous to Cys 1–Cys 4 and Cys 14–Cys 17 are missing in endo II. Cys 21 is conserved, whereas one more cysteine residue occurs between Cys 21 and Cys 22 in endo II.

Some progress in the nucleotide sequencing of the endo II gene has been made. The complete amino acid sequence of endo II would definitely help to localize all disulphide bridges of the two cellulases.

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References

- Bhikhabhai, R. & Pettersson, G. (1984) *FEBS Lett.* **167**, 301–308
- Edman, P. & Henschen, A. (1975) in *Protein Sequence Determination* (Needleman, S. B., ed.), 2nd edn., pp. 232–279, Springer-Verlag, Berlin, Heidelberg, New York
- Fägerstam, L. G., Pettersson, L. G. & Engström, J. Å. (1984) *FEBS Lett.* **167**, 309–315
- Gross, E. & Witkop, B. (1961) *J. Am. Chem. Soc.* **83**, 1510–1511
- Gross, E. & Witkop, B. (1962) *J. Biol. Chem.* **237**, 1856–1860
- Hirs, C. H. W. (1956) *J. Biol. Chem.* **219**, 611
- Inglis, A. S. (1983) *Methods Enzymol.* **91**, 324–332
- Maizel, J. V. (1969) in *Fundamental Techniques in Virology* (Habel, K. & Salzman, N. P., eds.), pp. 334–362, Academic Press, New York
- Rydén, L. & Norder, H. (1981) *J. Chromatogr.* **215**, 341–350
- Shoemaker, S., Schweickart, V., Ladner, M., Gelfand, D., Kwok, S., Myambo, K. & Innis, M. (1983) *Bio/Technology* **1** (8), 691–695
- Thornton, J. M. (1981) *J. Mol. Biol.* **151**, 261–287
- Vasseur, E. (1948) *Acta Chem. Scand.* **2**, 693–701
- Ward, C. W., Colman, P. M. & Laver, W. G. (1983) *FEBS Lett.* **153**(1), 29–33