The amino acid sequence of chymopapain from *Carica papaya*

David C. WATSON, Makoto YAGUCHI and Kenneth R. LYNN*

Division of Biological Sciences, National Research Council of Canada, Ottawa, Ont. K1A 0R6, Canada

Chymopapain is a polypeptide of 218 amino acid residues. It has considerable structural similarity with papain and papaya proteinase Ω , including conservation of the catalytic site and of the disulphide bonding. Chymopapain is like papaya proteinase Ω in carrying four extra residues between papain positions 168 and 169, but differs from both papaya proteinases in the composition of its S_2 subsite, as well as in having a second thiol group, Cys-117. Some evidence for the amino acid sequence of chymopapain has been deposited as Supplementary Publication SUP 50153 (12 pages) at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies may be obtained on the terms indicated in Biochem. J. (1990) 265, 5. The information comprises Supplement Tables 1–4, which contain, in order, amino acid compositions of peptides from tryptic, peptic, CNBr and mild acid cleavages, Supplement Fig. 1, showing re-fractionation of selected peaks from Fig. 2 of the main paper, Supplement Fig. 2, showing cation-exchange chromatography of the earliest-eluted peak of Fig. 3 of the main paper, Supplement Fig. 3, showing reverse-phase h.p.l.c. of the later-eluted peak from Fig. 3 of the main paper, and Supplement Fig. 4, showing the separation of peptides after mild acid hydrolysis of CNBr-cleavage fragment CB3.

INTRODUCTION

Of the major cysteine proteinase components of *Carica papaya* (Jansen & Balls, 1941) the primary structures of two, papain (Cohen *et al.*, 1986) and papaya proteinase Ω (Dubois *et al.*, 1988), have been reported. In 1985 we described a preliminary sequence for the third proteinase, chymopapain (Lynn *et al.*, 1985), and we now describe the primary structure of that molecule. While this work was being prepared for publication we learned of a parallel study of chymopapain (A. G. Schnek, personal communication). The results obtained in the two laboratories have been compared and are identical, though different methodologies were employed by the two groups involved.

MATERIALS AND METHODS

Enzyme purification

Chymopapain was prepared from crude type I papaya latex from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) by using methods previously described (Lynn, 1979). Extraction of the latex was in 'activation' buffer (Sluyterman & Widjenes, 1970), and, after centrifugation at 20000 *g* for 15 min at 4 °C, the extract was immediately applied to the agarose–mercurial affinity column (Sluyterman & Widjenes, 1970), when it was bound in an inactive form. Subsequent purification steps (Lynn, 1979) were with the inactive mercurial derivative of the enzyme. The chymopapain was homogeneous on polyacrylamide-gel electrophoresis.

Reduction and alkylation

A 10 mg (400 nmol) portion of chymopapain was dissolved in 1.0 ml of 0.1 M-Tris/HCl buffer, pH 8.3,

containing 6 M-guanidinium chloride and 2.5 mg of disodium EDTA. After reduction by the addition of 1.24 mg (0.8 μ mol) of dithiothreitol (Calbiochem, La Jolla, CA, U.S.A.) under N_2 at room temperature for 1 h, *S*-carboxy[14 C]methylation was effected by the dropwise addition of 5 μ Ci of iodo[2- 14 C]acetic acid (56 mCi/mol; Amersham Corporation, Arlington Heights, IL, U.S.A.) in 50 μ l of ethanol. After incubation at room temperature in the dark for 1 h, complete alkylation was assured by the addition of 15 mg (10 equiv./mol of thiol) of iodoacetic acid. The pH of the solution was immediately re-adjusted to pH 8.3 with 0.1 M- NH_3 solution and incubated for a further 60 min in the dark at room temperature. The *S*-carboxymethylated protein was desalted on a Sephadex G-25 (fine grade) column (2 cm $\times$ 50 cm) in 10 mM-HCl and freeze-dried.

Tryptic cleavage

Trypsin [bovine pancreas, twice crystallized, treated with *L*-*N*-tosylphenylalanylchloromethane ('TPCK')] was obtained from Sigma Chemical Co. and was further purified by reverse-phase h.p.l.c. before use according to the method of Titani *et al.* (1982). A 500 μ g portion of *S*-carboxymethylated chymopapain was dissolved in 1 ml of 50 mM-ammonium bicarbonate buffer, pH 8.0, trypsin was added in a 1:100 (w/w) ratio and the mixture was incubated for 3 h at 37 °C, after which the addition of trypsin and the incubation were repeated. The reaction mixture was then freeze-dried. The resultant tryptic-digest peptides were fractionated by elution with an acetonitrile (BDH Chemicals, Toronto, Ont., Canada) gradient (1 %/min) in 0.05 % (v/v) trifluoroacetic acid (BDH Chemicals) at a flow rate of 1 ml/min from a Synchropak RP-8 column (4.1 mm $\times$ 250 mm) (Syn-

* To whom correspondence and reprint requests should be addressed.

chrom, Linden, IN, U.S.A.) previously equilibrated in 0.05% trifluoroacetic acid, pH 2.0. The effluent was monitored at 220 nm and individual peptides were collected and freeze-dried. Those peptides that displayed signs of heterogeneity, either by distortion in peak shape or after amino acid composition analysis and sequencing, were re-fractionated on an identical Synchropak RP-8 column at pH 7.0 in 10 mM-ammonium acetate. Elution of the peptides was achieved by a linear gradient (0–50%) generated from 50% (v/v) propan-1-ol in water at a flow rate of 0.8 ml/min.

Limited peptic cleavage

A 4 mg portion of *S*-carboxymethylated chymopapain was dissolved in 1 ml of 5% (v/v) acetic acid. Pepsin (1:2000 w/w) was added to the chymopapain solution and digestion was allowed to continue for 1 h at 4 °C. At the end of this time the reaction was stopped by the addition of pepstatin (10 times the weight of pepsin used). The digestion mixture was applied to a Sephadex G-50 (superfine grade) column (1.5 cm × 400 cm) equilibrated in 10 mM-HCl. Elution was with that solvent, and the fractions of interest were pooled and freeze-dried. Re-fractionation was performed by either cation-exchange chromatography or reverse-phase h.p.l.c. Ion-exchange chromatography was performed on Whatman CM-52 CM-cellulose. The freeze-dried material was redissolved in 2 ml of 10 mM-sodium acetate buffer, pH 3.8, and applied to a column (0.9 cm × 30 cm) previously equilibrated in the same buffer. The column was washed with 50 ml of buffer, followed by a salt gradient (2 × 200 ml, 0.1–1.0 M-NaCl) in the same buffer at a flow rate of 20 ml/h. Eluted peptides were detected at 230 nm, and pooled fractions were freeze-dried and desalted on a Sephadex G-25 (fine grade) column (2 cm × 50 cm) in 10 mM-HCl. Reverse-phase h.p.l.c. was performed on an Altex Ultrapore RPSC C3 column (Beckman Instruments, Palo Alto, CA, U.S.A.) with a Varian 5000 liquid chromatograph. The freeze-dried material, dissolved in 500 µl of 0.05% trifluoroacetic acid, was injected on to the column, which had been previously equilibrated in 0.05% trifluoroacetic acid. Elution was with a linear acetonitrile gradient [0–60% (v/v) at 1%/min] in 0.05% trifluoroacetic acid at a flow rate of 1.0 ml/min.

Cleavage with CNBr at methionine and tryptophan residues

Cleavage of peptide bonds adjacent to both methionine and tryptophan residues was effected by using CNBr in the presence of heptafluorobutyric acid and formic acid according to the method of Ozols & Gerard (1977). A 1.5 mg portion of *S*-carboxymethylated chymopapain was dissolved in 1.0 ml of 88% (v/v) formic acid. Then 1 ml of anhydrous heptafluorobutyric acid (Beckman Instruments) was added followed by the addition of 25 mg of solid CNBr. The reaction vial was flushed with Ar, sealed, and incubated in the dark at room temperature for 24 h with continual stirring. The reagent and solvent were removed under a stream of N₂, and the remaining material was resuspended in 10 ml of Milli-Q water (Millipore, Bedford, MA, U.S.A.) and freeze-dried. That process was repeated before h.p.l.c. fractionation. The resultant CNBr-cleavage peptides were separated by elution with an acetonitrile gradient (0–60% at 1%/min) in 0.05% trifluoroacetic acid at a flow rate of 1.0 ml/min from an Altex Ultrapore RPSC C3 column (Beckman

Instruments) previously equilibrated in 0.05% trifluoroacetic acid. The effluent was monitored at 220 nm, and individual peptides were collected and freeze-dried.

Mild acid cleavage

Cleavage of the large CNBr-cleavage fragment CB3 at aspartic acid/asparagine residues was performed by incubation in 0.25 M-acetic acid under vacuum [2.7 kPa (20 mTorr)] at 110 °C for 8 h (Schroeder *et al.*, 1963) followed by freeze-drying. The peptides produced were fractionated by elution from an Ultrapore RPSC C3 column in the same manner as described for the CNBr-cleavage mixture.

Disulphide bridge assignment

Disulphide bridge assignments were made after the isolation and identification of disulphide-bonded peptides by the mapping of tryptic digests. To minimize disulphide bond cleavage and re-arrangement, the tryptic digestion was performed at pH 5.8. A 1.0 mg portion of chymopapain was dissolved in 1 ml of 50 mM-ammonium acetate buffer, pH 5.8, and trypsin was added to give a final concentration of 1:100 (w/w). The mixture was incubated at 37 °C for 3 h, after which the addition of trypsin and incubation were repeated. The digest was freeze-dried, redissolved in 0.05% trifluoroacetic acid and fractionated as described above for tryptic peptides. Re-fractionation of individual peaks was performed at pH 5.8 in 10 mM-ammonium acetate buffer by reverse-phase h.p.l.c. One-fifth of each peak was taken for sequence analysis to identify those containing disulphide-bonded peptides. These fractions were subsequently oxidized with performic acid, freeze-dried and subjected to sequence analysis.

Automated gas-phase and liquid-phase sequence analysis

Automated gas-phase sequencing was performed on an Applied Biosystems (Foster City, CA, U.S.A.) 475A protein sequencing system with 0.1–0.5 nmol quantities of purified peptides. The samples were dissolved in 30 µl of Milli-Q water and applied to a glass-fibre disc containing 1.5 mg of precycled Polybrene (Applied Biosystems). In automated liquid-phase sequencing a Beckman 890C sequencer was used with 5–10 nmol of purified peptides, and sequential Edman degradation was performed with the use of a 0.5 M-Quadrol program (Beckman program no. 111978). The phenylthiohydantoin derivatives of amino acids were identified by h.p.l.c. on a Varian Vista-56 liquid chromatograph with an IBM-cyano column (4.5 mm × 25 cm) (IBM Instruments, Meriden, CT, U.S.A.) maintained at 37 °C in 5% (v/v) tetrahydrofuran/30 mM-sodium acetate buffer, pH 5.1, with gradient elution using 100% acetonitrile.

Amino acid composition analysis

Amino acid composition analyses of protein and of purified peptides were performed with a Durrum D-500 analyser. Protein samples (70 µg) were hydrolysed *in vacuo* [2.7 kPa (20 mTorr)] at 110 °C in 6 M-HCl (Aristar; BDH Chemicals) for 24 h, 48 h and 72 h to correct for hydrolytic losses. Tryptophan was determined after hydrolysis in 4 M-methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole (Pierce Chemical Co., Rockford, IL, U.S.A.) at 110 °C for 20 h *in vacuo*. The

combined cystine and cysteine content of chymopapain was determined after oxidation to cysteic acid (Hirs, 1967) and acid hydrolysis in 6 M-HCl at 110 °C for 24 h. Purified peptides were hydrolysed as described above except that no corrections were made for hydrolytic losses.

RESULTS

Chymopapain is a 218-amino acid-residue polypeptide, as determined by sequence analysis (Fig. 1) and confirmed by amino acid composition analysis (Table 1). It contains a total of eight cysteic acid residues after oxidation. To facilitate the identification of these residues, we used S-carboxyl [¹⁴C]methylated chymopapain, which provided us with a secondary means of identification of cysteine residues in addition to the phenylthiohydantoin elution profiles. N-Terminal sequence analysis of the intact protein allowed unambiguous identification up to amino acid residue 40, at which point the sequence was no longer discernible from the background noise.

Cleavage of chymopapain with trypsin

Amino acid composition analysis (Table 1) showed the presence of 21 lysine and five arginine residues, giving a total of 26 potential trypsin-cleavage sites. A 500 μg portion of protein was subjected to tryptic digestion and the resulting peptides were separated by reverse-phase h.p.l.c. (Fig. 2). Samples of each purified peptide were removed for amino acid composition analysis (Table 1 of Supplementary Publication SUP 50153) and sequence analysis.

By comparison with the known primary structure of papain (Cohen *et al.*, 1986) and actinidin (Carne & Moore, 1978), tentative alignment of most of the tryptic-digest peptides of chymopapain was possible. This included tentative assignment of peptide T22 to the C-terminal position because of its lack of a C-terminal lysine or arginine residue.

Limited peptic cleavage

Limited peptic cleavage was performed on chymo-

Fig. 1. Amino acid sequence and peptides of chymopapain

T denotes peptides obtained by tryptic digestion, CB those obtained by CNBr cleavage, A those obtained by mild acid hydrolysis, and P those obtained by limited peptic digestion. + indicates residues identified directly by amino acid phenylthiohydantoin derivatives obtained by automated Edman degradation.

Table 1. Amino acid composition of chymopapain

Half-cystine was determined after performic acid oxidation, and tryptophan was determined after hydrolysis in 4 M-methanesulphonic acid.

	Amino acid composition (mol of residue/mol)				
	Determined			Assumed	Sequence
	24 h hydrolysis	48 h hydrolysis	72 h hydrolysis		
Asx	18.34	18.46	18.69	18–19	18
Thr	13.78	12.21	11.54	14	14
Ser	17.66	14.84	14.13	17	17
Glx	18.69	18.95	18.84	18–19	18
Pro	11.30	10.60	10.75	11	11
Gly	27.69	27.71	27.51	27–28	27
Ala	14.41	13.85	13.98	14	14
CyS	8.32			8	8
Val	15.51	15.63	15.85	16	16
Met	0.77	0.95	0.98	1	1
Ile	7.11	7.56	7.83	8	8
Leu	12.46	12.57	12.66	12–13	12
Tyr	14.77	14.28	13.01	15	15
Phe	6.37	6.11	6.15	6	6
His	2.95	3.18	3.46	3	3
Lys	20.62	21.28	21.32	21	21
Trp	3.88			4	4
Arg	4.75	5.32	5.47	5	5
Total				218–222	218

papain to obtain relatively large peptide fragments capable of overlapping the tryptic-digest peptides. Given the non-specificity of pepsin, and that the most susceptible sites are those formed by the aromatic amino acids,

Fig. 2. Separation of peptides after tryptic digestion of S-carboxymethylated chymopapain

Elution was with an acetonitrile gradient (1%/min) at a flow rate of 1 ml/min from a Synchropak RP-8 column equilibrated in 0.05% trifluoroacetic acid. The letter T denotes tryptic cleavage. Amino acid compositions of these peptides are given in Table 1 of Supplementary Publication SUP 50153.

leucine and to a lesser extent alanine, the digestion was subjected to mild conditions (see the Materials and methods section) in an attempt to limit the cleavage. Initial fractionation of the reaction mixture by gel filtration on Sephadex G-50 (superfine grade) resulted in two peaks (Fig. 3). The earlier-eluted of these peaks was re-fractionated by ion exchange on CM-cellulose and resolved into two components (Fig. 2 of Supplementary Publication SUP 50153), each of which proved to be homogeneous after two cycles of automated Edman degradation. The later-eluted peak from the Sephadex G-50 column was re-fractionated by reverse-phase h.p.l.c. (Fig. 3 of Supplementary Publication SUP 50153) and

Fig. 3. Separation of peptides from limited peptic digestion of S-carboxymethylated chymopapain

The digestion mixture was applied to a 1.5 cm × 400 cm Sephadex G-50 (superfine grade) column, and eluted with 10 mM-HCl. The fractions indicated by the bars were pooled and freeze-dried. —, A_{230} ; ----, ^{14}C radioactivity.

Fig. 4. Fractionation of CNBr-cleavage products of chymopapain

The cleavage products were separated on an Altex Ultrapore RPSC column equilibrated in 0.05% trifluoroacetic acid by elution with a linear acetonitrile gradient (1%/min). The column effluent was monitored at 220 nm and individual peptides were collected and freeze-dried. Amino acid compositions of these peptides are given in Table 3 of Supplementary Publication SUP 50153.

Fig. 5. Reverse-phase h.p.l.c. separation of tryptic-digest peptides from native chymopapain for disulphide bridge assignment

Elution conditions were identical with those described in the legend to Fig. 2.

Fig. 6. Fractionation of disulphide-bonded peptides from chymopapain after performic acid oxidation

Fractions isolated in Fig. 5 and determined by sequence analysis to be disulphide-bonded were oxidized and individually fractionated by reverse-phase h.p.l.c. on a Synchropak RP-8 column in 0.05% trifluoroacetic acid. Elution was with an acetonitrile gradient (1%/min) at a flow rate of 1 ml/min. (a) Fraction 8; (b) fraction 12; (c) fraction 11; (d) fraction 14.

resolved into five components, which again, from automated Edman degradation, proved to be homogeneous. Subsequent sequence analysis of the peptic peptides (P1-P7) resulted in the overlapping of most of the tryptic-digest peptides (Fig. 1).

CNBr cleavage

As sequence analysis of the tryptic-digest and peptic-digest peptides did not give total overlap, the CNBr-cleavage reaction at both methionine and tryptophan residues (Ozols & Gerard, 1977) was employed. After separation by h.p.l.c. on an Altex Ultrapore RPSC column in 0.05% trifluoroacetic acid, six homogeneous fragments were obtained (Fig. 4). Subsequent sequence analysis of fragments CB1-CB6 completed the overlapping procedure for the entire 218 amino acid residues. However, the largest CNBr-cleavage fragment, CB3 (Fig. 4), which spanned residues 27-181, was further cleaved by mild acid hydrolysis (Schroeder *et al.*, 1963). The fragments were fractionated by h.p.l.c. in 0.05% trifluoroacetic acid on an Ultrapore RPSC column (Fig. 4 of Supplementary Publication SUP 50153). Sequence analysis of these peptides provided confirmatory overlaps.

Disulphide bridge assignments

To minimize disulphide re-arrangements, cleavage with trypsin was performed at pH 5.8 in 50 mM-ammonium

acetate buffer. The sequence analysis of chymopapain shows the presence of eight half-cystine and cysteine residues within the molecule. Assuming that one of these, namely Cys-25, is the active-site cysteine residue and cannot be involved in disulphide bridge formation, probably six of the remaining residues form disulphide bridges and the seventh is a secondary free thiol moiety. By comparison of the elution profiles of the tryptic maps of *S*-carboxymethylated chymopapain (Fig. 2) and that of native chymopapain (Fig. 5) it can be seen that differences in elution times and elution patterns exist. The suspected disulphide-bonded peptide fractions were isolated and re-fractionated at pH 5.8 in ammonium acetate buffer, to ensure homogeneity.

Analysis of fraction 8 (Fig. 5) gave sequences of two peptides corresponding to residues 146–156 and residues 196–208, indicating that the half-cystine residues at positions 153 and 204 are disulphide-bonded. Likewise, analysis of fraction 11 (Fig. 5) gave two sequences corresponding to residues 18–39 and residues 59–64: hence cystine residues 22 and 63 are disulphide-bonded. Analysis of fraction 12 (Fig. 5) allowed the assignment of the third disulphide bond between half-cystine residues 56 and 95, as this fraction generated two sequences corresponding to residues 40–58 and residues 95–96. Finally, analysis of fraction 14 (Fig. 5) gave a single sequence corresponding to residues 113–139, indicating that the cysteine residue at position 117 constitutes a secondary free thiol group that is additional to the active-site cysteine residue at position 25. Fractions 8, 11 and 12 were further subjected to performic acid oxidation

and individually separated by reverse-phase h.p.l.c. (Fig. 6). Sequence analysis of these separated disulphide-bonded peptides confirmed the designation of half-cystine residues in disulphide bridges.

DISCUSSION

The sequence reported here is identical with that found by A. G. Schnek and co-workers (personal communication), even though the chymopapains were isolated from papaya latexes from completely different sources, and were purified by somewhat different means. It is thus reasonable to conclude that the two laboratories have each isolated the major form of chymopapain in papaya latex. A number of other chymopapains have been found in that source, and some are closely related, in size and amino acid composition, to the molecule discussed here (Ebata & Yasunobu, 1962; Kunimitsu & Yasunobu, 1967; Lynn, 1979; Kahn & Polgár, 1983). However, defining that relationship requires structural studies not yet available. Buttler & Barrett (1984) have previously shown that the multiple forms of chymopapain that they separated are immunologically identical, and concluded that chymopapain is a single enzyme.

We have restricted comparison of the sequence of chymopapain to papain and papaya proteinase Ω , which are also isolated from papaya latex. In general terms, the similarities between the three sequences (Fig. 7) are greatest near the *N*- and *C*-terminals, but structural similarities occur throughout. Residues generally agreed to be essential for the activity, namely Cys-25, Asp-158

Fig. 7. Comparison of amino acid sequences of chymopapain, papain and papaya proteinase Ω

— indicates that no amino acid is present at that position and that the sequence continues. The numbers above the chymopapain sequence are the residue numbers of chymopapain.

Fig. 8. Cysteine and half-cysteine residues and disulphide bonds in chymopapain, papain and papaya proteinase Ω

25* denotes the catalytic cysteine residue.

and His-159, with Trp-177 and Asn-175, are all conserved in chymopapain and papaya proteinase Ω. There are between chymopapain and papain 127 identities out of 212 possibilities, and between chymopapain and papaya proteinase Ω 144 identities in 216 residues.

On examining the sequences of Fig. 7, three notable areas of difference are found. Chymopapain has eight cysteine residues whereas papain and papaya proteinase Ω each has seven. The three disulphide bonds of the molecules are formed in identical ways (Fig. 8), the seventh cysteine residue being the residue essential for activity, at position 25. In chymopapain, uniquely, residue 117 is a cysteine. By using the three-dimensional model published by Drenth and co-workers (Kamphuis *et al.*, 1984) for papain as guide, position 117 of chymopapain is probably on the surface of the molecule. In the sequence it is remote from the closest cysteine residue (Cys-95) and is, furthermore, on a different domain, so interaction between them is unlikely to occur. Similarly, the other cysteine residues of chymopapain are distant from position 117, which is thus not expected to be an alternative site for intramolecular disulphide bond formation, which could produce other forms of chymopapain (Ebata & Yasunobu, 1962; Kunimitsu & Yasunobu, 1967; Lynn, 1979). The exterior position of Cys-117 would facilitate its engagement in intermolecular disulphide bonds, but we have found no evidence in the literature for the existence of dimeric forms of chymopapain. The exposed site of Cys-117 would make it susceptible to oxidation, which has been reported in studies of the second free thiol group of chymopapain (Baines & Brocklehurst, 1982). It may be noted that the *N*-terminal side of the sequence leading to this cysteine residue resembles that reported by Tsunoda & Yasunobu (1966) as occurring about the reactive thiol group of chymopapain. Apparently their derivative-forming reagent preferentially bound to the more exposed free thiol group and not, as they assumed, to that essential for catalysis.

Another difference between chymopapain and papain is that the former (like papaya proteinase Ω) contains four residues (169–172) more between residues 168 and 169 of the papain sequence. Similar insertions are also

found in actinidin, the cysteine proteinase from *Actinidia chinensis*, for which the three-dimensional structure has been published (Baker, 1980) and compared with that of papain (Kamphuis *et al.*, 1985). The insertions are then seen to extend two adjacent β-sheets.

The S_2 subsite in papain may be defined (Drenth *et al.*, 1976) by Tyr-67, Pro-68, Val-133, Val-157, Ala-160 and Ser-205. These residues are identical in papaya proteinase Ω, but in chymopapain are Tyr-67, Gln-68, Leu-133, Leu-157, Ala-160 and Ser-205. The substitution of the more bulky groups in chymopapain should affect the reactivities of the enzyme such that smaller groups at P_2 will provide more effective substrates, and the replacement by Gln-68 of proline is also expected to alter the reactivity of this subsite. There are not sufficient kinetic data available from reactions of chymopapain to test these predictions.

Although the data of Fig. 7 show that other differences between the enzymes exist, their significance cannot be established until high-resolution structures of all three are defined.

This paper is National Research Council of Canada Publication no. 30943.

REFERENCES

- Baines, B. S. & Brocklehurst, K. (1982) *J. Protein Chem.* **1**, 119–139
- Baker, E. N. (1980) *J. Mol. Biol.* **141**, 441–484
- Buttle, D. J. & Barrett, A. J. (1984) *Biochem. J.* **223**, 81–88
- Carne, A. & Moore, C. H. (1978) *Biochem. J.* **173**, 78–83
- Cohen, L. W., Coghlan, V. M. & Dihel, L. C. (1986) *Gene* **48**, 219–227
- Drenth, J., Kalk, K. H. & Swen, H. M. (1976) *Biochemistry* **15**, 3731–3738
- Dubois, T., Kleinschmidt, T., Schnek, A. G., Looze, I. & Braunitzer, G. (1988) *Biol. Chem. Hoppe-Seyler* **369**, 741–754
- Ebata, M. & Yasunobu, K. T. (1962) *J. Biol. Chem.* **237**, 1086–1094
- Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 197–199
- Jansen, E. F. & Balls, A. K. (1941) *J. Biol. Chem.* **137**, 459–460
- Kahn, I. V. & Polgár, L. (1983) *Biochim. Biophys. Acta* **760**, 350–356
- Kamphuis, I. G., Kalk, K. H., Swarte, M. B. A. & Drenth, J. (1984) *J. Mol. Biol.* **179**, 233–257
- Kamphuis, I. G., Drenth, J. & Baker, E. N. (1985) *J. Mol. Biol.* **182**, 317–329
- Kunimitsu, D. K. & Yasunobu, K. T. (1967) *Biochim. Biophys. Acta* **139**, 405–417
- Lynn, K. R. (1979) *Biochim. Biophys. Acta* **569**, 193–201
- Lynn, K. R., Yaguchi, M. & Watson, D. C. (1985) *Abstr. Int. Congr. Biochem.* 13th, Amsterdam, 513
- Ozols, T. & Gerard, C. (1977) *J. Biol. Chem.* **252**, 5986–5989
- Schroeder, W. A., Shelton, J. R., Shelton, J. B., Cormick, J. & Jones, R. T. (1963) *Biochemistry* **2**, 992–1008
- Sluyterman, L. A. E. & Widjenes, J. (1970) *Biochim. Biophys. Acta* **200**, 595–597
- Titani, K., Sasagawa, T., Resing, K. & Walsh, K. A. (1982) *Anal. Biochem.* **123**, 408–412
- Tsunoda, J. S. & Yasunobu, K. T. (1966) *J. Biol. Chem.* **241**, 4610–4625