The Amino Acid Sequence Around the Active-Site Cysteine and Histidine Residues, and the Buried Cysteine Residue in Ficin

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Ficin that had been prepared from the latex of *Ficus glabrata* by salt fractionation and chromatography on carboxymethylcellulose was completely and irreversibly inhibited with 1,3-dibromo[2-¹⁴C]acetone and then treated with *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide in 6*M*-guanidinium chloride. After reduction and carboxymethylation of the labelled protein, it was digested with trypsin and α -chymotrypsin. Two radioactive peptides and two coloured peptides were isolated chromatographically and their sequences determined. The radioactive peptides revealed the amino acid sequences around the active-site cysteine and histidine residues and showed a high degree of homology with the amino acid sequence around the active-site cysteine and histidine residues in papain. The coloured peptides allowed the amino acid sequence around the buried cysteine residue in ficin to be determined.

Ficin belongs to a group of plant proteinases that depend for their enzymic activity on a cysteine residue. It is similar in many respects to papain, which is by far the most extensively studied enzyme of this family. Papain is known from chemical (Husain & Lowe, 1968*a,b,c*) and X-ray studies (Drenth, Jansonius, Koekoek, Swen & Wolthers, 1968) to have the imidazole group of a histidine residue in close proximity to the active-site cysteine and probably hydrogen-bonded to it, and there is good evidence that this plays an essential role in papain-catalysed hydrolyses (Lowe, 1970). Similarly ficin has been shown, by the same chemical methods, to contain the imidazole group of a histidine residue in close proximity and probably hydrogen-bonded to the thiol group of the active-site cysteine residue (Husain & Lowe, 1968*d*).

Ficin is irreversibly inhibited through a reactive cysteine residue by iodoacetamide (Hollaway, Mathias & Rabin, 1964), by iodoacetate (Wong & Liener, 1964), by the chloromethyl ketones derived from *N*-toluene-*p*-sulphonyl-L-lysine and *N*-toluene-*p*-sulphonyl-L-phenylalanine (Stein & Liener, 1967), by *N*-methylmaleimide (Hollaway *et al.* 1964) and by DDPM* (Metrione, Johnston & Seng, 1967). Using iodo[¹⁴C]acetate-inhibited ficin Wong & Liener (1964) isolated a radioactive peptide, after enzymic digestion, that showed a high degree of homology with the amino acid sequence around the active-site cysteine of papain. It seems reasonable to regard this residue as the active-site cysteine, but

it is known that ficin contains two thiol groups, one that is reactive in the active enzyme and one that is only titratable with *p*-chloromercuribenzoate (Liener, 1961) or alkylated with iodoacetate (Englund, King, Craig & Walti, 1968) after the tertiary structure of the enzyme has been disrupted. Using DDPM to label ficin, Metrione *et al.* (1967) isolated a coloured peptide, after peptic digestion of the DDPS-ficin, that was quite different in amino acid composition and amino acid sequence from that obtained by Wong & Liener (1964) and showed no homology with the amino acid sequence around the active-site cysteine of papain. Two possible explanations were offered by Metrione *et al.* (1967). First, since a different purification procedure had been used this may have resulted in the isolation of a different enzyme, or, secondly, if the same enzyme was used the two reagents had reacted with different thiol groups. The fact that inhibition was observed does not of course guarantee that alkylation of an active-site residue had occurred. With the hope of resolving this problem and with a view to determining the amino acid sequence around the active-site histidine residue, ficin prepared by the method of Englund *et al.* (1968) was inhibited with 1,3-dibromo[2-¹⁴C]acetone and then treated with DDPM. Radioactive and coloured peptides were isolated and their sequences determined.

EXPERIMENTAL

1,3-Dibromo[2-¹⁴C]acetone. This reagent (specific radioactivity 0.815 mCi/mmol) was prepared from bromo-[1-¹⁴C]acetic acid as described by Husain & Lowe (1968*b*).

* Abbreviations: DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; DDPS, *N*-(4-dimethylamino-3,5-dinitrophenyl)succinimidyl.

DDPM. This reagent was obtained from K & K Laboratories Inc., Plainview, N.Y., U.S.A.

Enzymes. α -Chymotrypsin (thrice recrystallized), trypsin (thrice recrystallized) and leucine aminopeptidase ($C_1=12.5$) were obtained from Seravac Laboratories Ltd., Maidenhead, Berks, U.K. Carboxypeptidase A-DFP (carboxypeptidase A treated with di-isopropyl phosphorofluoridate, dialysed and recrystallized) was obtained from Sigma (London) Chemical Co., London S.W.6, U.K.

Cellulose derivatives for chromatography. Microgranular Whatman CM-cellulose CM-52 (mean ion capacity 1.0m-equiv./g dry wt.) and Whatman cellulose phosphate P11 (nominal total capacity 7.4m-equiv./g) were obtained from H. Reeve and Co., London E.C.4, U.K.

Anhydrous hydrazine. This was made from hydrazine hydrate by refluxing with CaO in toluene followed by distillation (Fraenkel-Conrat & Tsung, 1967).

Sodium tetrathionate. This was made by the method of Gilman, Philips, Koelle, Allen & St John (1946).

Other reagents. These were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Phenyl isothiocyanate was distilled under reduced pressure and stored at -20°C . Guanidinium chloride was treated with Norit and recrystallized from methanol.

Polyamide layers. Polyamide layers for chromatography of DNS-amino acids were obtained from the Cheng-Chin Trading Co. Ltd., Taipei, Taiwan.

All buffers were made from A.R.-grade reagents in de-ionized water and were deaerated before use.

Scintillation counting. Scintillation counting of radioactive solutions was performed on an automatic Beckman DPM-100 scintillation counter. The scintillation solution was made by dissolving 2,5-diphenyloxazole (6g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.06g) in A.R.-grade toluene (700ml) and methanol (300ml). The scintillation solvent (6ml) was mixed with the aqueous solution of the sample (25–50 μl) for counting.

Cellulose phosphate chromatography. Cellulose phosphate was thoroughly washed with 0.5M-NaOH, water, 0.5M-HCl, water and 0.05M-pyridine-acetate buffer, pH3.91. The slurry was packed under gravity in a column (35cm $\times$ 1cm) and thoroughly equilibrated with 0.05M-pyridine-acetate buffer, pH3.91. Peptide solutions were applied in 10–20% acetic acid solution (5–10ml) and eluted either with an eight-chamber gradient, each chamber containing 100ml of pyridine-acetate buffer as described by Canfield & Anfinsen (1963), or with a three-chamber gradient, the first chamber containing 0.05M-pyridine-acetate buffer, pH3.91 (150ml), the second 0.10M-pyridine-acetate buffer, pH4.02 (150ml), and the third 0.15M-pyridine-acetate buffer, pH4.21 (150ml).

Paper chromatography. Paper chromatography was performed on Whatman no. 3MM paper with butanol-acetic acid-water (40:6:15, by vol.) by descending flow. After development, guide strips were cut and sprayed with a 0.1% ethanolic solution of ninhydrin, with a 0.5% solution of *p*-dimethylaminobenzaldehyde solution in ethanol containing 2% concentrated hydrochloric acid (Ehrlich reagent) and counted for radioactivity on an I.D.L. chromatogram scanner 2029 in conjunction with an automatic scaler 1700 and a recorder. Peptides required for further study were cut out and eluted off the paper with aq. 10% acetic acid for at least 24h. Peptides thus eluted from paper were filtered through a column (140cm $\times$ 1cm)

of Sephadex G-25 by using water as eluent, or occasionally with aq. 50% acetic acid, to remove amino acids and other contaminants eluted from the paper.

Isolation of ficin. Crude ficin from the latex of *Ficus glabrata* (Lot 3486; Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.) was purified by salt fractionation and chromatography on CM-cellulose essentially as described by Englund *et al.* (1968) except that the method was adapted for a larger-scale preparation.

Crude ficin (120g) was extracted with a solution (1.2 litres) containing 1mM-EDTA and 10mM-sodium tetrathionate and fractionated with NaCl as described by Englund *et al.* (1968). After dialysis, chromatography was performed on a column (60cm $\times$ 5cm, 1litre) of CM-cellulose CM-52. The sample (600ml) was applied to the column and eluted with 5mM-sodium phosphate buffer, pH7.1 (1.8litres), and then with a gradient buffer obtained by connecting two vessels, one containing 5mM-sodium phosphate buffer, pH7.1 (6litres, containing 1mM-EDTA and 1mM-sodium tetrathionate), and the second containing 185mM-sodium phosphate buffer, pH6.9 (6litres, containing 1mM-EDTA and 1mM-sodium tetrathionate). The column was run at 300ml/h and 15ml fractions were collected. The proteolytic activity was determined by the method of Kunitz (1947) as described by Englund *et al.* (1968). The chromatogram is shown in Fig. 1.

Fractions 304–356 were pooled and the enzyme was precipitated by adding sodium chloride (260g). After standing at 4°C for several days the precipitated enzyme was centrifuged. The total yield of ficin from the pooled fractions was 0.82g as judged from the extinction of the solution taking $E_{280}^{1\%}^{0.1\text{cm}}$ as 2.1 (Englund *et al.* 1968).

Inhibition of ficin with 1,3-dibromo[2- ^{14}C]acetone. Purified ficin (0.526g) (approx. 20 μmol) was dissolved in 0.05M-sodium acetate buffer, pH5.6, containing 1mM-EDTA (60ml), and activated by adding cysteine hydrochloride (95mg). The activator was removed by gel filtration through a column (3.5cm $\times$ 45cm) of Sephadex G-25 with 0.05M-sodium acetate buffer, pH5.6, containing 1mM-EDTA, which had been purged of dissolved air by bubbling with N_2 . The protein-containing fractions were pooled and the activity assayed on a Radiometer pH-stat with methyl hippurate as substrate (cf. Lowe & Williams, 1965). The enzyme was found to be completely active, since no enhancement of the activity was observed after adding cysteine. Inhibition of the activator-free ficin was performed immediately by adding 1,3-dibromo[2- ^{14}C]acetone (specific radioactivity 0.815mCi/mmol, 7mg, 32 μmol). After the preparation had been kept at 18°C for 1h, no activity was detectable when assayed with methyl hippurate on a Radiometer pH-stat. Excess of inhibitor was then removed by gel filtration through a column (45cm $\times$ 3.5cm) of Sephadex G-25 eluted with 0.05M-sodium acetate buffer, pH5.6.

Denaturation of the dibromo[2- ^{14}C]acetone-inhibited ficin and reaction with DDPM. The inhibited ficin fractions after removal of the excess of inhibitor were evaporated to dryness in a rotary evaporator. The residue was redissolved in 6M-guanidinium chloride solution (25ml, containing 0.05M-sodium acetate buffer, pH5.6, 1mM-EDTA and 5ml of methanol). DDPM (12.4mg, 40 μmol) was added and after 20min at 18°C the excess of maleimide derivative was separated on a column (45cm $\times$ 3.5cm) of Sephadex G-25 (fine grade) equilibrated with aq. 50%

acetic acid. The coloured radioactive protein fractions were pooled and the acetic acid was removed in a rotary evaporator.

Reduction and carboxymethylation of the dibromo[2-¹⁴C]-acetone-DDPS-ficin. The dibromo[2-¹⁴C]acetone-DDPS-ficin was dissolved in 5.5M-guanidinium chloride [25ml, containing 1mM-EDTA, 12.5ml of tris-HCl buffer, pH 8.6, made from tris (5.23g) and 1M-HCl (9.0ml), diluted to 30ml with water]. Mercaptoethanol (0.3ml) was added and the solution kept under N₂ at 18°C. After 4h, recrystallized iodoacetic acid (0.8g) dissolved in 1M-NaOH (4.3ml) was added. After a further 20min the reagents were separated from the reduced and carboxymethylated protein by gel filtration through a column (45 cm × 3.5 cm) of Sephadex G-25 with aq. 50% acetic acid.

Digestion of the inhibited, reduced and carboxymethylated ficin with chymotrypsin and trypsin. The acetic acid was removed from the protein fractions in a rotary evaporator by repeatedly adding water and concentrating. The volume of the solution was adjusted to 100ml and the pH brought to 8.0 by adding 2M-NaOH, which caused precipitation of some of the protein. Trypsin (5mg) was added and the pH maintained at 8.0 by adding 0.1M-NaOH in a Radiometer pH-stat. After 4h at 25°C chymotrypsin (7mg) and more trypsin (2mg) were added and the digestion was allowed to proceed at 25°C for a further period of 3.5h, the pH being maintained automatically at 8.0. The digestion was terminated by adding 12M-HCl to bring the pH to 3.5.

Sephadex G-25 chromatography of the digest. The digest was concentrated to 5ml in a rotary evaporator and applied to a column (160 cm × 2 cm) of Sephadex G-25 (fine grade), and the peptides were eluted with 25% acetic acid containing 0.2% thiodiglycol. Fractions (4ml) were collected at a flow rate of 12ml/h. Peptides containing aromatic residues were located by measuring the extinction at 280nm, the coloured peptides by extinction at 440nm and the radioactive fractions by scintillation counting. The chromatogram is shown in Fig. 2.

Purification of the radioactive peptides. The radioactive fractions 46–56 (Fig. 2) were combined, concentrated to 5ml in a rotary evaporator, applied to a column (35 cm × 1 cm) of cellulose phosphate and eluted with the eight-chamber gradient described above. Fractions (4ml) were collected at a flow rate of 25 ml/h. The radiochromatogram is shown in Fig. 3.

Fractions 21–22 (Peptide 1) and 32–36 (Peptide 2) were combined separately. Each of the pooled radioactive peaks were concentrated to about 1ml in a rotary evaporator and further purified by paper chromatography. The radioactive peptide from fractions 21–22 (distance moved 5.5 cm in 15h) was well separated from small amounts of non-radioactive peptides. The radioactive peptide in fractions 32–36 moved 2.5 cm in 15h and was also well separated from the small amount of non-radioactive peptides. Each of the radioactive peptides gave a positive reaction with Ehrlich reagent. The radioactive peptides after elution from the paper were filtered on a column (140 cm × 1 cm) of Sephadex G-25 with water.

Baeyer-Villiger oxidation of the radioactive peptide 2 and separation of the cysteine and the histidine chains. Radioactive peptide 2 (1.0 μmol) was dissolved in 99% formic acid (0.2ml) and anhydrous methanol (0.02ml). The solution was cooled to –10°C, performic acid [0.4ml; made

from formic acid (9.5ml) and 30% hydrogen peroxide (0.5ml)] was added and the mixture was incubated for 2h. After 6h at –5 to –10°C the solution was diluted with cold water (10ml) and freeze-dried (cf. Hirs, 1956).

For the cleavage of the ester bond formed on performic acid oxidation, the peptide was treated with 2M-hydroxylamine, pH 9.6, at 35°C for 6h. The solution was chromatographed on a column (140 cm × 1 cm) of Sephadex G-25 with aq. 50% acetic acid, whereby two radioactive peaks were obtained (Fig. 4). Fractions 54–58 were rechromatographed on the same column (Fig. 4). Fractions 46–53 were purified by paper chromatography.

Purification of the coloured peptides. The coloured fractions 76–86 (Fig. 2) were combined and purified by paper chromatography. The major coloured peptide (*R_F* 0.72) was a broad band well clear of contaminating peptides, the nearest having *R_F* 0.36. The yellow peptide was eluted off the paper with water and rechromatographed on a column (140 cm × 1 cm) of Sephadex G-25 with deionized water. The coloured peptide (DDPS-peptide 2) was strongly held on Sephadex, requiring 160ml of water to elute it.

The coloured fractions 67–74 (Fig. 2) were also combined and chromatographed on paper. The major yellow band (*R_F* 0.43) was cut out and eluted off the paper with water. Further purification was performed on cellulose phosphate with a three-chamber gradient as described above. The only coloured peptide emerged after 400ml (DDPS-peptide 1). It was further purified on a column (140 cm × 1 cm) of Sephadex G-25 with deionized water; the coloured peptide emerged after 105ml.

Amino acid analyses. Aqueous solutions of protein or peptides (0.05–0.1 μmol in 1ml) were mixed with electronic-grade HCl (12M, 1ml) in a glass tube and evacuated to 0.01mmHg pressure. The tubes were sealed and kept at 110°C for 18–48h, after which the contents were removed and evaporated to dryness in a rotary evaporator. The residue was taken up in 0.05M-HCl (1ml) and analysed on a Technicon AutoAnalyzer with the buffer system of Thomson & Miles (1964).

Leucine aminopeptidase digestions. To the peptide (0.05–0.10 μmol) in 0.05M-tris-HCl buffer, pH 8.55, containing 5mM-MgCl₂ (0.5ml) was added leucine aminopeptidase (0.16mg) in the same buffer (0.03ml). The solution was incubated at 35°C for 36h and then acidified by adding 1M-HCl (0.1ml); the solution was then analysed for amino acids.

Carboxypeptidase digestions. Carboxypeptidase A (0.005ml of a suspension containing 0.01mg) was solubilized by adding 0.5% Na₂CO₃ solution (0.05ml) and immediately added to a solution of the peptide (0.05–0.20 μmol) in 0.10M-sodium phosphate buffer, pH 7.8 (1ml). The solution was incubated at 25–35°C and the digestion stopped by acidifying with 1M-HCl to pH 3. The solution was analysed for amino acids.

Hydrazinolysis. Hydrazinolysis of the radioactive peptide from fractions 32–36 (Fig. 3) that had been purified on paper was performed by the method of Akabori, Ohno & Narita (1952). The peptide (0.4 μmol) was dissolved in anhydrous hydrazine (0.5ml), sealed in a glass tube under vacuum and heated at 95°C for 15h. Hydrazine was removed under vacuum over H₂SO₄, and the residue analysed for amino acids.

Sequence determination by direct Edman degradation.

The peptides ($0.2\mu\text{mol}$) were degraded as described by Gray (1967). The phenylthiohydantoin derivatives were extracted with *n*-butyl acetate, and the residual peptide was analysed after acid hydrolysis.

Sequence determination by 'dansyl'-Edman degradations. 'Dansyl'-Edman degradations were performed as described by Gray (1967). DNS-amino acids were chromatographed on polyamide layers as described by Woods & Wang (1967), the solvent systems being formic acid-water (3:200, v/v) and benzene-acetic acid (9:1, v/v) at right-angles to the first development followed by ethyl acetate-methanol-acetic acid (20:1:1, by vol.) in the same direction.

RESULTS AND DISCUSSION

Salt-fractionated ficin had been shown in an earlier study to be completely and irreversibly inhibited by one molar equivalent of 1,3-dibromoacetone (Husain & Lowe, 1968*d*). The molecular weight and optical-rotatory-dispersion curve of the inhibited enzyme were indistinguishable from those of the active enzyme. By using 1,3-dibromo[2- ^{14}C]-acetone it was shown that this reagent cross-linked a cysteine and a histidine residue. It is known, however, that salt-fractionated ficin is not homogeneous, and so for the purpose of structural studies ficin was prepared from the crude latex of *Ficus glabrata* by salt fractionation and chromatography on CM-cellulose. The elution pattern (Fig. 1) was

almost identical with that reported by Englund *et al.* (1968). The material in fractions 304–356 was pooled and used for structural studies. Englund *et al.* (1968) also used this fraction in their studies as it appeared to be homogeneous. For the subsequent discussion, this material is referred to as ficin.

Ficin was inhibited with 1,3-dibromo[2- ^{14}C]-acetone at pH 5.6 and after removal of excess of inhibitor the enzyme was found to have incorporated 0.6 mol of the radioactive inhibitor/mol of ficin. Amino acid analysis indicated that 0.64 residue of histidine had been lost/molecule, the analysis for cysteine was less reliable, but all other residues were essentially the same. Thus it appears that the best preparations of ficin, like papain, contain only about 60% active enzyme. The inhibited enzyme in 6*M* guanidinium chloride was treated with DDPM. The reagents were separated from the protein by gel filtration through Sephadex G-25 and the incorporation of DDPM as determined from the extinction at 440 nm (using ϵ_{440} 3000; Gold & Segal, 1964) was 77%. Amino acid analysis of the protein hydrolysate showed 0.75 residue of *S*-succinylcysteine/molecule of enzyme.

The disulphide bridges of the labelled ficin were reduced and carboxymethylated and the protein was digested with trypsin and α -chymotrypsin. Gel filtration of the digest on Sephadex G-25 in aq. 25% acetic acid gave almost all the radioactivity in a

Fig. 1. Chromatography of salt-fractionated ficin on a column (60 cm $\times$ 5 cm) of CM-cellulose CM-52 with a gradient of sodium phosphate buffer containing 1 mM-EDTA and 1 mM-sodium tetrathionate. Fractions (15 ml) were collected at a flow rate of 300 ml/h. —, E_{280} ; ----, caseinolytic activity (E_{280}).

Fig. 2. Chromatography of the tryptic-chymotryptic digest of 1,3-dibromo[2-¹⁴C]acetone-inhibited DDPM-treated and carboxymethylated ficin on a column (160 cm × 2 cm) of Sephadex G-25 in aq. 25% acetic acid. Fractions (4ml) were collected at a flow rate of 12ml/h. —, E_{280} ; ----, E_{440} ; ····, radioactivity.

Fig. 3. Chromatography on cellulose phosphate of the pooled radioactive fractions (46-56, Fig. 2) with a pyridine-acetate buffer gradient. Fractions (4ml) were collected at a flow rate of 25ml/h.

Table 1. *Amino acid analyses of the acid hydrolysates (24 h) of the purified radioactive peptides*

Both peptides gave a positive reaction with Ehrlich reagent. No correction for decomposition has been applied. CMCys, *S*-carboxymethylcysteine.

Amino acid	No. of residues	
	Radioactive peptide 1	Radioactive peptide 2
CMCys	2.0	1.9
Asp	1.2	1.2
Thr	2.0	1.9
Ser	1.7	2.0
Glu	3.1	3.0
Pro	1.1	1.0
Gly	4.0	3.8
Ala	2.1	1.9
Val	1.1	1.0
Leu	2.0	1.9
Trp	+	+

single sharp peak; there were, however, several coloured peptides (Fig. 2).

The radioactive material from the Sephadex G-25 column was separated into two sharp radioactive peaks, peptide 1 (fractions 21-22) and peptide 2 (fractions 32-36), on cellulose phosphate (Fig. 3).

Both peptides were purified by paper chromatography and finally passed through a column of Sephadex G-25 to remove contaminants arising from the paper. The amino acid analyses of the hydrolysates were found to be the same (Table 1). Digestion of the radioactive peptide 2 with carboxypeptidase A for various times released the amino acids shown in Table 2. Digestion of the radioactive

Table 2. *Amino acid analyses of the carboxypeptidase A digests of the radioactive peptides 1 and 2*

The period of digestion is given for each analysis.

Amino acid	No. of residues			
	Radioactive peptide 2			Radioactive peptide 1 24 h
	1.5 min	10 min	24 h	
Ala	0.3	1.0	1.0	1.1
Val	—	0.7	1.0	1.0
Leu	0.7	1.0	0.9	1.0
Trp	0.6	1.0	1.0	1.0

Table 3. *Amino acid analysis of the leucine aminopeptidase digest (36 h) of the radioactive peptide 2*

Amino acid	No. of residues Radioactive peptide 2
CMCys	2.0
Asp	1.0
Thr	2.0
Ser } Gln }	4.5
Pro	1.0
Gly	4.0
Ala	1.8
Val	1.0
Leu	2.0
Trp	1.0

peptide 1 with carboxypeptidase A for 24 h showed that the peptides were identical at the *C*-terminus (Table 2). Hydrazinolysis of the radioactive peptide 2 for 15 h released tryptophan (0.8 residue/molecule) and leucine (0.2 residue/molecule) indicating these to be the *C*-terminal residues of the cross-linked peptide chains. Digestion of the radioactive peptide 2 with leucine aminopeptidase released the amino acids shown in Table 3. Digestion of the radioactive peptide 1 with leucine aminopeptidase did not release glutamine. Since glutamine was expected from the work of Wong & Liener (1964) to be the *N*-terminal residue of the cysteine chain, it appeared probable that radioactive peptide 1 contained a rearranged or pyroglutamic acid residue at the *N*-terminus of the cysteine chain. Advantage was taken of this observation to determine the sequence of the cysteine-containing and histidine-containing chains in the radioactive peptides. Successive Edman degradations of radioactive peptide 1 removed only one residue at each cycle, whereas with

Table 4. *Successive 'dansyl'-Edman degradation of radioactive peptides 1 and 2*

No. of Edman cycles	<i>N</i> -Terminal residue of peptide 1	<i>N</i> -Terminal residues of peptide 2
0	Thr	Glx, Thr
1	Gly	Glx, Gly
2	Pro	Gly, Pro
3	CMCys†	Glx, CMCys†
4	Gly	CMCys,† Gly
5	Thr	Gly, Thr
6	Ser	Ser, Ser
7	Leu	—,* Leu
8	Asp	—,* Asp
9	—*	—,* —*
10	Ala	—,* Ala
11	Val	—,* Val

* No *N*-terminal residues identified.

† DNS-CMCys could not be detected on the polyamide layer with the solvent system used and so was detected by electrophoresis as described by Gray (1967).

radioactive peptide 2 two residues were removed, one of which corresponded to that removed from radioactive peptide 1 (Table 4). By using the 'dansyl'-Edman technique nine successive cycles on each peptide were accomplished without complication. After nine cycles, no *N*-terminal residue was found, but after the tenth and eleventh cycles alanine and valine were the predominant *N*-terminal residues. As it was possible to assign the cysteine and histidine residues to the respective chains from the known sequence around the active-site cysteine residue (Wong & Liener, 1964), it only remained therefore to assign the *C*-terminal tryptophan and leucine to their respective chains. The radioactive peptide 2 was oxidized with performic acid and the ester bond formed from the cross-linking ketone was cleaved with hydroxylamine. The product was separated on Sephadex G-25 into two radioactive peaks (Fig. 4, —). The faster-moving radioactive peptide could not be completely purified by paper chromatography. The peak that was more strongly retained, however, was rechromatographed on the same column (Fig. 4, ----) and was shown to be pure by paper chromatography. The amino acid analysis of the acid hydrolysate of this peptide (Table 5) showed it to be derived from the histidine chain and contained both the leucine residues. The structure of radioactive peptide 2 is therefore as shown in Fig. 5, radioactive peptide 1 being a modification of this in which the *N*-terminal glutamine is rearranged.

The coloured fractions (67–74) from the Sephadex G-25 column (Fig. 2) were chromatographed on paper and the major coloured component (R_F 0.43)

was extracted and chromatographed on a column of cellulose phosphate with a three-chamber buffer gradient. Final purification of the peptide (coloured peptide 1) was achieved on a column of Sephadex G-25, by eluting with water. The purification of the coloured peptide in fractions 76-84 from the Sephadex G-25 column (Fig. 2) was achieved by

Fig. 4. Chromatography of the radioactive peptide (fractions 32-36, Fig. 3), after treatment with performic acid followed by hydroxylamine, on a column (140 cm $\times$ 1 cm) of Sephadex G-25. ----, Rechromatography on the same column of fractions 54-58 in the original (—) chromatogram. The column was eluted with aq. 50% acetic acid and fractions (1 ml) were collected at a flow rate of 6 ml/h.

Table 5. Amino acid analysis of the acid hydrolysate (24 h) of the radioactive peptide obtained (Fig. 4) after performic acid oxidation of radioactive peptide 2

1-CMHis, 1-carboxymethylhistidine, colour value unknown.

Amino acid	No. of residues
Asp	1.0
Thr	1.0
Ser	0.9
Gly	1.2
Ala	2.0
Val	1.1
Leu	1.9
1-CMHis	present

paper chromatography (R_F 0.72) and gel filtration on Sephadex G-25 by using water as the eluent. This peptide (coloured peptide 2) was strongly held on Sephadex G-25. The amino acid analyses of the acid hydrolysates of the two coloured peptides are shown in Table 6 and are seen to be identical. Digestion of the coloured peptide 2 with carboxypeptidase A for 8 h released lysine (1.1 residues/molecule) and valine (0.6 residue/molecule). The *N*-terminal residue of both coloured peptides was found to be DDPS-cysteine. Two cycles of 'dansyl'-Edman degradation established the sequence of the two coloured peptides as:

DDPS-Cys-Leu-Tyr-Pro-Val-Lys

The differences in structure may be associated with the generation of a new asymmetric centre when the cysteine residue adds to the maleimide derivative. If this addition is not stereospecific two diastereoisomers would be formed, which could account for the two coloured peptides (cf. Smyth, Blumenfeld & Konigsberg, 1964).

The amino acid sequences around the cysteine and histidine residues cross-linked by dibromo-[2- 14 C]acetone in ficin show a high degree of homology with the active-site cysteine and histidine residues in papain (Fig. 6), leaving no doubt that these are the active-site residues in ficin. The

Table 6. Amino acid analyses of the coloured peptides 1 and 2 of the acid hydrolysates (24 h)

No correction for decomposition has been applied. SucCys, *S*-succinylcysteine.

Amino acid	No. of residues	
	Coloured peptide 1	Coloured peptide 2
SucCys	0.7	0.7
Pro	1.0	1.0
Val	1.0	1.1
Leu	1.0	1.0
Tyr	0.9	0.9
Lys	0.9	1.0

Fig. 5. Structure of the radioactive peptide 2.

Ficin	Gln-Gln-Gly-Gln-Cys-Gly-Ser-Cys*-Trp
Papain	Asn-Gln-Gly-Ser-Cys-Gly-Ser-Cys*-Trp
Ficin	Thr-Gly-Pro-Cys-Gly-Thr-Ser-Leu-Asp-His*-Ala-Val-Ala-Leu
Papain	Val-Gly-Pro-Cys-Gly-Asn-Lys-Val-Asp-His*-Ala-Val-Ala-Ala

Fig. 6. Comparison of the amino acid sequences around the cysteine and histidine residues crosslinked by 1,3-dibromo[2-¹⁴C]acetone in ficin and the active-site cysteine and histidine residues in papain.

amino acid sequence of the coloured peptide shows no homology with the active-site cysteine of papain, and it is clear that DDPM has reacted with the buried cysteine residue in ficin. The DDPM-labelled cysteine, however, is at the *N*-terminus of the peptide, whereas the labelled cysteine is at the *C*-terminus of the peptide (Trp-Ser-Gly-Val-Cys) isolated from DDPS-ficin by Metrione *et al.* (1967), who pointed out that the peptide they isolated showed a high degree of homology with the amino acid sequence around one of the cysteine residues in papain (now known to be cysteine-200; Husain & Lowe, 1969). However, this forms a disulphide bridge with cysteine-153 in papain and the amino acid sequence around the cysteine labelled with DDPM reported here shows good homology with residues 206–211 (i.e. Ser-Phe-Tyr-Pro-Val-Lys; for numbering see Husain & Lowe, 1969) in papain. Although it is conceivable that the residues corresponding to 201–206 in papain are absent from ficin and that the cysteine residue labelled by DDPM in the experiment reported here and that of Metrione *et al.* (1967) are the same, the lack of overlap between these peptides leaves this undecided. The tertiary structure of papain, however, makes this unlikely, since residues 200 and 207 are a considerable distance apart, residues 201–206 forming a chain running through the molecule and not a loop on the surface. It seems more probable therefore that the additional cysteine residue in ficin (which is known to contain three disulphide bridges, like papain, and two cysteine residues; Englund *et al.* 1968) would correspond to serine-206 in papain, and that the material used by Metrione *et al.* (1967) was different from that used in the experiment reported here.

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