Evidence for Histidine in the Active Sites of Ficin and Stem-Bromelain

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1. Ficin and stem-bromelain are irreversibly inhibited by 1,3-dibromoacetone, a reagent designed to react first with the active-site cysteine residue and subsequently with a second nucleophile. Evidence is presented that establishes that a histidine residue is within a 5 Å locus of the active-site cysteine residue in both enzymes. The histidine residue in both enzymes is alkylated at N-1 by dibromoacetone. It is suggested that, as with papain, the thiol and imidazole groups act in concert in the hydrolysis of substrates by these enzymes. 2. The inhibition of thiol-subtilisin with 1,3-dibromoacetone is shown to be due to the alkylation of a cysteine residue only.

The irreversible inhibition of ficin by iodoacetamide is known to be due to the alkylation of a cysteine residue (Hollaway, Mathias & Rabin, 1964). The amino acid sequence around this residue has been established (Wong & Liener, 1964) and is shown in Table 1. Papain, which contains only one free cysteine residue, is irreversibly inhibited by iodoacetate (Light, Frater, Kimmel & Smith, 1964) and by the active-site-directed irreversible inhibitor, chloromethyl toluene-*p*-sulphonamido-methyl ketone (Husain & Lowe, 1965), through the alkylation of this residue. Although ficin contains at least two thiol groups/mol. (Liener, 1961) only one reacts with iodoacetate. It seems clear from the amino acid sequence around the alkylated cysteine residues (Table 1) that iodoacetate alkylates the active-site cysteine residue of ficin.

Stein & Liener (1967) reported that the chloromethyl ketones derived from *N*²-toluene-*p*-sulphonyl-L-lysine and *N*-toluene-*p*-sulphonyl-L-phenylalanine also inhibit ficin through a cysteine residue. This result, however, was apparently contrary to their expectations, since it was envisaged that these inhibitors would react with a histidine residue if it were present in the active site, as was found with trypsin (Shaw & Springhorn, 1967) and α -chymotrypsin (Ong, Shaw & Schoellmann, 1964). Their expectations, however, seem to be unwarranted, since, if both a thiol and an imidazole group are present in the catalytic site, the more nucleophilic thiol group is likely to react faster and so prevent alkylation of the imidazole group.

The irreversible inhibition of stem-bromelain by iodoacetate has also been investigated and the amino acid sequence around the alkylated cysteine residue established (see Table 1) (Chao & Liener, 1967). Although this peptide is rather small and

the serine residue adjacent to the alkylated cysteine residue in papain and ficin is replaced by alanine, it seems likely that this is the active-site cysteine residue. Further work with this enzyme is, however, desirable.

The problem of locating a nucleophile close to a reactive thiol group in the active site of an enzyme was solved with papain by using the bifunctional reagent 1,3-dibromoacetone (Husain & Lowe, 1968*a,b,c*). This reagent was designed to react first with the active-site thiol group and then intramolecularly with another nucleophile within a range of 5 Å. A histidine residue in papain was successfully alkylated with this reagent and this, together with other evidence, established the presence of this residue in the active site of the enzyme. 1,3-Dibromoacetone has now been used to investigate the active site of ficin, stem-bromelain and thiol-subtilisin.

MATERIALS AND METHODS

1,3-Dibromo[2-¹⁴C]acetone. This reagent was prepared as described by Husain & Lowe (1968*b*).

Ficin. Ficin was prepared from crude fig latex (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) by the method of Hammond & Gutfreund (1959). Enzyme activity was assayed by the rate of hydrolysis of methyl hippurate in a Radiometer pH-stat (cf. Lowe & Williams, 1965).

Inhibition of ficin. Ficin (0.18 g.) in 1 mM-EDTA-0.03 M-sodium acetate buffer, pH 5.6 (18 ml.), was treated with 1,3-dibromo[2-¹⁴C]acetone (1.5 mg.) in acetone (1 ml.) at 20°. No enzymic activity remained after 5 min. The excess of inhibitor was removed by dialysis against several changes of water for 24 hr.

Stem-bromelain. Bromelain was prepared from crude pineapple stem-bromelain (a generous gift from Enzyme Development Corp., New York, N.Y., U.S.A.; 1200 gelatin

Table 1. *Amino acid sequence around the cysteine residues alkylated by iodoacetate in some plant proteinases*

Papain contains only 1 free thiol group/mol. (Light *et al.* 1964), and this is also alkylated by the active-site-directed inhibitor chloromethyl toluene-*p*-sulphonamidomethyl ketone (Husain & Lowe, 1965).

Papain	Lys-Asn-Gln-Gly-Ser-Cys-Gly-Ser-Cys*-Trp
Ficin	Arg-Gln-Gln-Gly-Gln-Cys-Gly-Ser-Cys*
Stem-bromelain	Cys-Gly-Ala-Cys*-Trp

* This residue is alkylated by iodoacetate.

digestion units) by the method of Ota, Moore & Stein (1964). Most of the enzyme activity appeared as a single peak from Sephadex G-100 and sulphoethyl-Sephadex C-25. Purified bromelain (75 mg.) in 0.3 M-sodium citrate buffer (10 ml.) was activated with cysteine hydrochloride (32 mg.) and 0.1 M-EDTA (0.1 ml.). The activator was separated by passing the solution through a column of Sephadex G-25 equilibrated with 0.1 mM-EDTA-0.05 M-sodium acetate buffer, pH 5.6, at 4°. The enzyme activity was assayed by the rate of hydrolysis of *N*^α-benzoyl-L-arginine ethyl ester on a Radiometer pH-stat.

Inhibition of stem-bromelain. Activated stem-bromelain (34 mg.) in 0.1 mM-EDTA-0.05 M-sodium acetate buffer, pH 5.6 (25 ml.), was treated with 1,3-dibromo[2-¹⁴C]acetone (0.43 mg.) in acetone (0.5 ml.) at 20°. Inhibition was complete in less than 5 min. The excess of inhibitor was removed by dialysis against several changes of water for 2 days.

Molecular weights of the inhibited enzymes. The molecular weights of the inhibited enzymes were determined on a column of Sephadex G-75, which was calibrated with haemoglobin (mol.wt. 68000) and the active enzymes (ficin mol.wt. approx. 26000 and stem-bromelain mol.wt. approx. 33000; Murachi, Yasui & Yasuda, 1964) (see Fig. 1).

Optical rotatory dispersion of the inhibited enzymes. The optical-rotatory-dispersion curves of ficin, stem-bromelain and the 1,3-dibromoacetone-inhibited enzymes were measured in 0.1 mM-EDTA-0.05 M-sodium acetate buffer, pH 5.6, in a 1 cm. cell in a Bendix Polaromatic 62 instrument, between 303 and 240 mμ. By using λ₀ = 220, the Moffitt *b*₀ parameters were: for ficin (0.34 mg./ml.) -69 ± 3; for inhibited ficin (0.38 mg./ml.) -66 ± 4; for stem-bromelain (0.47 mg./ml.) -62 ± 6; for inhibited stem-bromelain (0.42 mg./ml.) -60 ± 8.

Determination of thiol groups. The thiol content of ficin, stem-bromelain and the inhibited enzymes were determined by the method of Ellman (1959). The results are shown in Table 2.

Amino acid analysis of the inhibited enzymes. Each enzyme (0.1 μmole) and each inhibited enzyme (0.1 μmole) was hydrolysed with constant-boiling HCl (2 ml.) in a sealed evacuated tube at 110° for 22 hr. The acid was removed under reduced pressure, and the residue was taken up in 0.1 N-HCl (1 ml.) and analysed for amino acids on a Technicon AutoAnalyzer. The results are shown in Table 3.

Performic acid oxidation. The inhibited enzymes (1 μmole) were oxidized with performic acid at -10° for 6 hr. by the method of Hirs (1956).

Radioactivity. All radioactive measurements were made in solution in an Isotopes Development Ltd. scintillation counter, type 6012, with a scaler 1700.

Thiol-subtilisin. This semi-synthetic enzyme was

prepared from crystalline subtilisin Novo (we are grateful to Dr M. H. Nielson of Novo Industri A/S, Copenhagen, Denmark, for a generous gift of this material) as described by Polgar & Bender (1967), and contained 0.69 thiol group/mol., determined by the method of Ellman (1959).

Inhibition of thiol-subtilisin. Thiol-subtilisin (45 mg.) in 0.05 M-sodium phosphate buffer, pH 7.5 (30 ml.), was treated with 1,3-dibromo[2-¹⁴C]acetone (0.86 mg.) in acetone (1 ml.) at 20°. After 1 hr., 70% of the enzyme activity was inhibited when assayed with *p*-nitrophenyl acetate (cf. Polgar & Bender, 1967). Further addition of the inhibitor and incubation for 4 hr. did not change the enzyme activity. The excess of inhibitor was removed by dialysis against several changes of water for 2 days.

RESULTS AND DISCUSSION

The irreversible inhibition of ficin and stem-bromelain by a molar equivalent of 1,3-dibromoacetone at pH 5.6 was complete in less than 5 min. The molecular weights of the inhibited enzymes, determined on a column of Sephadex G-75, were indistinguishable from those of the active enzymes, and there was no evidence of dimeric or oligomeric species (Fig. 1). The optical-rotatory-dispersion curves of ficin and 1,3-dibromoacetone-inhibited ficin were also indistinguishable, as were those of stem-bromelain and the 1,3-dibromoacetone-inhibited enzyme.

The thiol content and amino acid analyses of ficin and stem-bromelain, together with those of the 1,3-dibromoacetone-inhibited enzymes, are shown in Tables 2 and 3. The loss of 0.62 thiol group/mol. and 0.6 residue of histidine in the inhibited ficin together with incorporation of 0.59 mole of dibromo[¹⁴C]acetone/mole indicates that a cysteine residue and a histidine residue had been cross-linked by the inhibitor. The low thiol content of the enzyme may mean that some oxidation of the enzyme had occurred or that the preparation was not homogeneous (Sgarbieri, Gupte, Kramer & Whitaker, 1964). Stem-bromelain lost 0.74 thiol group and 0.75 histidine residue/mol. when inhibited by 1,3-dibromoacetone and incorporated 0.72 mole of the ¹⁴C-labelled reagent/mole, again indicating that these groups had been cross-linked. The histidine content of stem-bromelain is uncertain: Ota *et al.* (1964), assuming mol.wt. 35500, found

Fig. 1. Gel filtration of haemoglobin (●), ficin (▲), dibromoacetone-inhibited ficin (▼), stem-bromelain (■) and dibromoacetone-inhibited stem-bromelain (◆), on a column (38 cm. × 2 cm.) of Sephadex G-75 equilibrated and eluted with 0.05 M-sodium acetate buffer, pH 5.6. The rate of elution was 25 ml./hr. at 20°. Fractions (1 ml.) were collected.

Table 2. *Thiol content of ficin, stem-bromelain and the 1,3-dibromoacetone-inhibited enzymes*

The thiol contents were determined by the method of Ellman (1959).

Protein	Thiol group/mol.
Ficin	0.67
Inhibited ficin	0.05
Stem-bromelain	0.85
Inhibited stem-bromelain	0.11

2 histidine residues/mol., whereas Murachi (1964), assuming mol.wt. 33 000, found only 1.3 histidine residues/mol.

To identify histidine positively as one of the alkylated amino acid residues in ficin and stem-bromelain and to determine the site of alkylation (i.e. N-1 or N-3), ficin and stem-bromelain inhibited with 1,3-dibromo[2-¹⁴C]acetone were oxidized with performic acid and hydrolysed. The hydrolysates were applied to a Technicon AutoAnalyzer and the column was eluted with the buffer system of Thomson & Miles (1964). The eluate from the column not required by the analytical system was collected, and the radiochromatogram shown in Fig. 2 was determined for the inhibited ficin. An essentially similar radiochromatogram was obtained from inhibited stem-bromelain. The radioactivity in fractions 10–40 was due to *S*-carboxymethylcysteine sulphone or its acid-catalysed degradation

products or both. The only other radioactive peak in the whole amino acid chromatogram emerged with alanine. This radioactive peak was identified as 1-carboxymethylhistidine (Husain & Lowe, 1968*a,b*) and there was no evidence of 3-carboxymethylhistidine. To confirm the identity of this radioactive material further, fractions 149–155 (Fig. 2) were applied to an Evans Electroselenium Ltd. amino acid analyser, which is based on the method of Spackman, Stein & Moore (1958). In this system 1-carboxymethylhistidine is well resolved from glycine and alanine and emerges with proline (Crestfield, Stein & Moore, 1963). The analysis showed three peaks, glycine, alanine and a peak in the position of proline, thus confirming the amino acid as 1-carboxymethylhistidine.

These results provide direct evidence for the presence of an imidazole group of a histidine residue within a range of 5 Å from the active-site thiol group of ficin and stem-bromelain. This almost certainly means that a histidine residue is present in the active site of these enzymes. The structural and kinetic similarity between these enzymes and papain suggests that the histidine residue plays an essential catalytic role in the mechanism of action of both ficin and stem-bromelain. The concerted action of the active-site thiol and imidazole groups that was proposed in the mechanism of action of papain (Husain & Lowe, 1968*a,b*) appears to be equally probable in the mechanism of action of ficin and stem-bromelain.

Table 3. *Amino acid analyses of ficin, stem-bromelain and the 1,3-dibromoacetone-inhibited enzymes*

The amino acid analyses were performed in triplicate, the buffer system of Thomson & Miles (1964) being used, and the means were taken. Ficin was assumed to contain 6 phenylalanine residues/mol. (Stein & Liener, 1967), and stem-bromelain 9 leucine residues/mol. (Murachi, 1964).

Amino acid	Amino acid composition (residues/mol.)			
	Ficin	Inhibited ficin	Stem-bromelain	Inhibited stem-bromelain
Asp	20.0	20.6	28.9	29.1
Thr	9.7	10.0	12.4	12.7
Ser	16.1	15.6	24.9	24.2
Glu	28.1	27.6	23.0	23.7
Pro	12.5	13.3	13.1	13.4
Gly	33.9	33.3	30.5	30.2
Ala	25.6	25.8	32.4	31.5
Val	18.2	18.7	20.7	20.2
Cys	7.5	6.9	7.4	7.0
Met	3.8	3.9	5.0	4.8
Ile	7.4	7.3	18.4	18.7
Leu	16.7	17.0	9.0	9.0
Tyr	14.9	15.0	18.2	17.6
Phe	6.0	6.0	8.0	8.1
Lys	8.0	8.4	20.2	20.1
His	2.0	1.4	1.34	0.59
Arg	10.8	11.0	10.2	9.6

Fig. 2. Radiochromatogram of the acid hydrolysate of performic acid-oxidized dibromoacetone-inhibited ficin run on a Technicon AutoAnalyzer. The positions for the elution of amino acids is shown on the top scale. CysO_3H , cysteic acid; 1-CM-His and 3-CM-His, 1- and 3-carboxymethylhistidine. The pumping rate was 1.2 ml./min.

On the basis of kinetic evidence an imidazole group of a histidine residue has been suggested as essential for the mechanism of action of subtilisin (Glazer, 1967). Although several attempts have been made to alkylate this residue with active-site-directed irreversible inhibitors, so far these have failed (Smith *et al.* 1966). Polgar & Bender (1967) converted the active-site serine residue of subtilisin into a cysteine residue and the resulting thiol-subtilisin retained enzymic activity. In an attempt to alkylate the presumed active-site histidine residue, thiol-subtilisin, which contained 0.69 thiol group/mol., was inhibited with 1,3-dibromoacetone at pH 7.5. The inhibited enzyme was indistinguish-

able in molecular weight from the native enzyme by gel filtration on Sephadex G-75. The amino acid analysis of the inhibited enzyme showed no loss of histidine. The acid hydrolysate of performic acid-oxidized 1,3-dibromo[^{14}C]acetone-inhibited enzyme was applied to a Technicon AutoAnalyzer and the radiochromatogram was determined as with ficin and stem-bromelain. Radioactivity was found only in the fractions associated with *S*-carboxymethylcysteine sulphone or its acid-catalysed degradation products or both, and no other radioactive peaks were found.

It would be unwise to conclude from this negative evidence that a histidine residue does not play an

essential role in the mechanism of action of subtilisin or thiol-subtilisin. It could be that a conformational change takes place in the enzyme as the substrate binds (Koshland, 1956, 1958), which brings together the active-site residues, whereas in the absence of substrate they are more than 5 Å apart.

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REFERENCES

- Chao, L.-P. & Liener, I. E. (1967). *Biochem. biophys. Res. Commun.* **27**, 100.
- Crestfield, A. M., Stein, W. H. & Moore, S. (1963). *J. biol. Chem.* **238**, 2413.
- Ellman, G. L. (1959). *Arch. Biochem. Biophys.* **82**, 70.
- Glazer, A. N. (1967). *J. biol. Chem.* **242**, 433.
- Hammond, B. R. & Gutfreund, H. (1959). *Biochem. J.* **72**, 349.
- Hirs, C. H. W. (1956). *J. biol. Chem.* **219**, 611.
- Hollaway, M. R., Mathias, A. P. & Rabin, B. R. (1964). *Biochim. biophys. Acta*, **92**, 111.
- Husain, S. S. & Lowe, G. (1965). *Chem. Commun.* p. 345.
- Husain, S. S. & Lowe, G. (1968a). *Chem. Commun.* p. 310.
- Husain, S. S. & Lowe, G. (1968b). *Biochem. J.* **108**, 855.
- Husain, S. S. & Lowe, G. (1968c). *Biochem. J.* **108**, 861.
- Koshland, D. E. (1956). *J. cell. comp. Physiol.* **47** (Suppl. 1), 217.
- Koshland, D. E. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 98.
- Liener, I. E. (1961). *Biochim. biophys. Acta*, **53**, 332.
- Light, A., Frater, R., Kimmel, J. R. & Smith, E. L. (1964). *Proc. nat. Acad. Sci., Wash.*, **52**, 1276.
- Lowe, G. & Williams, A. (1965). *Biochem. J.* **96**, 199.
- Murachi, T. (1964). *Biochemistry*, **3**, 932.
- Murachi, T., Yasui, M. & Yasuda, Y. (1964). *Biochemistry*, **3**, 48.
- Ong, E. B., Shaw, E. & Schoellmann, G. (1964). *J. Amer. chem. Soc.* **86**, 1271.
- Ota, S., Moore, S. & Stein, W. H. (1964). *Biochemistry*, **3**, 180.
- Polgar, L. & Bender, M. L. (1967). *Biochemistry*, **6**, 610.
- Sgarbieri, V. C., Gupte, S. M., Kramer, D. E. & Whitaker, J. R. (1964). *J. biol. Chem.* **239**, 2170.
- Shaw, E. & Springhorn, S. (1967). *Biochem. biophys. Res. Commun.* **27**, 391.
- Smith, E. L., Markland, F. S., Kasper, C. B., DeLange, R. J., Landon, M. & Evans, W. H. (1966). *J. biol. Chem.* **241**, 5974.
- Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.
- Stein, M. J. & Liener, I. E. (1967). *Biochem. biophys. Res. Commun.* **26**, 376.
- Thomson, A. R. & Miles, B. J. (1964). *Nature, Lond.*, **203**, 483.
- Wong, R. C. & Liener, I. E. (1964). *Biochem. biophys. Res. Commun.* **17**, 470.