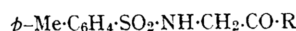
The Location of the Active Centre Cysteine Residue in the Primary Sequence of Papain

By S. S. HUSAIN and G. LOWE
(*The Dyson Perrins Laboratory, Oxford University*)

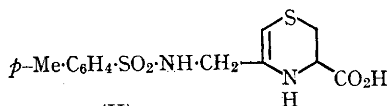
THE hydrolysis of a substrate by the proteolytic enzyme, papain, has recently been shown to involve the formation of an acyl–enzyme intermediate through the sulphydryl group of a cysteine

residue.[1] This cysteine residue must therefore be in the catalytic site of the enzyme and a knowledge of its environment would greatly facilitate our understanding of the mechanism of action of this enzyme. By designing an inhibitor for papain which is related structurally to a specific substrate and containing a reactive group with a predilection for sulphydryl groups, it has been possible to inhibit papain through its active centre cysteine residue and identify its location in the amino-acid sequence of the enzyme.

We have previously reported[2] the kinetic evidence which indicates that the four ketones (I; $R=CH_2.Cl$; $R=CH_2.Br$; $R=CH_2.I$; $R=CHN_2$) which are related to the substrate *N*-tosyl-glycine methyl ester (I; R=OMe),[3] react irreversibly with the active site of the enzyme. The [^{14}C]-chloro-ketone (I; $R=CH_2.Cl$) has now been prepared from [^{14}C]-glycine by a route similar to that used for the preparation of L-1-tosylamido-2-phenylethyl chloromethyl ketone, a specific inhibitor of α-chymotrypsin.[4]



(I)



(II)

Activated papain (0·68 g.) was inhibited with about twofold molar excess of [^{14}C]-chloro-ketone (I; $R=CH_2.Cl$; 15 mg.) at pH 7·0 and the excess of inhibitor removed when inhibition was complete, by filtration through a short column of Sephadex G-25. The inhibited papain was dissolved in 5M-guanidinium chloride and reductive cleavage of the disulphide bonds accomplished with mercapto-ethanol. Carboxymethylation with iodoacetic acid at pH 8·6 followed by exhaustive dialysis gave insoluble protein which was digested with 4% of its weight of α-chymotrypsin at pH 8·0 and 25° for 15 hr. The digest was concentrated (to 4 ml.) at 35°, centrifuged and the supernatant fluid applied to a Sephadex G–25 column (140 × 1 cm.) and eluted with water containing 0·2% thiodiglycol. Most of the radioactive material emerged as a single peak which was concentrated and re-chromatographed on the same column but using 5N-acetic acid containing 0·2% thiodiglycol as eluant. The radioactive material again emerged as a single peak which was concentrated and applied to a carboxymethylcellulose column (70 × 1 cm.) and eluted with 0·01M-pyridine–acetate buffer, of pH 4·6 containing 0·2% thiodiglycol. The radioactive fraction was shown to contain two peptides by chromatography on Whatman No. 4 paper using butanol–acetic acid–water (v/v 40:6:15) containing 0·2% thiodiglycol. The pure [^{14}C]-peptide, which gave a purple colour with Ehrlich reagent, was eluted from the paper with 1·7N-acetic acid containing 0·2% thiodiglycol and a portion of the eluate was hydrolysed with 6N-HCl at 110° for 24 hr. Amino-acid analysis (Technicon AutoAnalyzer) gave the following results (corrected for decomposition during hydrolysis and contamination from the paper): CM–Cys (1·04), Asp (0·94), Glu (1·00), Ser (1·99), Gly (2·38). Inspection of the amino-acid sequence of papain[5] revealed that the peptide must be derived from residues 18 to 26 inclusive (the numbers represent the position with respect to the *N*-terminus of the enzyme), and the inhibitor attached to either cysteine-22 or -25, or much less likely the tryptophan residue.

Asn–	Gln–	Gly–	Ser–	Cys–	Gly–	Ser–	Cys–	Try
18	19	20	21	22	23	24	25	26

Partial digestion of the [^{14}C]-peptide with carboxypeptidase A at pH 7·8 and 25° for 18 hr. followed by amino-acid analysis on the AutoAnalyzer led to frontal elution of the radioactive peak, which gave no colour with ninhydrin and contained 57% of the activity of the peptide. A synthetic product derived from the chloro-ketone (I., $R=CH_2.Cl$) and cysteine, which was shown to be the dihydro-1,4-thiazine (II), gave an intractable product on treatment with dilute acid, which gave no colour with ninhydrin. The amino-acid analysis gave the following results (the numbers in parentheses being the molar ratios relative to the amount of peptide used): Try (0·88), Ser (0·1), Gly (*ca.* 0·005), and thus confirmed the sequence at the carboxy-terminus of the peptide and identified cysteine-25 as the active centre cysteine residue.

(*Received, June 24th*, 1965; *Com.* 394.)

[1] G. Lowe and A. Williams, *Proc. Chem. Soc.*, 1964, 140; G. Lowe and A. Williams, *Biochem. J.*, 1965, **96**, 189. M. L. Bender and L. J. Brubacker, *J. Amer. Chem. Soc.*, 1964, **86**, 5333.
[2] G. Lowe and A. Williams, First Meeting of the Federation of European Biochemical Societies, London, 1964, Abstract A32, p. 27.
[3] G. Lowe and A. Williams, *Biochem. J.*, 1965, **96**, 194.
[4] E. B. Ong, E. Shaw, and G. Schoellmann, *J. Biol. Chem.*, 1965, **240**, 694, and earlier references there cited.
[5] A. Light, R. Frater, J. R. Kimmel, and E. L. Smith, *Proc. Nat. Acad. Sci. U.S.A.*, 1964, **52**, 1276.