

Studies on the Active Site of Papain. V.¹⁾ Photooxidation of Histidine Residues²⁾

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- 1) The present research has been planned to demonstrate the importance of histidine residues on enzyme activity of papain by means of photooxidation.
- 2) Papain was rapidly inactivated by methylene blue-sensitized photooxidation.
- 3) The rate of photo-sensitized inactivation was pH dependent, and about one histidine residue per molecule of papain was lost when complete inactivation took place.
- 4) Mercuripapain was not inactivated by methylene blue-sensitized photooxidation, and the histidine residue was not affected in the illuminated mercuripapain.
- 5) These results indicate that the modification of a histidine residue by photooxidation cause loss of the enzyme activity for hydrolysis of benzoyl-L-arginine amide.
- 6) The state of the histidine residue in the active site of papain is discussed to explain the results of the photooxidation studies described here.

Numerous studies⁴⁾ of papain [3.4.4.10.] have shown that a SH group is a part of the active site. Recently, the bifunctional reagent, 1,3-dibromoacetone, was reported to crosslink the SH group of cysteine and the NH group of histidine residue in papain and to irreversibly inhibit the enzyme activity by Husain *et al.*⁵⁾ From this report, it was suggested that a histidine residue may be important for enzyme activity of this enzyme.

The photooxidation of protein in the presence of methylene blue have been known to cause a rapid modification of histidine and tryptophan residues and a slower modification of tyrosine, methionine and cysteine residues.⁶⁾ The studies on the pH effects on the inactivation of enzyme by photooxidation have been known to be useful to distinguish a histidine residue from other residues.⁷⁾

The present research has been planned to demonstrate the importance of histidine residues on enzyme activity of papain by means of photooxidation.

Experimental

Materials—Crystalline papain and α -benzoyl-L-arginine amide (BAA) were prepared by the procedure of Kimmel and Smith.⁸⁾ Mercuripapain was purchased from Sigma Chemical Co., St. Louis. Methylene blue and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Nakarai Chemicals Co. Ltd., Kyoto.

Assay Procedure of Enzyme Activity—The assay procedure described in the previous paper⁹⁾ was employed.

- 1) Part IV: H. Kanazawa and A. Ohara, *Chem. Pharm. Bull.* (Tokyo), **21**, 1832 (1973).
- 2) A part of this research was presented at the 94th Annual Meeting of the Pharmaceutical Society of Japan in Sendai, April 1974.
- 3) Location: 5 Nakauchicho, Misasagi, Yamashina, Higashiyama, Kyoto 607, Japan.
- 4) A.N. Glazer and E.L. Smith, "The Enzymes," 3rd ed., Vol. 3, Academic Press, New York and London, 1974, p. 501.
- 5) S.S. Husain and G. Lowe, *Biochem. J.*, **108**, 855 (1968).
- 6) L. Weil, *Arch. Biochem. Biophys.*, **110**, 57 (1965).
- 7) E.W. Westhead, *Biochemistry*, **4**, 2139 (1965).
- 8) J.R. Kimmel and E.L. Smith, "Biochemical Preparation," Vol. 6, John Wiley and Sons, Inc., New York, 1957, p. 61.
- 9) H. Kanazawa, S. Uchihara, A. Ohara and M. Yoshioka, *Chem. Pharm. Bull.* (Tokyo), **18**, 195 (1970).

Photooxidation—To 2.7 ml of a 0.05 M phosphate buffer solution (pH 6.0–8.0) containing 6 mg of papain (or mercuripapain), 0.3 ml of 0.02% methylene blue was added and the mixture was illuminated with a 500 W incandescent lamp placed at a distance of 25 cm from the reaction vessel which was thermostatted at 30°.

Amino Acid Analyses—Amino acid analyses were performed by the method of Moore, *et al.*¹⁰⁾ with a Hitachi 034 Liquid Chromatograph. Samples for analysis were dialyzed with the cellulose tubing in the dark and hydrolyzed with 6 N HCl at 110° for 24 hr in evacuated sealed tubes. Tryptophan contents were determined by the method of Spies and Chambers.¹¹⁾

Assay Procedure of SH Contents—SH contents were analyzed essentially according to the procedure of Ellman.¹²⁾

Results and Discussion

Papain was rapidly inactivated by methylene blue-sensitized photooxidation. The initial rate of inactivation was found to be the first-order reaction. The rate of photo-sensitized inactivation was pH dependent in the phosphate-buffered solutions from pH 6.0 to pH 8.0, as shown in Fig. 1. The results indicated that a group having a pK_a of about 6.5–7.0 is concerned with the enzymatic activity, and that this plot closely resembles the titration curve of histidine.

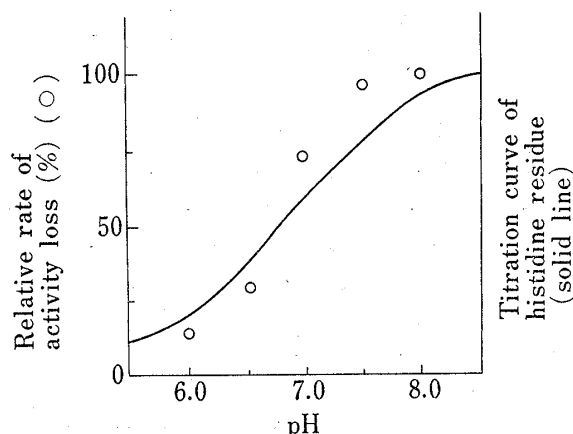


Fig. 1. The pH Dependence of the Rate of Inactivation of Papain by Methylene Blue-sensitized Photooxidation

Photooxidation was performed at 30° with 0.2% protein and 0.002% methylene blue in 0.05M phosphate buffer. After the activation, activities of photooxidized papain were assayed by alkalimetric titration in alcohol. Reaction mixtures contained papain (5×10^{-6} M), substrate (BAA) (5×10^{-3} M), potassium cyanide (3×10^{-3} M) and EDTA (1×10^{-3} M) in a total volume of 2.5 ml. The data obtained (initial rate) were expressed in per cent of the value for the intact sample. The solid line represents the titration curve of histidine residue.

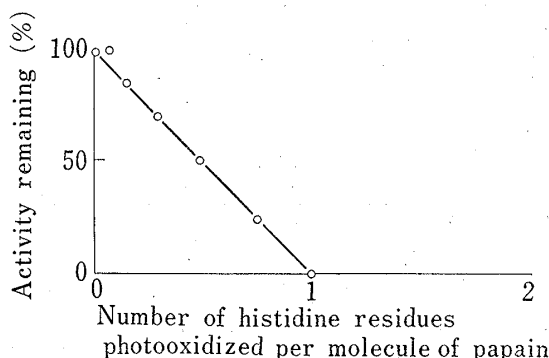


Fig. 2. The Correlation between the Decrease in Histidine Content and the Degree of Inactivation by Photooxidation at pH 7.5

Experimental conditions were the same as Fig. 1.

Amino acid analyses of the inactivated papain showed that the histidine and tryptophan residues were most significantly affected by the photooxidation, as shown in Table I (column I, II, III and IV). The correlation between the decrease in histidine content and the degree of inactivation by photooxidation is shown in Fig. 2. About one histidine residue per molecule of papain was lost when complete inactivation took place. Table I (column I, II, III and IV) also shows that about two tryptophan residues appear to be oxidized at complete inactivation. This results prevent us from definitely concluding that the modification of only a histidine

10) S. Moore, D.H. Spackman and W.H. Stein, *Anal. Chem.*, **30**, 1190 (1958).

11) J.S. Spies and D.C. Chambers, *Anal. Chem.*, **21**, 1249 (1949).

12) G.L. Ellman, *Arch. Biochem. Biophys.*, **74**, 443 (1958); *idem, ibid.*, **82**, 70 (1959).

TABLE I. Amino Acids Composition of Photooxidized Papain

Amino acid ^{a)}	Intact papain	Illuminated ^{b)} papain		Intact mercuripapain	Illuminated ^{b)} mercuripapain	
Histidine	2.0	1.9	1.5	1.0	1.9	1.9
Tryptophan ^{c)}	4.8	3.6	3.5	2.4	4.8	3.0
Half-cystine	6.9	6.8	7.1	6.9	6.4	6.5
Methionine	0	0	0	0	0	0
Tyrosine	17.9	17.7	17.6	18.0	18.3	18.1
-SH (free) ^{d)}	0.20	0.18	0.19	0.18	—	—
Residual activity (%)	100	85	50	0	100	100

a) All the amino acids which are present in papain, were examined, but the table reports only the results for those which are known to be susceptible to photooxidation. No change was found in the content of the other amino acids. The values in the table denote number of residues per protein molecule are given in terms of the molar ratio of the amino acids, assuming the number of leucine residue to be 11.0. No correction was made for decomposition during acid hydrolysis.

b) Illumination at column II, III, IV and VI was achieved at pH 6.0, 7.0, 8.0 and 8.0.

c) determined by the method of Spies and Chambers¹¹⁾

d) determined by the method of Ellman¹²⁾

residue is the cause of the activity loss of papain by the photooxidation. However, the clear pH dependence of the loss of enzyme activity show in Fig. 1 may indicate that only a histidine residue is responsible for the loss of enzyme activity by photooxidation. This conclusion is further supported by the following result. In spite of the decrease of tryptophan content of mercuripapain by photooxidation, the enzyme activity did not decrease, as shown in Table I (column V and VI).

On the other hand, the illuminated and non-activated papain had about 0.2 mole of SH group per mole of papain, as shown in Table I (column I, II, III and IV). In the previous paper¹⁾ it was reported that non-activated papain have 0.18 mole of SH group per mole of papain and cyanide-activated papain have 0.52 mole of SH group. It is supported by the results in this paper and in the previous paper¹⁾ that the "active" SH group was not affected by methylene blue-sensitized photooxidation.

These results in this paper, the clear pH dependence of the loss of enzyme activity for papain and the amino acid analyses of photooxidized papain, may be taken as good evidence for believing that only a histidine is exclusively modified by methylene blue-sensitized photooxidation with the loss of enzyme activity.

The state of the histidine residue in the active site of papain is proposed to explain the results of the photooxidation studies described here (Fig. 3). Based on this model, the present observation can be explained, as follows:

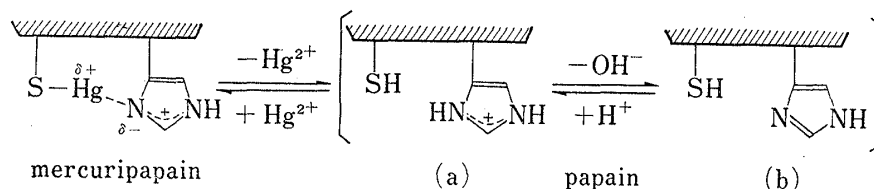


Fig. 3. The Schematic Illustration of the State of the Histidine Residue in the Active Site of Papain

(a): protonation form, (b): non-protonation form

1) When a histidine residue is deprotonated, the photooxidation of this residue take place. Therefore, as a histidine residue is part of the active site, the rate of photo-sensitized inactivation is pH dependent.

2) As a histidine residue is near by a "reactive" SH group, in the mercuripapain, the mercuric ion which combines with SH group affects the state of the histidine residue.

3) The mercuripapain, in which the state of histidine residue is protected by mercurion, is not inactivated by methylene blue-sensitized photooxidation.

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Added in Proof After this manuscript had been completed, we received an extensive paper (T. Murachi and K. Okumura, *FEBS Letters*, **40**, 127 (1974)) in which work similar to ours has been described. In their report, the apparent pK_a value for the ionization of the histidine residue as determined by the pH dependence of the rate of the photooxidation in papain is 6.7, which are in the normal range for an imidazole group. We are grateful to Dr. T. Murachi for communicating this result to us.