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Chymopapain. II.[1] Photooxidation of Histidine Residues[2]

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1) The rate of photo-inactivation of chymopapain A was pH-dependent.
2) About three histidine residues were oxidized and the enzyme activity was lost during the disappearance of a first oxidizable histidine residue.
3) The essential SH group was not affected by photooxidation.
4) About two tryptophan residues were also oxidized at pH 8.0 with the loss of activity and at pH 4.0 with no loss of activity.
5) Two photooxidizable tryptophan residues were found to be different from the essential tryptophan residue by means of NBS oxidation.
6) From these results, it is considered that the modification of only a histidine residue is the cause of activity loss by the photooxidation.

Chymopapain possesses a single reactive SH group which is thought to play an essential role in the enzyme action.[4] The identification of the other amino acid residues involved in the active site of chymopapain has been performed by N-bromosuccinimide (NBS) oxidation.[1] From this result, a tryptophan residue is considered to play an essential role in the enzyme action. However, on the active site of chymopapain, little information is available beyond the fact that this enzyme possesses a single reactive SH group[4] and an essential tryptophan residue.[1] The present research has been planned to demonstrate the importance of histidine residues on enzyme activity of chymopapain A by means of photooxidation.

Experimental

Materials——Chymopapain A was prepared by the procedure of Ebata and Yasunobu.[5] α-N-Benzoyl-L-arginine amide (BAA) was prepared by the procedure of Kimmel and Smith.[6] Methylene blue, *p*-chloromercuribenzoate (PCMB), *p*-dimethylaminobenzaldehyde (PDAB), cysteine-HCl and ethylenediaminetetraacetic acid 2Na salt (EDTA) were purchased from Nakarai Chemicals, Co., Ltd., Kyoto.

Enzyme Assay——After activation with cysteine and EDTA at pH 6.0, enzyme activity was examined using BAA as substrate by alkalimetric titration in alcohol at pH 6.0. Reaction mixtures contained chymopapain A (6 μM), substrate (BAA) (50 mM), crysteine (20 mM) and EDTA (8 mM) in a total volume of 2.5 ml.

Photooxidation——To 2.7 ml of a 0.05 M phosphate buffer (pH 6.0—8.0) or 0.05 M acetate buffer (pH 4.0—5.5) containing 6 mg of chymopapain A (57 μM), 0.3 ml of 0.02% methylene blue solution was added and the mixture was illuminated with a 500 W incandecent lamp placed at a distance of 25 cm from the reaction vessel at 30°.

NBS Oxidation——NBS oxidation described by Spande, *et al.*[7] was employed with slight modification. The molar concentration of the oxidized tryptophan residues was calculated from the decrease in absorbance at 280 nm and empirical factor, 1.31.[8]

1) Part I: H. Kanazawa, S. Ishimitsu, M. Sakane, K. Kawamata, R. Tsunekuni, and A. Ohara, *Chem. Pharm. Bull.* (Tokyo), **24**, 2369 (1976).
2) A part of this research was presented at the 96th Annual Meeting of the Pharmaceutical Society of Japan in Nagoya, April 1976.
3) Location: 5 *Nakauchicho, Misasagi, Yamashina, Kyoto, 607, Japan.*
4) A.N. Glazer and E.L. Smith, "The Enzymes," Vol. 3, (3 rd ed.), Academic Press, New York and London, 1971, p. 501.
5) M. Ebata and K.T. Yasunobu, *J. Biol. Chem.*, **237**, 1086 (1962).
6) J.R. Kimmel and E.L. Smith, "Biochemical Preparations," Vol. 6, ed. by C.S. Vestling, John Wiley and Sons, Inc., New York and London, 1957, p. 61.
7) T.F. Spande and B. Witkop, "Method in Enzymology," Vol. 11, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1967, p. 498.
8) A. Patchornik, W.B. Lawson, and B. Witkop, *J. Am. Chem. Soc.*, **80**, 4747 (1958); *idem, ibid.*, **80**, 4748 (1958).

Amino Acid Analyses——Amino acid analyses were performed by the modified method of Moore, *et al.*[9] using Hitachi 034 Liquid Chromatograph, after hydrolysis with 6 N HCl in a sealed evacuated tube at 110° for 22 hr. Tryptophan contents were determined by the decrease in absorbance at 280 nm[8] and also by the method of Spies and Chambers with PDAB.[10]

SH Contents——SH contents were determined by the method of Boyer with PCMB.[11]

Results

Chymopapain A was rapidly inactivated by photooxidation in the presence of methylene blue. The rate of inactivation conformed to first-order reaction kinetics. The first-order rate constants measured at various pH's from 4.0 to 8.0 are plotted in Fig. 1, showing that the inactivation reaction is pH-dependent. The solid line in Fig. 1 is the theoretical titration curve with apparent pK_a value of 7.0. The experimental data are consistent with this titration curve. The results indicate that a group having a pK_a value of 7.0 participates in the inactivation of chymopapain A.

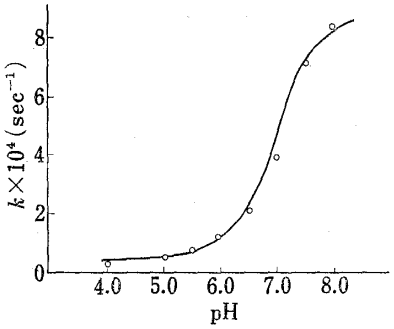

Fig. 1. The pH Dependence of the Rate of Inactivation of Chymopapain A by Methylene Blue-Sensitized Photooxidation

The rate of inactivation is presented in terms of the first-order rate consant, k (sec^{-1}). The solid line represents the theoretical titration curve with a pK_a value of 7.0.

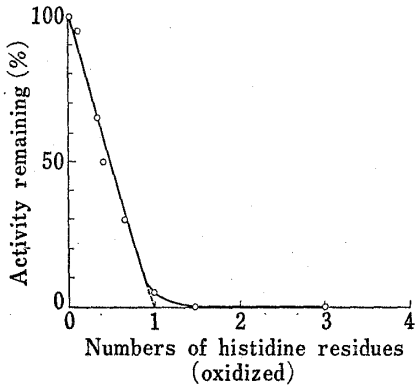

Fig. 2. The Correlation between the Decrease in Histidine Content and the Degree of Inactivation by Photooxidation at pH 8.0

Experimental conditions were the same as Fig. 1.

TABLE I. Amino Acid Composition of Photooxidized Chymopapain A

Amino Acid		His.	Tyr.	Met.	Trp.[a]	-SH[b]	Activity (%)
Intact		4.2	21.3	1.2	5.8	0.87	100
Oxidized	I	3.2	20.6	1.1	3.9	0.91	4.5
	II	1.1	20.9	0.4	3.8	0.90	0
	III	4.3	21.0	1.0	3.9	0.91	95

All amino acids which are present in chymopapain A were examined. Since no appreciable change was found in the content of the other amino acids, only those which are known to be susceptible to photooxidation are listed in the table. The values in the table denote number of residues per protein molecule, assuming the number of isoleucine to be 11.0 and the number of arginine to be 10.0.[c] No correction was made for decomposition during acid hydrolysis. Photooxidation at I, II and III was achieved at pH 8.0 for 60 min, at pH 8.0 for 120 min and at pH 4.0 for 60 min, respectively.

a) determined by the method of Spies and Chambers[10]
b) determined by the method of Boyer[11]
c) D.K. Kunimitsu and K.T. Yasunobu *Biochim, Biophys. Acta*, **139**, 405 (1967)

9) S. Moore, D.H. Spackman, and W.H. Stein, *Anal. Chem.*, **30**, 1190 (1958).
10) J.S. Spies and D.C. Chambers, *Anal. Chem.*, **21**, 1249 (1949).
11) P.D. Boyer, *J. Am. Chem. Soc.*, **76**, 4331 (1954).

The results of amino acid analyses are shown in Table I. Table I shows only the results for amino acid residues which are known to be susceptible to photooxidation. No appreciable change was found in the content of the other amino acids which are not listed in Table I. The most significant changes were seen in histidine and tryptophan contents after photooxidation. After photooxidation for 60 min at pH 8.0, causing 95.5% inactivation, 0.91 mole of SH per molecule was found. On the other hand, 0.87 mole of SH per molecule was found in the control experiment without illumination. These results indicate that the reactive SH group did not change into cysteic acid on photooxidation for 60 min at pH 8.0.

The remaining activity of photooxidized chymopapain A was plotted *versus* the loss of histidine residues (Fig. 2). About one histidine residue per molecule of enzyme was lost when complete inactivation took place. From Fig. 2, it was expected that only the oxidation of one histidine residue participated in the inactivation of chymopapain A, and in fact, as shown in Fig. 1, the pH profile of the loss of enzyme activity closely resembled the titration curve of histidine residue.

The results of amino acid analyses also showed that at pH 8.0 about two tryptophan residues were also oxidized at complete inactivation, together with the loss of one histidine residue mentioned above. Generally, the rate of photooxidation of histidine is pH dependent,[12] and at acidic pH, where the imidazole base is protonated, the reaction proceeds only at a very low rate.[13] But the rate of photooxidation of tryptophan is not pH dependent.[13] About two tryptophan residues only were modified by photooxidation at pH 4.0 with no loss of activity. These results suggest that the loss of histidine may cause the inactivation, while the loss of tryptophan does not participate in the inactivation. On the other hand, the results on chymopapain A in the previous study[1] suggested that the modification of one tryptophan residue by NBS oxidation was the cause of activity loss. On the basis of these data, subsequent experiments were carried out to examine moreover that only the photooxidation of one histidine residue participate in the inactivation of chymopapain A.

Both the photooxidation and the NBS oxidation of chymopapain A were performed. To begin with, the photooxidation of chymopapain A was performed at pH 8.0 and this illuminated enzyme was dialyzed in 0.1M acetate buffer (pH between 3.0 and 6.0). On this dialyzed

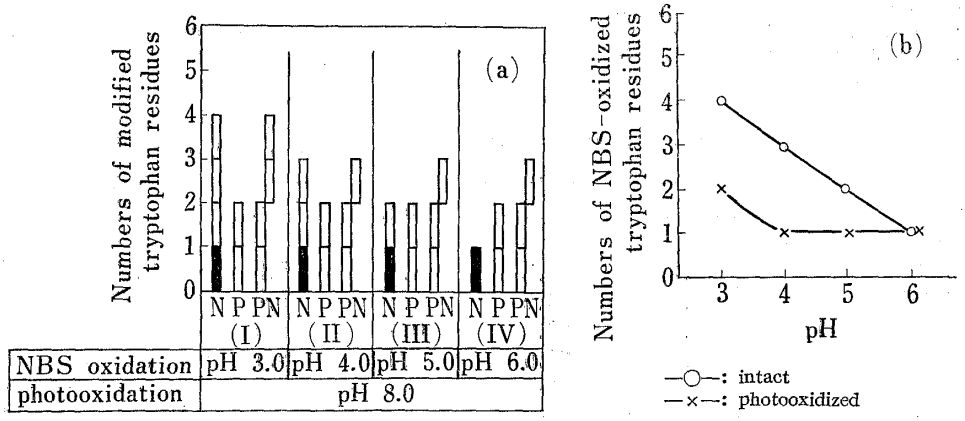

Fig. 3. Numbers of Modified Tryptophan Residues of Chymopapain A

(a) The numbers of tryptophan residues to be modified by photooxidation and NBS oxidation were reported here in their order of oxidation. Black square expresses NBS-oxidized tryptophan residue with the loss of enzyme activity. Photooxidations were performed at pH 8.0. NBS oxidations at column I, II, III and IV, were performed at pH 3.0, 4.0, 5.0 and 6.0, respectively. N; NBS oxidation, P; photooxidation, PN; photooxidation+NBS oxidation

(b) The data of Fig. 3(a) on the NBS-oxidized tryptophan residues were re-plotted.

12) E.W. Westhead, *Biochemistry*, 4, 2139 (1965).
13) L. Weil, *Arch. Biochem. Biophys.*, 110, 57 (1965).

enzyme solution, NBS oxidation was applied at each pH. The results on the numbers of the modified tryptophan residues of chymopapain A are summarized in Fig. 3(a). The results showed that two tryptophan residues were lost by the photooxidation at pH 8.0 and the other tryptophan residues were further lost by NBS oxidation. Since the NBS oxidation rate of each tryptophan residue in protein varies with pH, the reactivity of each tryptophan residue can be compared with NBS oxidation. On chymopapain A, the rate of oxidation by this reagent increased with the decrease of pH, and the enzyme activity was completely lost with the disappearance of the first oxidizable tryptophan residue at each pH.[1] The data of Fig. 3(a) on the NBS-oxidized tryptophan residues of the intact and the photooxidized enzymes are re-plotted in Fig. 3(b). On the intact enzyme, only one tryptophan residue was lost by NBS oxidation at pH 6.0 with the loss of enzyme activity.[1] On the other hand, on the photooxidized enzyme (two tryptophan residues oxidized), another residue was lost by NBS oxidation at pH 6.0. If one of the photooxidizable tryptophan residues is identical with the essential tryptophan residue, no residue of photooxidized enzyme must be oxidized by NBS oxidation at pH 5.0 and pH 6.0. However, another residue was oxidized at pH 5.0 as well as pH 6.0, as mentioned above. These facts lead us to assume that the photooxidizable tryptophan residues are different from the essential tryptophan residue. This conclusion is further supported by the following results (Table II). NBS oxidation was applied at pH

Table II. Numbers of Modified Tryptophan Residues

	Intact[a]	1 st NBS Oxidation at pH 6.0[b]	2 nd Photooxidation at pH 8.0[a]	3 rd NBS Oxidation at pH 4.0[b]
I	0(5.8)	—	2(3.9)	1(3.0)
	0(5.8)	—	—	3(2.9)
II	0(5.8)	1(4.9)	2(2.8)	0(2.8)
	0(5.8)	1(4.9)	—	2(3.0)

The values in the parentheses denote the numbers of tryptophan residues per protein molecule.
a) determined by the method of Spies and Chambers with PDAB[10]
b) determined by the decrease in absorbance at 280 nm[8]

6.0 on chymopapain A. By this NBS oxidation, enzyme activity was completely lost and only one tryptophan (which is the essential residue in the enzyme action[1]) and no histidin residues were lost. The other two tryptophan residues of this NBS-oxidized enzyme (the essential tryptophan residue oxidized) were oxidized by the photooxidation at pH 8.0. On this oxidized enzyme, NBS oxidation at pH 4.0 was applied. No tryptophan residues were affected. On the other hand, in the control experiment without illumination, the other two tryptophan residues of the NBS-oxidized (at pH 6.0) enzyme (the essential tryptophan residue oxidized) were affected by NBS oxidation at pH 4.0. These results on the tryptophan residues are schematically described in Fig. 4. From these results, it seems very reasonable to conclude that two photooxidized tryptophan residues are different from the essential residue and that only the oxidative loss of one histidine residue participate in the inactivation of this enzyme.

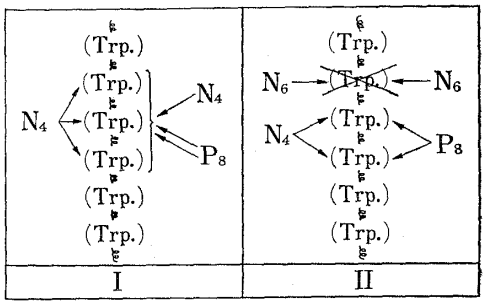

Fig. 4. The schematic Illustration of the Oxidizable Tryptophan Residues of Chymopapain A

The data of Table II were schematically described. P_8; photooxidation at pH 8.0, N_4 and N_6; NBS oxidation at pH 4.0 and pH 6.0, respectively

Discussion

Histidine and tryptophan residues were oxidized during the photo-inactivation of chymopapain A. A single reactive SH group was not affected by the photooxidation in the presence of methylene blue, and this indicates that cysteine was not oxidized under the conditions employed.

About three of four histidine residues were oxidized and the enzyme activity was lost during the disappearance of a first oxidizable histidine residue. These findings, together with the characteristic pH profile of the loss of enzyme activity, have led to the postulation that a histidine residue is in fact involved in catalysis, as it is the case in serine proteases.[14] The apparent pK_a value of 7.0 found for histidine residue in chymopapain A reflects the normal ionization of an imidazole moiety. From this finding, it is considered that the histidine residue concerned is positively charged at pH 6.0 but has no charge at pH 8.0, and yet the catalytic activity of this enzyme[5] is known to remain almost constant over the pH range 6 to 8. This is the experimental basis upon which the role of histidine residue is not completely understood, although it is evident from this research that the loss of the first photooxidizable histidine residue is responsible for the loss of enzyme activity.

Chymopapain A has six tryptophan residues per molecule. Only a tryptophan residue is considered to play an essential role in the enzyme action.[1] The photooxidation of about two tryptophan residues may imply that the essential tryptophan residue was the target of the oxidation. However, from this work, it is suggested that two photooxidizable tryptophan residues may be different from the essential tryptophan residue.

14) D.M. Blow, J.J. Birktoft, and B.S. Hartley, *Nature*, 221, 337 (1969).