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Studies on the Active Site of Papain. VII.[1] States of Tryptophan Residues[2]

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1) The present research has been planed to clarify the states of tryptophan residues in papain by means of N-bromosuccinimide (NBS) oxidation and photooxidation.

2) Only a tryptophan residue was exclusively modified by NBS oxidation with the loss of enzyme activity.

3) This residue was not affected by methylene blue-sensitized photooxidation.

4) Two of five tryptophan residues were not affected by NBS oxidation and photooxidation.

5) The first NBS-oxidizable tryptophan residue is considered to exist in or nearby the active site of papain.

6) The second and the third NBS-oxidizable tryptophan residues are considered to be identical with the photooxidizable residues.

7) The state of tryptophan residues of papain was illustrated schematically.

Papain [3.4.4.10.] possesses a single reactive SH group which is thought to play an essential role in the enzyme action. Numerous studies[4] has been carried out on the SH group at the active site of papain. The identification of the other amino acid residues involved in the active site of papain has been performed by photooxidation[5] and N-bromosuccinimide (NBS) oxidation.[1] From these results, we found that the histidine and tryptophan residues were involved in or nearby the active site of papain. The photooxidation of papain in the presence of methylene blue[5] indicated that the loss of histidine causes the inactivation and that the loss of tryptophan is not responsible for the inactivation. However, NBS oxidation of papain[1] indicated that the loss of tryptophan causes the inactivation.

The present research has been planed to clarify the state of tryptophan residues in papain by means of NBS oxidation and photooxidation.

Experimental

Enzymes——Crystalline papain was prepared by the procedure of Kimmel and Smith.[6] Mercuripapain was purchased from Sigma Chemical Co., St. Louis. Acetylpapain was prepared by the procedure described in the previous paper.[1]

Meterials——α-N-Benzoyl-L-arginine amide (BAA) was prepared by the procedure of Kimmel and Smith.[6] N-Bromosuccinimide (NBS), methylene blue, N-acetylimidazole, hydroxylamine, *p*-chloromercuribenzoate (PCMB) and *p*-dimethylamino benzaldehyde were purchased from Nakarai Chemicals Co. Ltd., Kyoto.

Assay Procedure of Enzyme Activity——The assay procedure described in the previous paper[7] was employed.

1) Part VI: M. Sakane, H. Kanazawa, and A. Ohara, *Chem. Pharm. Bull.* (Tokyo), **23**, 1741 (1975).
2) A part of this research was presented at the 95th Annual Meeting of the Pharmaceutical Society of Japan in Nishinomiya, April, 1975.
3) Location: 5, *Nakauchicho, Misasagi, Yamashina, Higashiyama, Kyoto, 607, Japan.*
4) *a*) E.L. Smith and J.R. Kimmel, "The Enzymes," Vol. 4, (2 nd ed.), Academic Press, New York and London, 1960, p. 133; b) A.N. Glazer and E.L. Smith, "The Enzymes," Vol. 3, (3 rd ed.), Academic Press, New York and London, 1971, p. 501.
5) A. Ohara, S. Fujimoto, H. Kanazawa, and T. Nakagawa, *Chem. Pharm. Bull.* (Tokyo), **23**, 967 (1975).
6) J.R. Kimmel and E.L. Smith, "Biochemical Preparations," Vol. 6, John Willey and Sons, Inc., New York, 1957, p. 61.
7) H. Kanazawa, S. Uchihara, A. Ohara, and M. Yoshioka, *Chem. Pharm. Bull.* (Tokyo), **18**, 195 (1970).

NBS Oxidation——As described in the preceding paper,[1] NBS oxidation described by Spande and Witkop[8] was employed with slight modification.

Photooxidation——Photooxidation was performed by the procedure in the previous paper.[5]

Amino Acid Analyses——As described in the preceding paper,[5] amino acid analyses were performed by the method of Moore, *et al.*[9]

SH Contents——SH contents were assayed according to the method of Boyer with PCMB.[10]

Tryptophan Contents——Tryptophan contents were determined by the method of Spies and Chambers.[11]

Results and Discussion

NBS oxidation was applied to papain at pH 3.0. Oxidation of tryptophan residues was measured by the decrease in absorption at 280 nm. The molar concentration of the oxidized tryptophan residues was calculated as the product of the decrease in absorption at 280 nm and the empirical factor, 1.31.[12] The course of NBS titration of tryptophan residues at pH 3.0 was shown in Fig. 1. About 3 tryptophan were oxidized at pH 3.0. Relationship

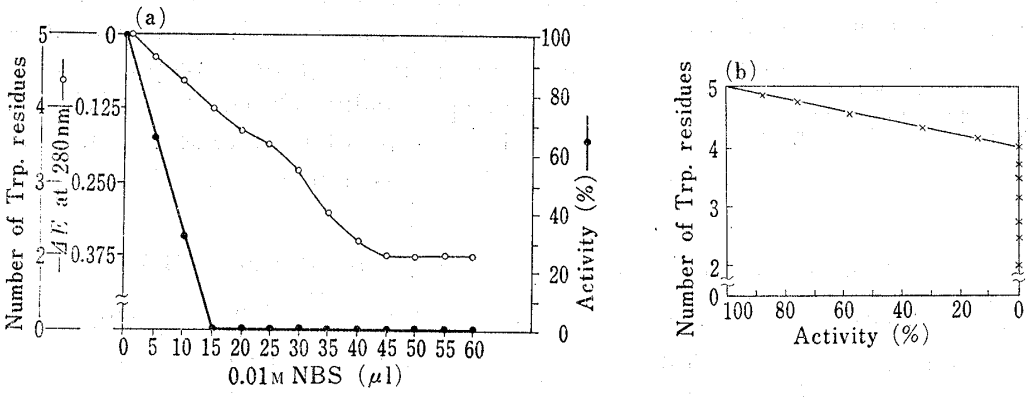

Fig. 1. The Relation between Tryptophan Residues oxidized and Enzyme Activity

(a) The reaction mixtures (for enzyme activity) contained papain (6 μM), substrate (BAA) (40 mM), cysteine-HCl (100 mM), EDTA (40 mM) and 0.1M citrate buffer (pH 6.0) in a total volume of 2.5 ml. Enzyme activities were assayed by alkalimetric titration in alcohol.

The NBS oxidation was performed at pH 3.0 (0.1M acetate buffer) using about 30 μM of papain. Five μl portions of a 0.01M NBS solution were added to the enzyme solution with rapid stirring. After about 20 sec., the absorbance at 280 nm was read. Addition of NBS was continued in the stepwise manner.

(b) The data Fig. 1 (a) were re-plotted.

between NBS oxidation of tryptophan and the change in the enzyme activity is shown in Fig. 1. Enzyme activity was examined at pH 6.0 using BAA as substrate. The degree of oxidation to a completely inactivated state indicated that about 1 mole of tryptophan was lost during inactivation by NBS oxidation. Therefore, this results may suggest that 1 out of 5 tryptophan residues is responsible for the loss of enzyme activity by NBS oxidation. At pH 4.0, about 2 tryptophan residues were oxidized,[1] and at pH 3.0, another one tryptophan residue was further oxidized. Then, tryptophan residues of papain were oxidized with NBS at pH between 3.0 and 6.0. The rate of oxidation by this reagent increased with the decrease of pH, as shown in Fig. 2. However, enzyme activity was completely lost during the disappearance of first oxidizable tryptophan residue at each pH.

Amino acid analyses of acid hydrolyzate of the NBS oxidized papain at pH 3.0 showed that only 1 tryptophan and 3 tyrosine residues were most significantly affected at completely

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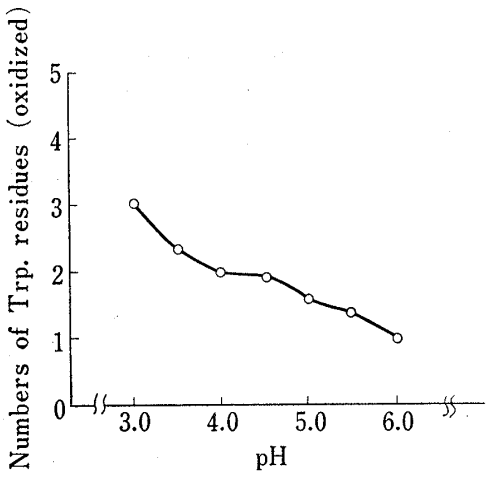

Fig. 2. Numbers of NBS Oxidized Tryptophan Residues at Each pH

The data obtained were expressed in terms of the moles of tryptophan oxidized per mole of protein at a given pH with 10—15-fold molar excess N-bromosuccinimide in papain.

inactivation, as shown in Table I. This results prevent us from definitly concluding that the modification of only a tryptophan residue is the cause of activity loss of papain by the NBS oxidation. As described in the preceding paper,[1] acetylation by N-acetylimidazole and deacetylation by hydroxylamine of papain do not affect the enzyme activity favorably, and the acetylation of papain prevent tyrosine from oxidation by NBS. Then, NBS oxidation was applied at pH 3.0 on acetylpapain. In the NBS-oxidized acetylpapain, about 3 tryptophan residues only oxidized, and enzyme activity was completely lost during the disappearance of first oxidizable tryptophan residue (Table I). This results at pH 3.0 is quite similar to that at pH 4.0.[1] Therefore, it seems very reasonable to conclude that 1 out of 5 tryptophan residues in papain specifically related the activity of papain.

The results of photooxidation in the previous paper[5] are as follows. (1) Papain was rapidly

Table I. Amino Acid Residues of NBS-oxidized Papain at pH 3.0

Amino Acid[a]		Trp.[b]	Tyr.	His.	–SH[c]	Met.	Activity (%)
Intact		5.0	19.0	1.8	1.00	0	100
Papain	I	4.0	16.0	1.9	0.93	0	0
NBS oxidation[d]	II	2.0	15.4	1.8	0.83	0	0
Intact		5.0	20.0	1.8	—	0	100
Acetylpapain[e]	I	4.0	19.5	1.8	—	0	0
NBS oxidation[d]	II	2.0	18.4	1.8	—	0	0
Intact		5.0	19.5	1.9	—	0	100
Hg-papain	I	4.0	18.1	1.9	—	0	0
NBS oxidation[d]	II	2.4	18.1	1.9	—	0	0

a) All amino acids which are present in papain, were examined, but the table reports the results for those which are known to be susceptible to NBS oxidation. No change was found in the content of the other amino acids. The values in the table denote number of residues per protein molecule, assuming the number of leucine residue to be 11.0 and the number of arginine residue to be 12.0. No correction was made for decomposition during acid hydrolysis.
b) determined by the methods of Spies and Chambers[11]
c) determined by the method of Boyer[10]
d) NBS oxidations at I and II were performed by 15 μl and 45 μl of 0.01M NBS solution, respectively.
e) prepared by the procedure described in the previous paper[1]

Table II. Amino Acid Residues of Photooxidized (pH 8.0) and NBS-oxidized (pH 4.0) Papain

		Trp.	Try.	His.	–SH	Met.	Activity (%)
Papain	Intact	5.0	19.0	1.8	1.0	0	100
	Photooxidation	3.5	18.0	1.0	—	0	0
	Photooxidation +NBS-oxidation	2.6	15.8	1.0	—	0	0
	NBS-oxidation	3.0	16.0	1.8	0.93	0	0
Hg-papain	Intact	5.0	19.5	1.9	—	0	100
	Photooxidation	3.0	18.3	1.9	—	0	100
	Photooxidation +NBS-oxidation	2.18	18.1	1.9	—	0	0
	NBS-oxidation	3.11	18.1	1.9	—	0	0

Assay conditions were the same as Table I.

inactivated by methylene blue-sensitized photooxidation. (2) The rate of inactivation was pH dependent. (3) Under the conditions employed, almost complete inactivation of papain occurred at pH 8.0, whereas practically no inactivation of mercuripapain took place at pH 8.0. (4) Amino acid analyses of the photooxidized mercuripapain at pH 8.0 showed that 2 tryptophan residues were significantly affected without inactivation, as shown in Table II.

The relationship between the modified tryptophan residues by NBS oxidation and photooxidation is not clear. In mercuripapain, as well as acetylpapain, tryptophan residues only were oxidized by NBS, and enzyme activity was completely lost during the disappearance of first oxidizable tryptophan residue (Table I). On the other hand, in the photooxidized mercuripapain, tryptophan residues only were also affected without inactivation (Table II). Then, the photooxidation and the NBS oxidation of mercuripapain was performed. To begin with, the photooxidation of mercuripapain was performed at pH 8.0 and this illuminated enzyme was dialyzed in 0.1M acetate buffer (pH 4.0). This phooxidation and this dialysis did not affect the enzyme activity. On this dialyzed enzyme solution, NBS oxidation was applied at pH 4.0. In this NBS oxidation, enzyme activity was completely lost. Amino acid analyses showed that by photooxidation 2 tryptophan residues was lost and another only one tryptophan residue was further lost by NBS oxidation with the loss of enzyme activity. These results on tryptophan residues for mercuripapain is summarized in Fig. 3.

Based on this summary (Fig. 3), the relationship between the modified tryptophan residues by NBS oxidation and photooxidation can be explained, as follows; 1) Only a tryptophan residue is exclusively modified by NBS oxidation with the loss of enzyme activity. 2) This first NBS-oxidizable tryptophan residue exists in or nearby the active site of papain. 3) This residue is not affected by methylene blue-sensitized photooxidation. 4) The second NBS-oxidizable tryptophan residue at pH 4.0 may be identical with the photooxidized one. 5) Another one tryptophan residue to be further oxidized at pH 3.0 may be also identical with the another photooxidized one. 6) It is considered that the residual two tryptophan residues are not affected by NBS oxidation and Photooxidation.

From the experiments described above, one of the msot possible illustration on the state of tryptophan residues of papain is schematically in Fig. 4.

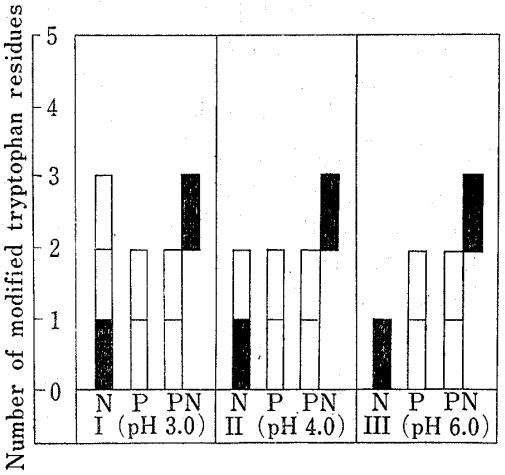

Fig. 3. Number of Tryptophan Residues of Mercuripapain to be modified by NBS Oxidation and Photooxidation

The numbers of the tryptophan residues were reported here in their order of oxidation. Black square expresses NBS-oxidized tryptophan residue with the loss of enzyme activity. NBS oxidations at column I, II and III were performed at pH 3.0, 4.0 and 6.0, respectively. Photooxidations were performed at pH 8.0. N; NBS Oxidation, P; Photooxidation, PN; Photooxidation+NBS Oxidation

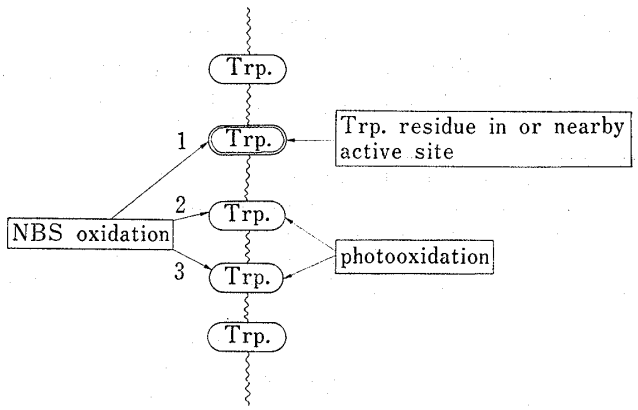

Fig. 4. The Schematic Illustration of the State of the Tryptophan Residues of Papain

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