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Studies on the Active Site of Papain. VI.[1] Chemical Modification of Tryptophan Residues by N-Bromosuccinimide[2]

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1) The present research has been planed to demonstrate the importance of tryptophan residues on enzyme activity of papain by means of NBS oxidation.

2) About 2 tryptophan residues were oxidized and the first oxidizable tryptophan was important for enzyme activity.

3) The relationship between tryptophan oxidation and enzyme activity for acetylpapain and mercuripapain are quite similar to that for papain.

4) About 1 tryptophan and 3 tyrosine residues in papain were modified by NBS oxidation. However, only 1 tryptophan residue and no tyrosine residue in acetylpapain were oxidized at complete inactivation.

5) The acetylation of tyrosine in papain prevented the tyrosine from oxidizing by NBS.

6) SH group and histidine residues in papain were not affected by NBS oxidation.

7) These results indicate that the modification of a tryptophan residue by NBS oxidation causes loss of the enzyme activity.

Papain [3.4.4.10.] is one of the most conspicuous plant proteinase contained in the latex of the immature fruits of papaya, *Carica papaya* L. Papain possesses a single reactive SH group,[4] aldehyde group[5] and imidazole group,[1] which are considered to play an essential role in the enzyme action. But the presence of the aldehyde group in papain has not been yet directly identified, although it was reffered by Morihara in his inhibition studies[6] that the indole ring of a tryptophan residue possesses some chemical properties as a carbonyl group. In our present investigation we undertook to study the reactivity of the tryptophan residue in the active site of papain with the specific reagent, N-bromosuccinimide (NBS).

Experimental

Materials——Crystalline papain and α-benzoyl-L-arginine amide (BAA) were prepared by the procedure of Kimmel and Smith.[7] Mercuripapain was purchased from Sigma Chemical Co., St. Louis. Acetylpapain was prepared by the procedure of Riordan, *et al.*[8] N-Bromosuccinimide (NBS), *p*-chloromercuribenzoate (PCMB), N-acetyl imidazole, *p*-dimethylaminobenzaldehyde, hydroxylamine, formic acid and hydrogen peroxide were purchased from Nakarai Chemicals Co., Ltd., Kyoto.

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2) A part of this research was presented at the 24th Meeting of Kinki Branch, Pharmaceutical Society of Japan, Osaka, Nov., 1974.
3) Location: 5 *Nakauchicho, Misasagi, Yamashina, Higashiyama, Kyoto, 607, Japan.*
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5) S. Maeda, *Bull. Chem. Soc. Japan*, **12**, 319 (1937); S. Okumura, *Bull. Chem. Soc. Japan*, **13**, 534 (1938); *idem, ibid.*, **14**, 161 (1939); M. Bergmann and W.F. Ross, *J. Biol. Chem.*, **111**, 659 (1935); *idem, ibid.*, **114**, 717 (1936).
6) K. Morihara, *J. Biochem.* (Tokyo), **62**, 250 (1967); K. Morihara and K. Nagami, *ibid.*, **65**, 321 (1969).
7) J.R. Kimmel and E.L. Smith, "Biochemical Preparations," Vol. 6, John Willy and Sons, Inc., New York, 1957, p. 61.
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Assay Procedure of Enzyme Activity——The assay procedure described in the previous paper[9] was employed.

NBS Oxidation——NBS oxidation described by Spande, *et al.*[10] was employed with slight modification, as followed: The reaction conditions were 3×10^{-5} M of papain at pH 4.0 (0.1 M acetate buffer) and 25°. The sample cell was placed in a thermostated bath ($25\pm0.5°$) and allowed to equilibrate, and the initial optical density at 280 nm was determined. The cell was replaced in the bath and 5 μl portions of a 0.01 M NBS solution were added with rapid stirring to the contents of the cell. After about 20 sec, the absorbance was read. Addition of NBS was continued in this stepwise manner until the absorbance did not decrease any longer and began to increase. The molar concentration of the oxidized tryptophan residues was calculated as the product of the decrease in absorbance at 280 nm and the empirical factor, 1.31.[11]

Assay Procedure of SH Contents——SH contents were analyzed essentially according to the procedure of Boyer.[12]

Amino Acid Analyses——Amino acid analyses were performed by the method of Moore, *et al.*[13] with a Hitachi 034 Liquid Chromatograph. Samples for analyses were dialyzed with the cellulose tubing and hydrolyzed with 6 N HCl at 110° for 22 hr in evacuated sealed tubes. Tryptophan contents were determined by the method of Spies and Chambers.[14]

Results and Discussion

NBS oxidation was applied on papain. Oxidation of tryptophan residues was measured by the decrease in absorption at 280 nm. The course of NBS titration of tryptophan residues at pH 4.0 was shown in Fig. 1. About 2 residues were oxidized and 3 residues were not oxidized. The relationship between the tryptophan oxidation by NBS and the change in the enzyme activity of papain was shown in Fig. 2. The degree of oxidation to a completely inactivated state indicated that about 1 mole of tryptophan was lost during inactivation by NBS oxidation. Therefore, it seems very reasonable to conclude that 1 out of 5 tryptophan residues in papain specificially related the activity of papain.

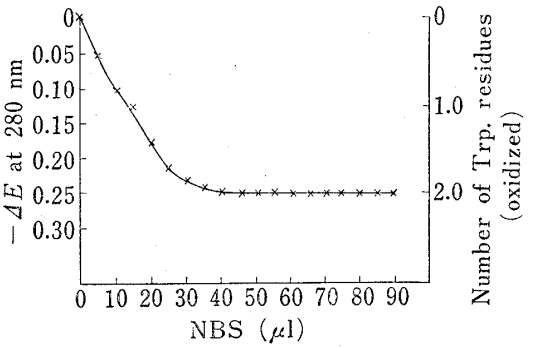

Fig. 1. The Degree of Oxidation of 30 μM Papain plotted against NBS Concentration

The oxidation was performed in 0.1M acetate buffer (pH 4.0) for 20 sec after each addition of 5 μl aliquots of NBS (0.01M).

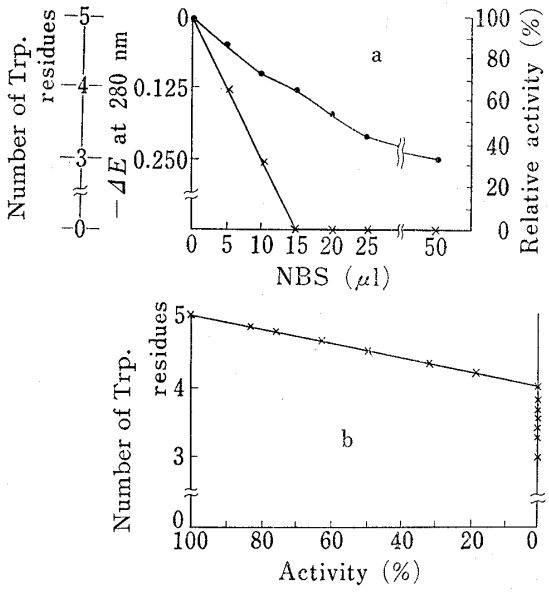

Fig. 2. The Correlation between the Decrease in Tryptophan Content and Degree of Oxidation by NBS Oxidation

(a) The NBS oxidation conditions were the same as Fig. 1. The reaction mixtures (for enzyme activity) contained papain (6 μM), substrate (BAA) (40 mM), cysteine-HCl (100 mM), EDTA (40 mM) and citrate buffer (pH 6.0, 32 mM). Enzyme activities were assayed by alkalimetric titration in alcohol. (b) The relation between tryptophan residues oxidized and enzyme activity. The data of Fig. 2-a were re-plotted.

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Table I. Amino Acids Composition of NBS-oxidized Papain

Amino acid[b]	Papain		Acetylpapain[a]		Mercuripapain	
	Intact (I)	NBS Oxidation (II)	Intact (III)	NBS Oxidation (IV)	Intact (V)	NBS Oxidation (VI)
Tryptophan[c]	5.0	4.0	5.0	4.0	5.0	3.8
Tyrosine	19.0	16.0	20.0	19.5	19.5	18.1
Histidine	2.0	2.0	1.9	1.8	1.8	1.8
Methionine	0	0	0	0	0	0
$CySO_3H$[d]	6.9	6.8	—	—	—	—
–SH(free)[e]	1.0	0.93	—	—	—	—
Residual activity (%)	100	0	100	0	100	0

a) Prepared by the procedure of Riordan, *et, al.*[8]
b) 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.
c) Determined by the methods of Spies and Chambers.[14]
d) Oxidized by performic acid.
e) Determined by the method of Boyer.[12]

Amino acid analyses of the inactivated papain showed that about 1 tryptophan and 3 tyrosine residues were oxidized at completely inactivation, as shown in Table I (Column I and II). This results prevent us from definitly concluding that the modification of only a tryptophan residue is the cause of the activity loss of papain by the NBS oxidation. Therefore, the NBS oxidation of acetylpapain has been planed to clarify the importance of tyrosine residues on the enzyme activity of papain.

Acetylpapain was prepared by the procedure of Riordan,[8] *et al.* with N-acetylimidazole (N-AI). The course of the reaction of N-AI with papain to form O-acetylpapain was monitored by the measurement of absorption at 278 nm ($-\Delta\varepsilon_{278}=1160\ \text{M}^{-1}\cdot\text{cm}^{-1}$). In this acetylation (about 7 tyrosine residues were acetylated), enzyme activity did not disappear. On treatment with hydroxylamine, acetylpapain gave rise to papain, the reaction being accompanied by the maintenance of enzyme activity. Then, NBS oxidation was applied on acetylpapain. About 2 tryptophan residues were oxidized and the first oxidizable tryptophan was important for enzyme activity. This results for acetylpapain is quite similar to that for papain. Amino acid analyses of the inactivated acetylpapain showed that only 1 tryptophan residue and no tyrosine residue were oxidized at complete inactivation unlike that of papain, as shown in Table I (Column III and IV). This results indicate that tyrosine residue does not affect the enzyme activity and the acetylation of tyrosine in papain prevents the tyrosine from oxidizing by NBS.

In the previous paper,[1] it was reported that mercuripapain was not inactivated by methylene blue-sensitized photooxidation unlike papain. However, by NBS oxidation, mercuripapain was inactivated and only 1 tryptophan residue was modified, as shown in Table I. These results for mercuripapain are quite similar to that for papain.

SH contents and half-cystine contents of NBS oxidized papain were determined by the method of Boyer[12] and by amino acid analyses as cysteic acid contents, respectively. No decrease of SH contents and half-cystine contents were observed (Table I; Column V and VI). These results and the inactivation of mercuripapain indicate that "active" SH group is not affected by NBS oxidation.

Table I reported only the results for those which are known to be susceptible to NBS oxidation. "Active" SH group and histidine residue are not affected by NBS oxidation, and no methionine residue is contained in papain. Tyrosine residues are oxidized in papain

and not oxidized in acetylpapain, the reaction being accompanied by the loss of enzyme activity. These results may be taken as good evidence for believing that only a tryptophan residue is exclusively modified by NBS oxidation with the loss of enzyme activity. However, about 2 tryptophan residues were modified by photooxidation with no loss of enzyme activity.[1] The relationship between the modified tryptophan residues by NBS oxidation and photooxidation is now under investigation.

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