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Chymopapain. I. Oxidation of Tryptophan Residues by N-Bromosuccinimide[1]

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1) The present research has been planned to clarify the importance of tryptophan residues in chymopapain A by means of N-Bromosuccinimide (NBS) oxidation.

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

3) The rate of oxidation by NBS increased with the decrease of pH.

4) SH group and histidine residues in chymopapain A were not affected by NBS.

5) These results indicate that the first oxidizable tryptophan residue is responsible for the loss of enzyme activity by NBS oxidation.

Studies of the protein constituents of papaya latex indicated the presence of four discrete proteinases.[3] Three of these correspond to papain, chymopapain and papaya peptidase A, and on the fourth component little information is available. Papain has been studied in detail, but the studies on the other proteinases are much less definitive.

Chymopapain was isolated from papaya latex by Jansen and Balls.[4] Like papain, it is a thiolenzyme, requiring cysteine or cyanide for activation, and is inhibited by thiol reagents.[3] The substrate specificity of papain and chymopapain shows a considerable degree of similarity.[5] In contrast to papain, chymopapain is very stable at acidic pH.[4] Smith and Kimmel reported that chymopapain isolated by the procedure of Jansen and Balls, was electrophoretically heterogeneous.[6] Recently, the chymopapain fraction can be separated into several major components by ion-exchange chromatography.[7] Two of these components, chymopapain A[5] and chymopapain B[7] have been obtained in crystalline and apparently homogeneous state.

Papain possesses a single reactive SH group,[3] a histidine residue[8] and a tryptophan residue,[9] which are considered to play an essential role in enzyme action. However, on the active sites of chymopapain, little information is available beyond the fact that it appears to be thiolenzyme.[5] In our present investigation we studied the reactivity of tryptophan residue in the active site of chymopapain A with a specific reagent, N-bromosuccinimide.

Experimental

Materials——Chymopapain A was prepared by the procedure of M. Ebata and K.T. Yasunobu.[5] α-Benzoyl-L-arginine amide (BAA) was prepared by the procedure of Kimmel and Smith.[10] N-Bromosuccini-

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1) A part of this research was presented at the 25th Meeting of Kinki Branch, Pharmaceutical Society of Japan, Kobe, Nov., 1975.
2) Location: *5 Nakauchicho, Misasagi, Yamashina, Higashiyama, Kyoto, 607, Japan.*
3) A.N. Glazer and E.L. Smith, "The Enzymes" (3 rd ed.) Vol. 3, 1971, pp. 501—546.
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9) M. Sakane, H. Kanazawa and A. Ohara, *Chem. Pharm. Bull.* (Tokyo), **23**, 1741 (1975); *idem, ibid.*, **24**, 22 (1976).
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mide (NBS), *p*-chloromercuribenzoate (PCMB), *p*-dimethylaminobenzaldehyde (PDAB), cysteine-HCl and ethylenediaminetetraacetic acid 2Na salt (EDTA) were purchased from Nakarai Chemicals, Co. Ltd., Kyoto.

Enzyme Assay——The assay procedure for papain described by Kimmel and Smith[10] was employed with slight modification as follows: After activation with cysteine and EDTA at pH 6.0, enzyme activity was examined using BAA as substrate at pH 6.0 by alkalimetric titration in alcohol.

NBS Oxidation——NBS oxidation described by Spande, *et al.*[11] was employed with slight modification. The molar concentration of the oxidized tryptophan residues was calculated from the product of the decrease in absorbance at 280 nm and empirical factor, 1.31.[12]

Amino Acid Analyses——Amino acid analyses were performed by the modified of Moore, *et al.*[13] using Hitachi 034 Liquid Chromatograph, after hydrolysis with 6 N HCl in a sealed evacuated tube at 110° for 22 hr.

Tryptophan Contents——Tryptophan contents were determined by the method of Spies and Chambers with PDAB.[14]

SH Contents——SH contents were determined by the method of Boyer with PCMB.[15]

Ultraviolet Difference Spectrometry——All spectra were measured using a Hitachi 624 spectrophotometer and a Hitachi 056 recorder using two matched quartz cell of 1 cm light path.

Results

NBS oxidation was applied at pH 4.0 on chymopapain A. Oxidation of tryptophan residues was monitored by the decrease in absorption at 280 nm. The course of NBS titration of tryptophan residues at pH 4.0 is shown in Fig. 1. About three tryptophan residues were oxidized at pH 4.0. Relationship between NBS oxidation of tryptophan at pH 4.0 and the change in enzyme activity is shown in Fig. 2. Enzyme activity was examined at pH 6.0 using BAA as sub-

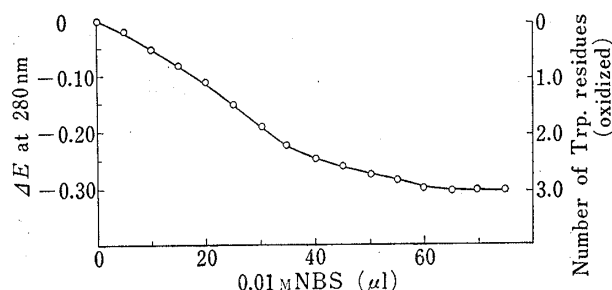

Fig. 1. The Degree of Oxidation of Chymopapain A (20 μM) plotted against NBS Concentration

This oxidation was performed in 0.1 M acetate buffer (pH 4.0) for 20 sec after each addition of 5 μl aliquot of NBS (0.01 M). Tryptophan contents were determined by the decrease in absorbance at 280 nm.[12]

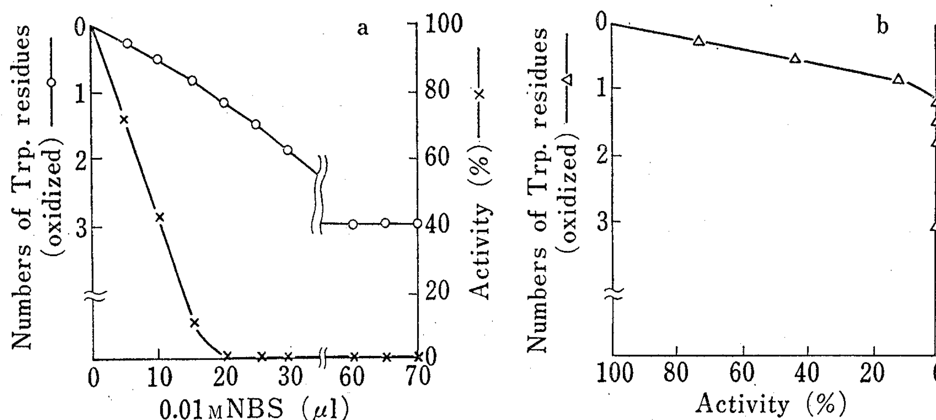

Fig. 2. The Correlation between the Decrease in Tryptophan Contents and the Degree of Inactivation by NBS Oxidation

a) The NBS oxidation conditions were the same as Fig. 1. The reaction mixtures (for enzyme assay) contained chymopapain A (20 μM), substrate (BAA) (40 mM), cysteine (100 mM), EDTA (40 mM) and citrate buffer (pH 6.0). Enzyme activities were assayed by alkalimetric titration.

b) The relation between the tryptophan residues oxidized and the enzyme activity. The data of Fig. 2 (a) were replotted.

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13) S. Moore, D.H. Spackman and W.H. Stein, *Anal. Chem.*, **30**, 1190 (1958).
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strate. The results indicated that about 1 mole of tryptophan was lost during inactivation by NBS oxidation at pH 4.0. On the basis of these data, subsequent experiments were carried out to examine the susceptibility of the active tryptophan residue to NBS at each pH.

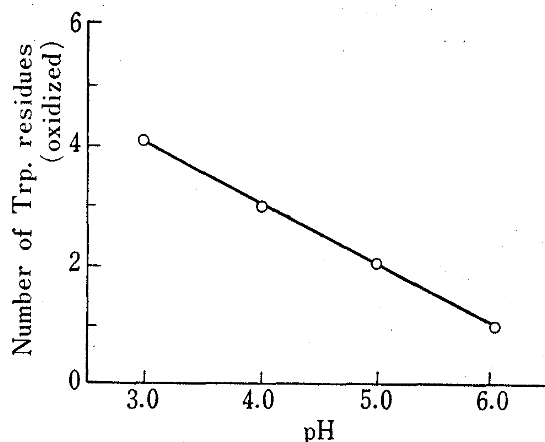

Fig. 3. Numbers of NBS-oxidized Tryptophan Residues at Each pH

Chymopapain A was treated 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. 3. However, enzyme activity was completely lost during the disappearance of the first oxidizable tryptophan residue at each pH. Therefore, this results suggest that one out of six tryptophan residues may be responsible for the loss of enzyme activity.

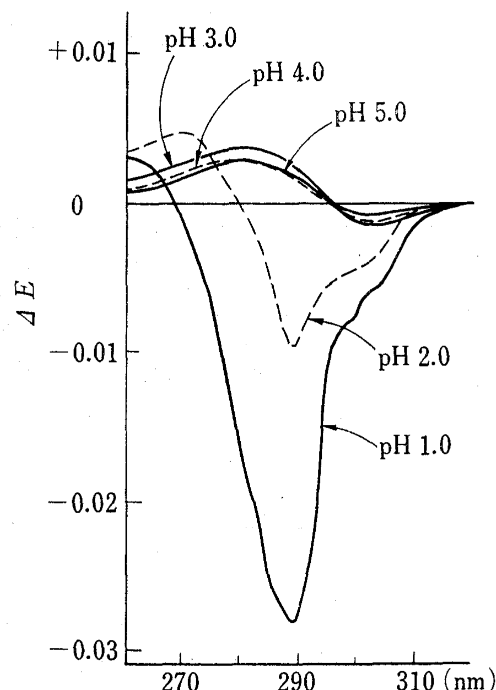

Fig. 4. Difference Spectra of Chymopapain A at Acidic pH

Difference spectrum of chymopapain A in acidic pH was measured using chymopapain A at pH 6.0 as reference. The final concentration was 1×10^{-5} M. The buffer solutions used were glycine-HCl buffer (0.1 M) for pH 1.0 and 2.0 and acetate buffer (0.1 M) for pH 3.0, 4.0, 5.0 and 6.0.

The results of amino acid analyses of the NBS-oxidized chymopapain A at 4.0 showed that only one tryptophan residue was significantly affected at complete inactivation, as shown in Table I. Table I shows only the results for amino acid residues which are known to be

Table I. Amino Acid Composition of NBS-oxidized Chymopapain A at pH 4.0

Amino acid	Trp.[a]	Tyr.	His.	Met.	-SH[b]	Activity
Intact	5.8	21.3	4.4	1.2	0.87	100%
Oxidized	4.9	22.8	5.1	1.3	0.84	0%

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 NBS-oxidation 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. No correction was made for decomposition during acid hydrolysis.
a) determined by the method of Spies and Chambers[14]
b) determined by the method of Boyer[15]

susceptible to NBS oxidation. No change was found in the content of the amino acids which are not listed in the Table I. The values in the Table I are expressed as the number of residues per molecule of mol.wt. 35000. SH contents of NBS-oxidized chymopapain A were determined by the method of Boyer. No decrease of SH contents is observed (Table I: column V). This findings indicate that "active" SH group is not affected by NBS oxidation. Therefore, it seems very reasonable to conclude that one out of six tryptophan residues in chymopapain A specificially relate to the activity of chymopapain A.

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To examine the change of state of tryptophan residues of chymopapain A, the difference spectra of this enzyme at acidic pH were taken using the spectrum at pH 6.0 as reference. One negative peak at 287 nm and two shoulders at 298 nm and 305 nm were observed, but these peak and shoulders were not observed at pH between 3 and 5 (Fig. 4). Therefore, it can be concluded that no conformational change of chymopapain A occurs at pH between 3 and 6, so far as checked by difference spectrum.

Discussion

In earlier experiments for papain[9,16] we suggested as follows: (1) The rate of NBS oxidation of the tryptophan residues of papain increased with the decrease of pH. (2) Enzyme activity was completely lost during the disappearance of a first oxidizable tryptophan residue by NBS at each pH. (3) The "active" SH group and the histidine residue of papain are not affected by NBS oxidation. (4) This first NBS-oxidizable tryptophan residue exists in or nearby the active site of papain.

The results on chymopapain A in present study was similar to the above results on papain. For the state of tryptophan residues, these findings may indicate as follows: (1) The intactness of the first NBS-oxidizable residue is essential for the catalytic action of chymopapain A. (2) Three tryptophan residues in addition to the first oxidizable tryptophan residue are modified by NBS oxidation at pH 3.0. To examine the change of state of tryptophan residues of chymopapain A, the difference spectra of chymopapain A at acidic pH were taken using the spectrum at pH 6.0 as reference. Generally, it could be reasonably assigned that the absorbancy difference at 280—300 nm is due to the change in state of tryptophan residues.[17] However, this absorbancy difference was not observed at pH between 3 and 6. From this findings, it could be concluded that no conformational change of chymopapain A occurs at pH above 3. Therefore, it is considerable that the oxidative degradation of these 3 tryptophan residues does not have any effect on the enzyme activity of chymopapain A. (3) Other two tryptophan residues are not affected by NBS oxidation. These 2 residues must be deeply burried in native protein in solution.

Since the rate of oxidation by NBS varies with pH, the reactivity of each tryptophan residue can be compared with NBS oxidation at each pH. The rate of oxidation by this reagent increased with the decrease of pH (Fig. 3) and enzyme activity was completely lost during the disappearance of the first oxidizable tryptophan residue at each pH. It is noticeable that at pH 6.0 only 1 mole of tryptophan was oxidized by NBS with the loss of enzyme activity. This findings indicate that only one tryptophan residue is most susceptible to NBS and may be responsible for the loss of enzyme activity by NBS oxidation.

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