[Chem. Pharm. Bull. 32( 8 )3111—3117(1984)]

# Chemical Modification of Tryptophan and Histidine Residues in Semi-alkaline Proteinase from *Aspergillus melleus*

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(Received October 24, 1983)

The amino acid residues responsible for the enzyme activity of semi-alkaline proteinase from *Aspergillus melleus* were identified by means of chemical modification studies. The modification of the enzyme with *N*-bromosuccinimide (NBS) resulted in the loss of the enzyme activity and a subtle alteration of conformation. NBS-modified enzyme still retained the antigenic structure, but became labile to heat and pH as the extent of modification of tryptophan increased. The relation between the extent of tryptophan oxidation and the enzyme stability suggested that 1 of the 3 tryptophan residues is important for the maintenance of structural integrity of the enzyme. The dye-sensitized photooxidation of the enzyme led to the loss of the enzyme activity with first-order kinetics. The rate of inactivation of this enzyme was pH-dependent and the rate constant-pH profile gave a sigmoidal curve with an inflection point at pH 6.5. Amino acid analysis of photooxidized enzyme indicated that the inactivation of this enzyme was directly proportional to the loss of histidine residue. Thus, these results suggested that at least 1 histidine residue is involved in the active site of the enzyme.

**Keywords**——semi-alkaline proteinase; *Aspergillus melleus*; chemical modification; *N*-bromosuccinimide; dye-sensitized photooxidation; stability; tryptophan; histidine

Semi-alkaline proteinase (SAP) purified from *Aspergillus melleus* is now clinically used as an anti-inflammatory agent. The specific catalytic activity of enzyme preparations depends on their molecular structure, and care is necessary in the storage of enzyme preparations due to the relative instability of the protein. Therefore, it is important to clarify the properties of SAP both to assess enzyme stability during storage and to evaluate the quality of enzyme preparations.

A series of studies on the purification and characterization of SAP from *Aspergillus melleus* was reported by Sugiura *et al.*[1–5] This enzyme was characterized by an optimum pH of 7.5—8.5, an optimum temperature of 45 °C and wide substrate specificity. The molecular weight of this enzyme as determined by gel filtration, sedimentation analysis and sodium dodecyl sulfate (SDS)-electrophoresis is $32000 \pm 2000$ and it consists of a single polypeptide chain with no disulfide bond.

However, little is known about the structure of the active center of this enzyme. Recently chemical modifications of enzymes have been extensively carried out to identify the critical residues involved in the active site or to assign a critical role to a particular amino acid.[6–8] In the present paper, dye-sensitized photooxidation was used to demonstrate the involvement of histidine in the catalytic function of SAP. The effect of oxidation of tryptophan residues by *N*-bromosuccinimide (NBS) on the stability of the enzyme is also discussed.

## Materials and Methods

**Reagents**——Casein, rose bengal and NBS were obtained from Wako Pure Chemical Ind. Co., Ltd. NBS was used after recrystallization from hot water. Methylene blue was from Kanto Chemical Co. Fluorescamine was purchased from Sigma Chemical Co. DEAE-Biogel A was a product of BIO-RAD Laboratories. All other reagents were of reagent grade.

**Purification of the Enzyme**——SAP was purified from *Aspergillus melleus* according to the method of Sugiura *et al.*[2] with a minor modification. Before use, the enzyme solution was applied to a DEAE-Biogel A column (3 × 30 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and eluted with a linear gradient of NaCl (0—0.2 M) in the same buffer. The active fraction was concentrated by ammonium sulfate precipitation. The purified enzyme gave a single protein band on disc electrophoresis at pH 9.4. It was reported that SAP contains 2 tryptophan and 2 histidine residues.[3] Amino acid analysis of SAP used in the present work demonstrated that 3 tryptophan and 6 histidine residues were present. This difference in amino acid compositions may be due to mutation of the bacteria. However, the enzyme properties were found to be the same as those of SAP previously reported.[2]

**Assay of Enzyme Activity**——The caseinolytic activity was measured by the method of Hagiwara *et al.*[9] with a minor modification. A 1.2% solution of Hammarstein casein in 0.1 M phosphate buffer (pH 7.0) was used as a substrate. One-half ml of enzyme solution was mixed with 2.5 ml of substrate and incubated for 10 min at 37 °C. The reaction was stopped by addition of 2.5 ml of a solution containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid. The mixture was left for 20 min at 37 °C and then filtered through No. 131 filter paper (Toyo Roshi). The absorbance of the filtrate at 280 nm was measured with a Hitachi 200-10 spectrophotometer.

**NBS Oxidation**——Small portions of 10 mM NBS solution (10—20 μl) were added to 3 mg of the enzyme dissolved in 3 ml of 0.1 M acetate buffer (pH 5.0) containing 0.25 mM $CaCl_2$ in a stepwise manner. Ultraviolet difference spectra were recorded with a Hitachi 557 spectrophotometer. The number of oxidized tryptophan residues was calculated from the decrease in absorbance at 280 nm according to the method of Spande *et al.*[10] The increase of amino-terminal residue during the modification with NBS was traced by the method of Fukihata *et al.*[11] The fluorescence intensity at 475 nm was measured with excitation at 390 nm using a Hitachi 650-10S fluorescence spectrophotometer.

**Dye-Sensitized Photooxidation**——The enzyme (4 mg) was dissolved in 8 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM $CaCl_2$ and 0.002% methylene blue or rose bengal. The reaction mixture was illuminated from a distance of 25 cm (methylene blue) or 20 cm (rose bengal) with a 150 W incandescent lamp at 37 °C. At regular intervals, 1 ml of the reaction mixture was withdrawn and applied to a Sephadex G-25 column (1.5 × 35 cm) equilibrated with 2 mM $CaCl_2$ solution in the dark. The protein fraction was collected, lyophilized and then used for amino acid analysis.

**Amino Acid Analysis**——Native, NBS-modified and photooxidized enzymes were hydrolyzed in 1 ml of 6 N HCl at 110 °C for 24 h in evacuated, sealed tubes. The contents of tryptophan, methionine and methionine sulfoxide were determined after hydrolysis with 3 N *p*-toluenesulfonic acid for 24 h at 110 °C in evacuated, sealed tubes. The amino acid compositions of hydrolysates were analyzed with a Hitachi 835 automatic amino acid analyzer. In addition, tryptophan was colorimetrically determined by the method of Dalby *et al.*[12]

**Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis**——SDS polyacrylamide gel electrophoresis was carried out according to the method of Suzuki.[13]

**Instrumental Analysis**——High-performance liquid chromatography (HPLC) was performed on a Hitachi 638-80 liquid chromatograph equipped with a Hitachi 635M multi-wavelength ultraviolet (UV) monitor. A TSK-GEL G-3000 SW column (7.5 × 600 mm) was obtained from Toyo Soda Co. As a mobile phase, 0.2 M phosphate buffer (pH 7.0) containing 0.1% SDS was used at a flow rate of 0.5 ml/min, and the effluent was monitored at 220 nm. Circular dichroism (CD) spectral measurement was carried out with a Jasco J-40C automatic recording spectropolarimeter at a protein concentration of 0.035 mg/ml in a cell of 10 mm light path at 20 °C.

**Preparation of SAP Antibody**——SAP antibody was obtained by immunization of rabbits with 1 ml of purified SAP (10 mg) emulsified in the same volume of Freund's complete adjuvant. The IgG fraction was prepared by fractionation (33% ammonium sulfate saturation), followed by passage through a DEAE-cellulose column equilibrated with 10 mM Tris-HCl buffer (pH 8.0).

## Results and Discussion

### NBS Oxidation of SAP

To elucidate the role of tryptophan residues, modification of the enzyme with NBS was performed. Figure 1 shows the correlation of the enzyme activity with the amount of oxidized tryptophan residue. The oxidation of tryptophan produced a substantial loss of enzyme activity; when 9 mol of NBS per mol of the enzyme was consumed, one tryptophan residue

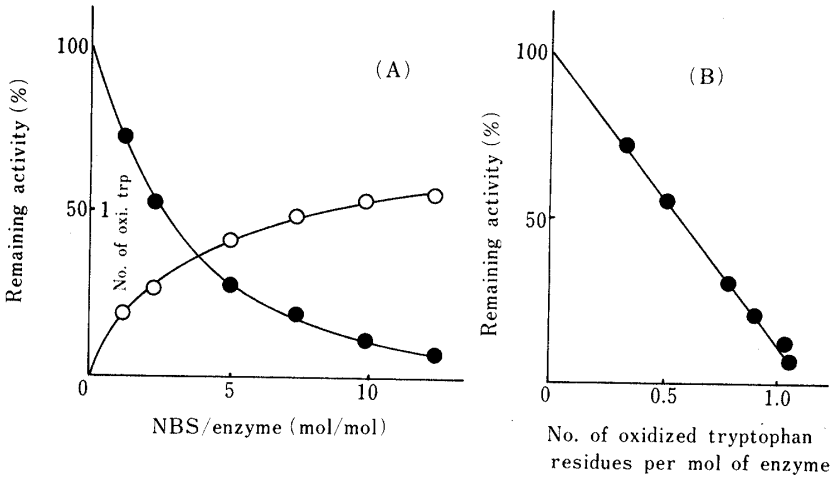


Fig. 1. Effects of Various Concentrations of NBS on Enzyme Activity and Oxidation of Tryptophan in SAP (A) and Change of Enzyme Activity as a Function of the Number of Oxidized Tryptophan Residues (B)

●, remaining activity; ○, number of oxidized tryptophan residues.

TABLE I. Proteinase Activity of NBS-Modified SAP

| | NBS/enzyme (mol/mol) | S.A.[a] | No. of tryptophan residue/mol of enzyme |
|---|---|---|---|
| Native SAP | | 24.8 | 3.0 |
| NBS-modified SAP (M-I) | 1.5 | 21.5 | 2.6 |
| NBS-modified SAP (M-II) | 3.0 | 15.9 | 2.4 |

*a*) Specific activity: caseinolytic activity per 10 min per mg of enzyme protein determined by HPLC.

was oxidized and the enzyme activity of SAP decreased to 10% of the original activity. Simultaneously, the number of N-terminal residues increased 3.8-fold. Spande and Witkop[14] reviewed the chemical cleavage of peptide bonds by NBS. Under our conditions, no cleavage of peptide bonds by NBS occurred, because NBS-oxidized phenylmethylsulfonyl SAP, which is modified with phenylmethylsulfonyl fluoride (a proteinase inhibitor), showed a single band on SDS polyacrylamide gel electrophoresis and the number of N-terminal residues did not increase. These results suggested that the increase in the N-terminal residues was due to autolysis of the SAP induced by NBS-oxidation of the tryptophan residue.

SAP is considered to decompose into several small peptides (or amino acids) owing to the autolysis during NBS-oxidation. Therefore, the concentration of the remaining enzyme protein during NBS-oxidation was determined by HPLC. The decrease in the concentration of enzyme protein was not directly proportional to the decrease in the enzyme activity (Fig. 2). This result suggested the production of a modified enzyme with lower specific activity during NBS-oxidation. Therefore, NBS-modified enzyme was purified on Sephadex G-100 and the properties of this enzyme were compared with those of the native enzyme. The activities of various NBS-modified enzyme preparations are summarized in Table I. The modified enzymes (13 and 36% reductions in specific activity as compared to that of the native enzyme) are referred to as M-I and M-II, respectively. As the elution volume of the NBS-modified enzymes (M-I and M-II) was the same as that of native SAP in the HPLC chromatograms, the

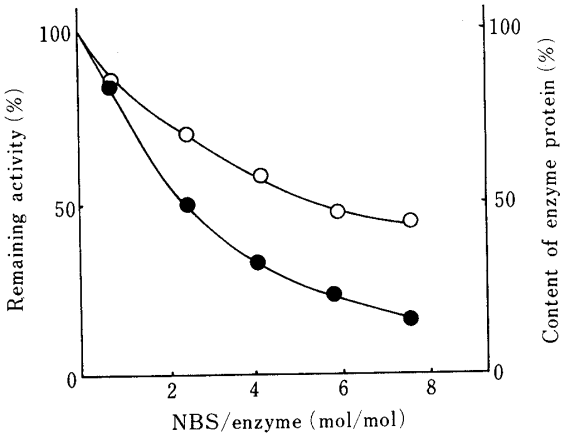


Fig. 2. Effects of Various Concentrations of NBS on Enzyme Activity and Content of Enzyme Protein

Content of enzyme protein was calculated from the peak area in HPLC.
●, remaining activity; ○, content of enzyme protein.

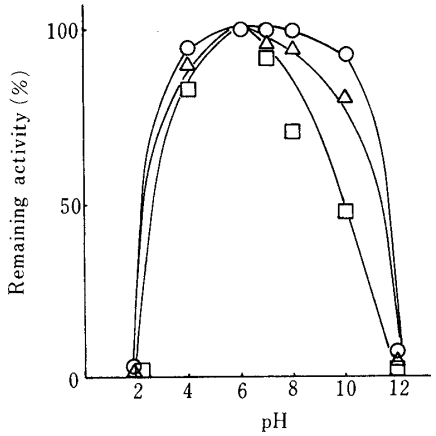


Fig. 3. Effect of pH on the Stability of Native and NBS-Modified SAP

The enzyme was incubated in Britton–Robinson buffer at 37 °C for 10 min, then the enzyme activity remaining in the incubation medium was assayed.
○, native SAP; △, M-I (13% loss in specific activity); □, M-II (36% loss in specific activity).

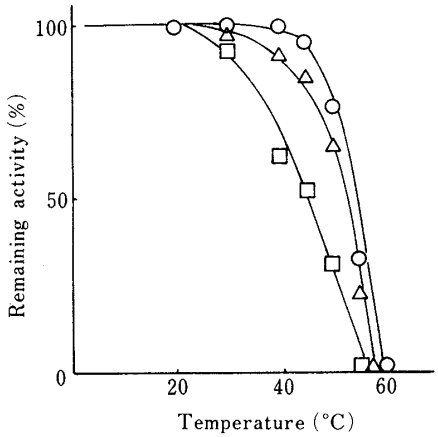


Fig. 4. Thermal Stability of Native and NBS-Modified SAP

The enzyme was incubated in 50 mM Tris-HCl buffer (pH 7.5) at various temperatures for 10 min, then the enzyme activity remaining in the incubation medium was assayed.
○, native SAP; △, M-I; □, M-II.

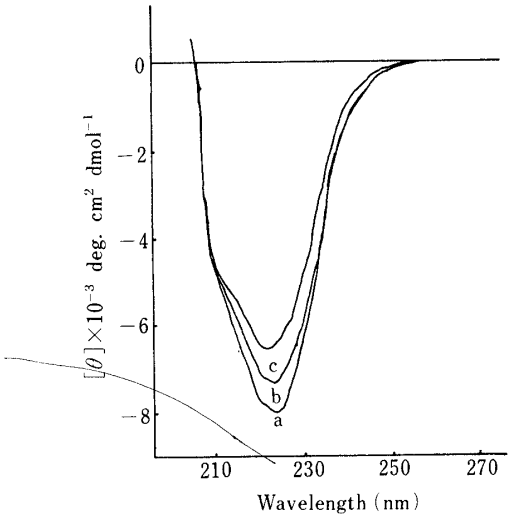


Fig. 5. CD Spectra of Native and NBS-Modified SAP

Data are expressed in terms of mean residue ellipticity [θ].
a, native SAP; b, M-I; c, M-II.

molecular weight of the modified enzyme was concluded to be unchanged. The amino acid analysis of the modified enzymes indicated that no amino acid other than tryptophan was affected by NBS-oxidation.

**Stability of NBS-Modified Enzyme**

The effect of pH on the stability of the NBS-modified enzymes was studied by carrying out incubation at 37 °C for 10 min at various pH values (Fig. 3). The native enzyme was stable in the pH range from 4 to 10, whereas the enzyme activity of the native enzyme was completely lost at pH 2 and 12. On incubation at pH 10, the enzyme activity of NBS-modified enzyme (M-I) decreased to 80% as compared to that of untreated M-I and the enzyme activity

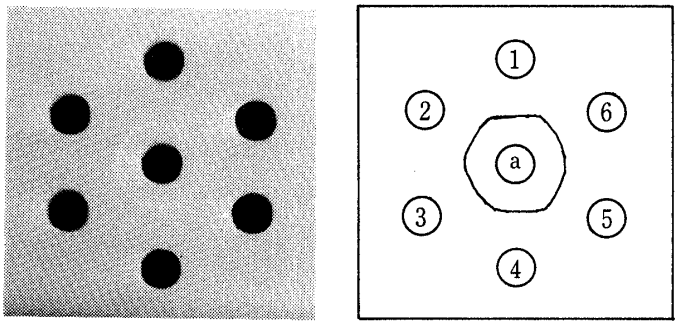


Fig. 6. Ouchterlony Immunodiffusion of Native and NBS-Modified SAP against Native SAP Antibody

Wells 1, 4: native SAP; wells 2, 5: M-II; wells 3, 6: M-I; well a: native SAP antibody.

of M-II decreased to 48% of the original activity of M-II. Thus, a remarkable difference in the stabilities of native and NBS-modified enzyme was observed in the alkaline pH range. Spande *et al.*[10] suggested that a protein containing oxindole residues would be more sensitive to pH change. Our results supported this view.

Figure 4 shows the thermal stability of NBS-modified enzymes after incubation in 50 mM Tris-HCl buffer (pH 7.5) at various temperatures for 10 min. In the case of native enzyme, no change was observed in its activity at 45 °C. The enzyme activities of M-I and M-II decreased to 85 and 65% of the original values after the treatment at 45 °C, respectively. These results suggest that the modified tryptophan residue may be important for the stabilization of SAP.

**CD Spectra and Immunological Properties of NBS-Modified Enzymes**

The conformational changes of native and NBS-modified enzymes were investigated by measuring the CD spectra in the ultraviolet region (Fig. 5). The native enzyme had a negative band at 222 nm. The trough at 222 nm decreased in proportion to the degree of oxidation of the tryptophan residue.

An immunological study of NBS-modified enzymes was performed by Ouchterlony's method (Fig. 6). Native and NBS-modified enzymes showed a single precipitation line against antibody to the native enzyme and the precipitation lines fused completely. This observation indicates that SAP still retains the antigenic structure despite the oxidation of tryptophan in the enzyme by NBS. Thus, it is assumed that the structural change observed in the CD spectra reflects a conformational change around the tryptophan residue, which affects the enzyme stability or activity.

SAP has 3 tryptophan residues, of which only 1 was oxidized by NBS under the conditions that we employed. The other two tryptophan residues were oxidized in the presence of such denaturating agents as urea or SDS. It therefore seems likely that the tryptophan residue oxidized by NBS must be located at the surface of the protein molecule, while the other two tryptophan residues may be buried.

In summary, 1 of the 3 tryptophan residues plays an important role in stabilizing the active conformation of SAP, and the modification of this tryptophan residue results in destabilization and induction of autolysis.

**Chemical Modification of Histidine Residue**

Photooxidation of SAP in the presence of methylene blue or rose bengal led to loss of the enzyme activity with first-order kinetics (Fig. 7). The first-order rate constant of inactivation in the presence of dye at various pH values was measured. The rate of inactivation was markedly dependent on pH (Fig. 8). The rate constant-pH profile showed a sigmoidal shape with an inflection point at pH 6.5. This value is almost identical with the p$K$ value of 6.6 estimated from the pH dependence of the association constant of benzeneboronic acid, a possible transition-state analog for serine protease, to SAP.[15] It is well known that the photooxidation of histidine is pH-dependent and that other photosensitive amino acids do

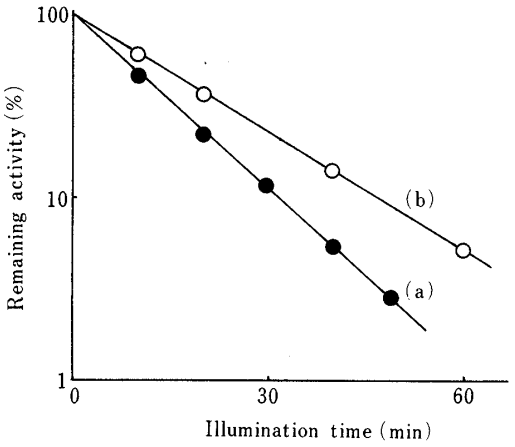


Fig. 7. Time Course of Inactivation of SAP by Photooxidation in the Presence of Methylene Blue (a) or Rose Bengal (b)

The reaction mixtures were composed of 0.047% enzyme, 0.002% methylene blue or rose bengal, 2 mM $CaCl_2$ and 50 mM Tris-HCl buffer (pH 7.5). The reaction mixtures were illuminated from a distance of 25 cm (methylene blue) or 20 cm (rose bengal) with a 150 W incandescent lamp at 37 °C.

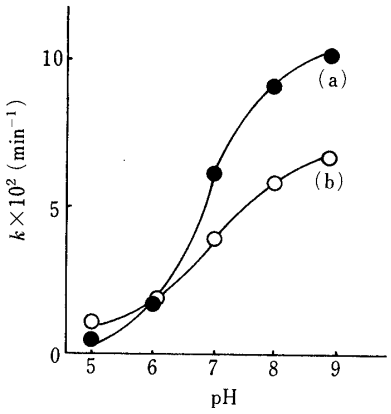


Fig. 8. The pH Dependence of Inactivation of SAP by Photooxidation in the Presence of Methylene Blue (a) or Rose Bengal (b)

$k$: first-order rate constant for inactivation.
The buffers used were 50 mM acetate buffer (pH 5.0), 50 mM phosphate buffer (pH 6.0 and 7.0) and 50 mM Tris-HCl buffer (pH 8.0 and 9.0).

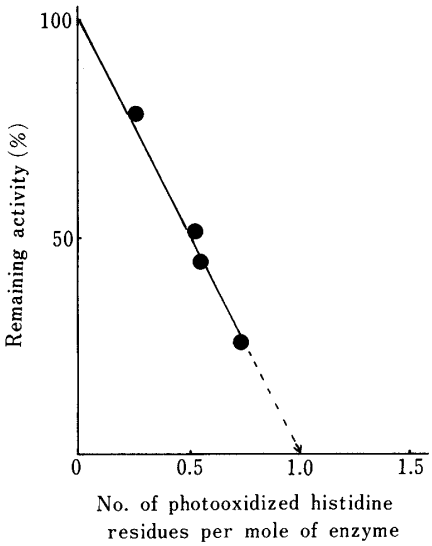


Fig. 9. Plot of the Extent of Modification of Histidine Residue against Loss of Enzyme Activity

not show this dependence.

No significant change in the CD spectra of the photooxidized enzyme was observed (not shown). Therefore, these results strongly suggested that modification of histidine residues was the major factor in the inactivation of the enzyme. This was confirmed by the results of amino acid analysis of the photooxidized enzyme. Figure 9 shows that the loss of the enzyme activity is related to the extent of modification of one histidine residue.

It has been reported that dye-sensitized photooxidation of proteins resulted in the destruction of methionine, tryptophan, histidine, tyrosine and cysteine residues.[16] The amino acid compositions of photooxidized enzyme having 10% activity indicated that only 1 of 6 histidine residues of the native enzyme was lost and that other amino acid residues were unaffected by the photooxidation.

It is suggested on the basis of these results that at least one histidine residue is involved in the active site of SAP and that that residue is essential for the enzyme activity.

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