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Reinvestigation of *N*-Bromosuccinimide Oxidation of Glucoamylases from *Aspergillus saitoi*

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The reaction of Gluc M_2, the minor glucoamylase [EC 3.2.1.3, α-D-(1→4)glucan glucohydrolase] from *Aspergillus saitoi*, with *N*-bromosuccinimide (NBS) was reinvestigated in order to elucidate the structure–activity relationship. Some data were also obtained for Gluc M_1. The tryptophan residues in Gluc M_2 were oxidized at various NBS/Gluc M_2 ratios. The enzymatic activity decreased to about 7.5% of that of native Gluc M_2 with the oxidation of the first 6 tryptophan residues at an NBS/Gluc M_2 ratio of 10. In the presence of 10% soluble starch, about 4.5 tryptophan residues in Gluc M_2 were oxidized at an NBS/Gluc M_2 ratio of 10. The remaining activity of Gluc M_2 at this stage of oxidation was about 75%. On further oxidation after removal of soluble starch, the enzymatic activity decreased to zero with the concomitant oxidation of 3 tryptophan residues. The results indicated that the essential tryptophan residue(s) is amongst these 3 tryptophan residues.

The ultraviolet difference spectrum induced by addition of maltose and maltitol to Gluc M_2 showed four troughs at 280, 288, 295 and 310—313 nm. The latter 3 troughs were probably due to tryptophan residues of Gluc M_2, and the magnitude of the troughs decreased with the progress of oxidation, but to a far lesser extent than the decrease in activity. These results indicate that there are two types of tryptophan residues is in Gluc M_2. One of them is located near subsites 1 and 2 and is related to catalysis, while the other is located at subsites 2 and 3, and contributes to the maltose binding.

Keywords——glucoamylase; *Aspergillus saitoi*; *N*-bromosuccinimide; NBS; tryptophan; active site; maltose binding site

Aspergillus saiotoi produces two glucoamylases, Gluc M_1 and Gluc M_2 [EC 3.2.1.3 α-D-(1→4)glucan glucohydrolase], sharing a common antigenicity.[1,2] Since the sequences of the N-terminal 4 amino acid residues of these enzymes are identical and the molecular weight of Gluc M_1 (M_r 90000) is larger than that of Gluc M_2 (M_r 70000), it has been suggested that Gluc M_2 is an enzyme species produced by removal of the carboxyl-terminal part of Gluc M_1 by proteolysis.[2]

As regards the active site of Gluc M_1, the major glucoamylase of *A. saitoi*, the participation of 1 or 2 carboxyl groups was proved by chemical modification of Gluc M_1 with a water-soluble carbodiimide.[3] A similar experiment on Gluc M_2 also showed that one carboxyl group is involved in the active site of this enzyme.[4]

As proposed by Hiromi *et al.*,[5] the active site of Gluc M_1 consists of about 7 subsites, each of which interacts with a glucose moiety of soluble starch.[6] Chemical modification of Gluc M_1 with *N*-bromosuccinimide (NBS) showed that one or two tryptophan residues located at subsite 1 are crucial for enzymatic activity.[7] In this experiment, the number of tryptophan residues oxidized was estimated from the change in absorbancy at 280 nm according to Spande and Witkop.[8] However, Iwama *et al.*[9] recently suggested that in a

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complex molecule such as glucoamylase, the NBS-oxidized tryptophan residues estimated from the change in absorbancy at 280 nm are less than the actually oxidized tryptophan residues, and thus the estimation should be performed by other methods, such as colorimetry[10] or measuring the fluorescence decrease of tryptophan residues in 6 M guanidine – HCl.[11] Therefore, in this paper we reinvestigated the relation between the enzymatic activity and tryptophan residues oxidized using Gluc M_2, a minor glucoamylase of *A. saitoi*[2] which has a smaller molecular weight than Gluc M_1 and is supposed to have the same active site as Gluc M_1. The tryptophan contents of Gluc M_1 and Gluc M_2 are 28 and 19 residues, respectively.

Materials and Methods

Substrates——Soluble starch was purchased from Wako Pure Chemicals and used as a substrate after exhaustive dialysis against distilled water followed by lyophilization. Maltose was obtained from Wako Pure Chemicals.

Other Reagents——NBS obtained from Wako Pure Chemicals was recrystallized from hot water before use. Maltitol was obtained from Tokyo Kasei. 5,5′-Dithiobis-(2-nitrobenzoic acid) was obtained from Wako Pure Chemicals. All other chemicals were of analytical grade.

Enzyme——Gluc M_1 and Gluc M_2 were purified from the commercial digestive "Molsin" (*Aspergillus saitoi*, Seishin Pharm. Co., Ltd.) according to the method reported previously.[1,2]

Determination of Glucoamylase Activity——The enzymatic activity was determined with soluble starch as a substrate at pH 5.0 and 37 °C as previously described.[1]

Protein Concentration——Gluc M_1 and Gluc M_2 concentrations were determined from the absorbance at 280 nm by taking $A_{cm}^{280\,nm}$ (1%) to be 14.97 and 14.18, respectively.

Amino Acid Analysis——Samples of *ca.* 0.3 mg of protein were hydrolyzed in evacuated tubes with 6 N HCl at 110 °C for 24 h. Amino acid analyses were performed by the method of Spackman *et al.*[12] with a Nihon Denshi JEOL 6AH amino acid analyzer. The sulfhydryl groups in the protein were titrated with 5,5′-dithiobis-(2-nitrobenzoic acid) according to the method of Ellman.[13] The amount of remaining tryptophan residues was estimated by the method of Pajot.[11] Samples were dissolved in 6 M guanidine–HCl solution containing 30 mM 2-mercaptoethanol, and then the fluorescence intensity at 355 nm of each sample (excited by 295 nm) was measured with a RF 502 Shimadzu spectrofluorometer.

Circular Dichroism (CD) Spectra——CD spectra were measured with a JASCO J-40 spectropolarimeter at room temperature in cells of 0.1- and 0.5-cm light path for the wavelength regions 200—240 and 240—320 nm, respectively. The enzyme concentrations used were 6.4—10.1 μM.

Difference Absorption Spectra of Glucoamylase and NBS-Oxidized Glucamylase Induced by Addition of Maltose and Maltitol——Ultraviolet (UV) difference spectra of glucoamylase and NBS-oxidized glucoamylase induced by addition of 4.5 mM maltose and maltitol were measured with a Shimadzu UV 240 spectrophotometer at pH 5.0 (0.1 M acetate buffer) and 25 °C using tandem cells. The enzyme concentration was *ca.* 12—18 μM for both enzymes. In the case of difference spectra induced by maltose, the spectra were measured 20 s after mixing the enzyme and maltose to exclude the effect of hydrolysis of maltose.

NBS-Oxidation——(a) To 3 ml of glucoamylase solution (9.5 μM in 0.1 M acetate buffer, pH 4.5) kept at 25 °C was added 5—20 μl of NBS stock solution (7.2—29 mM). The amount of tryptophan residues oxidized was estimated from the fluorescence intensity at 355 nm in 6 M guanidine–HCl excited at 295 nm as described by Iwama *et al.*[9] To determine the amino acid composition and CD spectra, NBS oxidation was stopped by addition of 30 μl of L-tryptophan solution (48 mM), and then the reaction mixture was dialyzed against 0.1 M acetate buffer (pH 4.5).

(b) NBS-oxidation of Gluc M_2 in the presence of soluble starch. NBS oxidation in the presence of 10% soluble starch was performed principally as described above. After stopping the reaction by addition of tryptophan, the reaction mixture was dialyzed against distilled water overnight. To eliminate the remaining soluble starch, the oxidized Gluc M_2 was adsorbed on a column of diethylaminoethyl (DEAE)-Sephadex A-50 (0.8 × 12 cm) equilibrated with 0.1 M phosphate buffer (pH 6.0). The enzyme was eluted with the same buffer containing 1 M NaCl. The eluate containing protein was desalted by dialysis against acetate buffer (0.1 M, pH 4.5).

Results

NBS Oxidation of Gluc M_2 in Relation to the Decrease in Enzymatic Activity

The tryptophan residues in Gluc M_2 were oxidized with increasing addition of NBS up to an NBS/Gluc M_2 ratio of 30, and the decrease in enzymatic activity during the course of NBS

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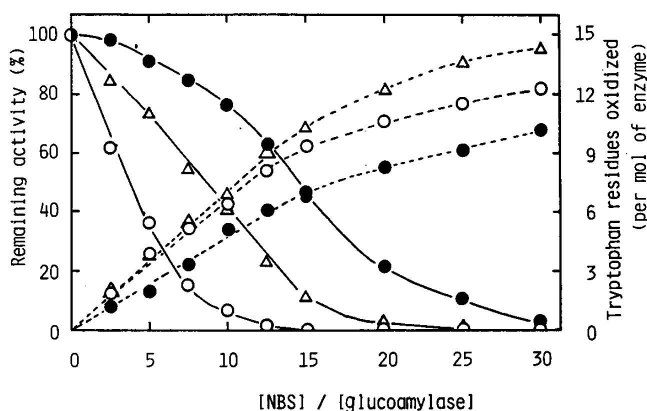

Fig. 1. NBS Oxidation of Gluc M_2 and Gluc M_1 in the Absence and Presence of Soluble Starch (10%) at pH 4.5

Gluc M_2 or Gluc M_1 in 3.0 ml of 0.1 M acetate buffer (pH 4.5) was oxidized with various concentrations of NBS in the presence of 10% soluble starch (●, Gluc M_2) and in the absence of soluble starch (○, Gluc M_2; △, Gluc M_1) at 25 °C. The other experimental conditions were as described in Materials and Methods. (——), remaining activity; (----) tryptophan residues oxidized with NBS per mol of enzyme.

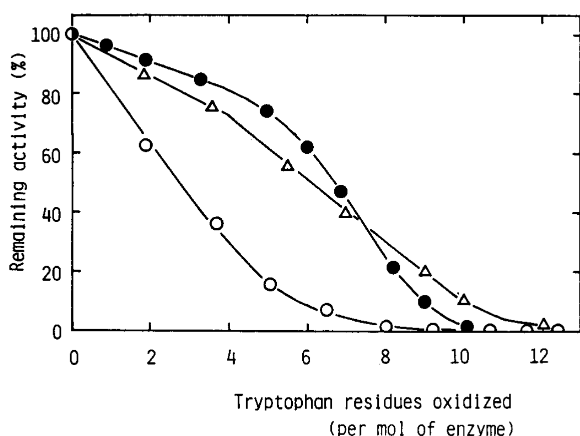

Fig. 2. Relation between Enzymatic Activity of Gluc M_2 and Tryptophan Residues Oxidized

The data in Fig. 1 were replotted. The symbols used are the same as in Fig. 1.

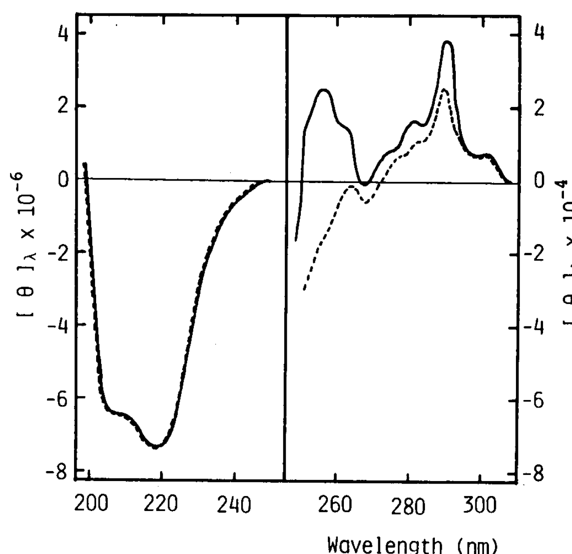

Fig. 3. CD Spectra of Gluc M_2 (——) and Gluc M_2 Oxidized with NBS (----)

Gluc M_2 oxidized at an NBS/Gluc M_2 ratio of 12.5, and having 3% residual activity, was used. The conditions for CD measurements were as described in the text.

oxidation was measured with soluble starch as a substrate at pH 4.5 (Fig. 1). The enzymatic activity decreased sharply with the oxidation of tryptophan residues. The number of tryptophan residues oxidized up to 4 is almost linearly correlated to the loss of enzymatic activity (Fig. 2).

CD Spectrum of Gluc M_2 Oxidized with NBS

The CD spectrum of Gluc M_2 oxidized with 12.5 mol eq of NBS (residual activity, *ca.* 3%) was measured between 200—320 nm. The results are shown in Fig. 3. The CD spectrum in the short wavelength region (200—240 nm), which is attributable to the peptide backbone conformation of the protein, was practically identical with that of the native Gluc M_2. Thus it could be concluded that, at least in terms of the CD spectrum, the peptide backbone structure of NBS-oxidized Gluc M_2 remained intact during the course of NBS oxidation. However, the three peaks at 256, 265 and 290 nm of the native Gluc M_2 attributed to the aromatic amino acids decreased markedly upon NBS oxidation.

Amino Acid Composition of NBS-Oxidized Gluc M_2

The amino acid composition of NBS-oxidized Gluc M_2 having 5% enzymatic activity was measured. The amino acid composition was pratically the same as that of native Gluc M_2 except for the tryptophan content. No decrease in tyrosine, histidine and SH contents was

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observed (data not shown). Thus, the decrease in enzymatic activity is due mainly to the loss of tryptophan residues.

NBS Oxidation of Gluc M_2 in the Presence of 10% Soluble Starch

The results of NBS oxidation of Gluc M_2 in the presence of substrate (10% soluble starch) are shown in Figs. 1 and 2. At an NBS/Gluc M_2 ratio of 10, about 6.5 tryptophan residues of Gluc M_2 were oxidized in the absence of substrate and the enzymatic activity decreased almost to zero. However, under the same conditions, about 4.5 tryptophan residues in Gluc M_2 were oxidized in the presence of soluble starch, and the decrease in enzymatic activity was only 25%. However, on further addition of NBS up to an NBS/Gluc M_2 ratio of 30, the enzymatic activity decreased to zero with the oxidation of 10 tryptophan residues. The Gluc M_2 which was oxidized at an NBS/Gluc M_2 ratio of 10 in the presence of soluble starch (residual activity 75%) was chromatographed on a column of DEAE-Sephadex A-50 to eliminate the soluble starch. The soluble starch-free enzyme was further oxidized in the absence of soluble starch. The results are shown in Fig. 4. The enzymatic activity decreased to zero with the simultaneous oxidation of about 2.5 tryptophan residues. When we consider that the difference in the tryptophan residues oxidized at an NBS/Gluc M_2 ratio of 10 in the presence and absence of soluble starch was about 2.0, the results described above may indicate that some enzymatically important tryptophan residue(s) are protected from oxidation by soluble starch. The effect of maltose, a poor substrate for Gluc M_2, on the oxidation of Gluc M_2 was examined at a concentration of 0.6 M. The oxidation progressed at almost the same rate as that in the absence of maltose. Since the maltose concentration in the reaction mixture after 15 min was about 97% of the initial concentration, the non-protective nature of maltose was not due to a decrease in the concentration of maltose due to enzymatic hydrolysis. Maltitol (0.1 M), an effective inhibitor of glucoamylase,[3] also exhibited a very poor protective effect.

The Effect of NBS Oxidation on the Fluorescence Intensity of Gluc M_2

The fluorescence emission of Gluc M_2 (excited at 295 nm, measured at 350 nm) decreased with the progress of NBS oxidation (Fig. 5). The contribution of NBS-reactive tryptophan residues to the fluorescence intensity seemed to be very high.

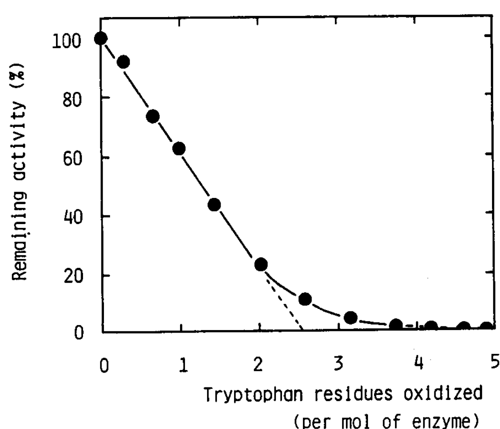

Fig. 4. NBS Oxidation of Gluc M_2 Which Had Previously Been Oxidized in the Presence of Soluble Starch

Gluc M_2 (9.76 μM) oxidized previously at an NBS/Gluc M_2 ratio of 12.5 in the presence of 10% soluble starch, was oxidized in the absence of soluble starch. The other experimental conditions were as described in the text and the legend to Fig. 1.

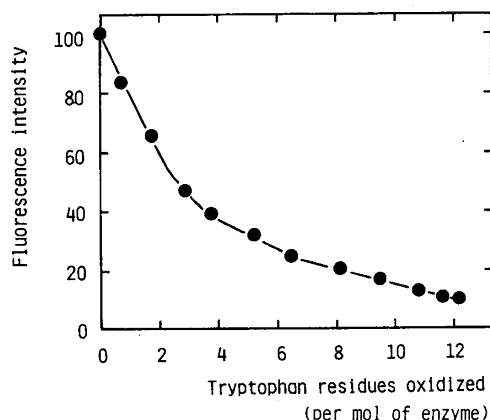

Fig. 5. Decrease in Fluorescence Intensity upon Modification of Tryptophan Residues of Gluc M_2 with NBS

The concentrations of Gluc M_2 and Gluc M_2 oxidized with NBS were 0.6 μM. The other experimental conditions were as described in the text.

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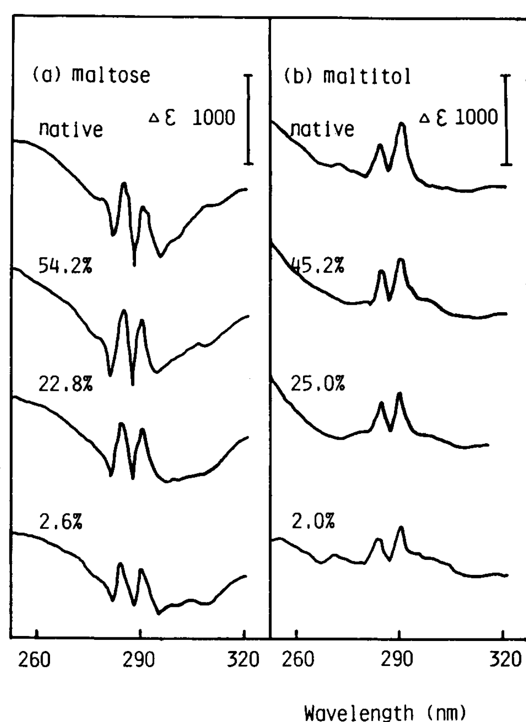

Fig. 6. UV Absorption Difference Spectra Induced by Mixing Maltose (a) and Maltitol (b) with NBS-Oxidized Gluc M_2 at pH 4.5 and 25 °C

Maltose and maltitol concentrations were 4 mM. The residual activities of NBS-oxidized Gluc M_2 used are indicated.

UV Difference Spectra of Gluc M_2 and NBS-Oxidized Gluc M_2 Induced by Addition of Maltose and Maltitol

When Gluc M_2 was mixed with maltose at pH 4.5, the UV difference spectra showed 4 troughs at 281, 288, 295 and 310 nm (Fig. 6). The UV difference spectra of Gluc M_2 and maltitol gave peaks at 284 and 290 nm and troughs at 280, 288, 295 and 313 nm. The concentrations of maltose and maltitol added were less than 0.2%. Thus, the difference spectra observed were not due to solvent perturbation of the enzyme. The UV difference spectra induced by addition of maltose were measured 20 s after mixing Gluc M_2 and maltose to minimize hydrolysis of the substrate. The difference spectra induced by maltose disappeared on prolonged incubation due to the hydrolysis of maltose. This phenomenon also indicated that the difference spectra observed are not due to simple solvent perturbation. The wavelengths of the troughs, 280—281, 288, 295 and 310—313 nm, suggested that these difference spectra are mainly caused by interaction of tryptophan residues with substrate or inhibitor. The magnitude of the difference spectra of NBS-oxidized Gluc M_2 induced by maltose or maltitol tended to decrease with the progress of oxidation with NBS. However, the intensites of the UV difference spectra of the enzyme species showing 2.6% activity were 50% and 40% of those of the native enzyme for maltose and maltitol, respectively. The results indicate that binding ability towards these substrates and substrate analogues is retained after loss of enzymatic activity due to NBS oxidation of Gluc M_2.

Reinvestigation of the NBS-Oxidation of Gluc M_1

The NBS oxidation of Gluc M_1 was reinvestigated by the same procedure as described above for Gluc M_2. Gluc M_1 has four rapidly oxidizable tryptophan residues and their oxidation reduces the enzymatic activity by only 20%. On further oxidation of Gluc M_1, *ca.* 8 tryptophan residues are oxidized with a simultaneous decrease in enzymatic activity. The results are also included in Figs. 1 and 2. When we compared the results for Gluc M_1 and Gluc M_2 having 10—60% remaining activity, it could be concluded that about 4—5 tryptophan residues are probably located on the C-terminal part of Gluc M_1 that is missing in Gluc M_2.

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Discussion

As described previously, the minor glucoamylase from *A. saitoi* having a molecular weight of 70000 is an N-terminal fragment of the major glucoamylase, Gluc M_1 (molecular weight 90000).[2] Gluc M_1 and Gluc M_2 share the same active site structure which consists of 7 subsites,[6] and subsites 2 and 3 each have high affinity for a glucose moity of the substrate. In this sense, both enzymes are typical glucoamylases, as has been suggested by Hiromi *et al.*[5] Therefore, it seems reasonable to assume that maltose and maltitol bind with Gluc M_2 mostly at subsites 2 and 3.

Even NBS-oxidized Gluc M_2 which has no activity, shows difference UV spectra upon addition of maltose or maltitol. This suggests the presence of a tryptophan residue(s) which is located at the active site (between subsites 1 and 2) and contributes to the enzymatic catalysis but not to the major maltose or maltitol binding site. The fact that maltose or maltitol did not protect Gluc M_2 against NBS oxidation is reasonable because the glucose analogues bind preferentially to subsites 2 and 3[5] and do not protect the tryptophan residue on subsites 1 and 2.

On the other hand, NBS-oxidized Gluc M_2 which has almost no activity still shows a maltose-induced difference UV spectrum similar to that of native Gluc M_2. These patterns of the difference spectra seem to indicate the contribution of some tryptophan residue(s) in subsites 2 and 3 to the binding of maltose and maltitol.

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