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Oxidation of Glucoamylase from a *Rhizopus* sp. with *N*-Bromosuccinimide

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Two glucoamylases from a *Rhizopus* sp., $Gluc_1$ and $Gluc_2$, were oxidized with *N*-bromosuccinimide (NBS). The number of tryptophan residues oxidized could be accurately estimated from the decrease of fluorescence intensity in 6 M guanidine hydrochloride, but not from the decrease in absorbancy at 280 nm. $Gluc_1$ and $Gluc_2$ were inactivated when about 4 to 5 tryptophan residues were oxidized. These numbers were similar to those of tryptophan residues perturbed with polyethylene glycol as a perturbant. One tryptophan residue, which existed on the surface at the *N*-terminal part of $Gluc_1$, was oxidized first, causing only a slight decrease in the enzymatic activity. Even when the enzymatic activities of $Gluc_1$ and $Gluc_2$ were destroyed by NBS oxidation, the binding abilities of $Gluc_1$ and $Gluc_2$ with maltitol were fairly well retained, because fluorescence quenching of tryptophan residues induced by the addition of maltitol was still observable. Therefore, it is suggested that these glucoamylases have at least two important tryptophan residues, one related to the catalytic action and the other related to the binding of substrates, and the latter one(s) reacts only slowly with NBS.

Keywords——glucoamylase; *Rhizopus*; *N*-bromosuccinimide; NBS; tryptophan; active site

We previously isolated three forms of glucoamylase, $Gluc_1$, $Gluc_2$ and $Gluc_3$ [EC 3.2.1.3α-D-(1→4)glucan glucohydrolase] from a *Rhizopus* sp.[1] The minor components, $Gluc_2$ (M_r 58600) and $Gluc_3$ (M_r 61400) lack N-terminal parts of $Gluc_1$ (M_r 74000).[2] In other papers, we reported a comparison of the enzymatic properties of these isozymes[3] and demonstrated the participation of carboxyl groups(s) in the active site of $Gluc_1$.[4]

N-Bromosuccinimide (NBS) oxidation of a glucoamylase from *Rhizopus niveus* was previously reported by Hiromi *et al.*[5] Their results can be summarized as follows. The glucoamylase contained two rapidly oxidizable tryptophan residues and the oxidation of these residues little affected the enzymatic activity. On further oxidation of an additional one tryptophan residue, the glucoamylase was inactivated. In their experiments, the number of tryptophan residues oxidized was estimated from the decrease in absorbance at 280 nm using $\Delta\varepsilon = 4200$.[6]

In this work, in order to re-investigate the state and the role of tryptophan residues of the glucoamylases from a *Rhizopus* sp. in relation to the enzymatic activity, NBS oxidation of $Gluc_1$ and $Gluc_2$ was studied.

Materials and Methods

Enzymes——$Gluc_1$ and $Gluc_2$ were purified from the commercial digestive, "Gluczyme" (*Rhizopus* sp., Amano Pharm. Co., Ltd.), according to the method reported previously.[1]

Determination of Protein Concentration——The concentrations of $Gluc_1$ and $Gluc_2$ were estimated on the basis

of A_{cm}^{280} (1%) 13.2 and 13.7, respectively.[1]

Chemicals——Soluble starch was purchased from Wako Pure Chemicals and used as a substrate after exhaustive dialysis against distilled water. Maltose and *p*-dimethylaminobenzaldehyde (DBA) were purchased from Wako Pure Chemicals. *p*-Nitrophenyl α-D-glucopyranoside (PNPG) was obtained from Koch-Light Lab. Ltd. Maltitol was obtained from Tokyo Kasei Kogyo Co. *N*-Acetyl-L-tryptophan ethyl ester (ATEE) and *N*-acetyl-L-tyrosinamide were obtained from Aldrich Chemical Co. and Sigma Chemical Co., respectively. NBS was obtained from Nakarai Chemical Co. and was recrystallized from hot water.

Determination of Glucoamylase Activity——The enzymatic activity was measured with soluble starch as a substrate by the D-glucose oxidase method described previously.[1]

Solvent Perturbation Difference Spectrum——Solvent perturbation difference spectra were measured according to the procedure of Herskovitz and Laskowski[7] with ethylene glycol and polyethylene glycol as perturbants. The model compounds used were ATEE and *N*-acetyl-L-tyrosinamide. All spectra were measured with a Shimadzu UV-200 spectrophotometer.

NBS Oxidation——(a) NBS oxidation of ATEE. To 2.5 ml of an ATEE solution (0.16 mM in 0.1 M acetate buffer) kept at 25°C was added 10—20 μl of an NBS stock solution (5—80 mM), and the change in absorbancy at 280 nm was followed for 5 min. The rate of oxidation was estimated by two methods. (i) The rate was determined from the decrease in absorbance at 280 nm taking $\Delta\varepsilon_{280nm}$ to be 4200, according to the method of Spande and Witkop.[6] (ii) The residual ATEE was estimated from the fluorescence spectrum excited at 295 nm with a Shimadzu spectrofluorometer, RF 502. The sample concentration was 32 μM. (b) NBS oxidation of $Gluc_1$ and $Gluc_2$. To 1—3 ml of each enzyme solution (7—10 μM in 0.1 M acetate buffer) at 25°C was added 10—20 μl of an NBS solution (0.3—25 mM), and the change in absorbance at 280 nm was followed for 5 min. The number of tryptophan residues oxidized was also estimated by the fluorescence method described in "Amino Acid Analysis (c)."

Amino Acid Analysis——(a) Samples of 0.1—0.2 mg of protein were hydrolyzed in evacuated, sealed tubes with 6 N HCl at 110°C for 24 h. Amino acid analyses were performed by the method of Spackman *et al.*[8] with a Nihondenshi JEOL JLC-200A amino acid analyzer. (b) Sulfhydryl groups in protein were titrated with 5,5′-dithiobis(2-nitrobenzoic acid) according to the method of Ellman.[9] (c) The amount of remaining tryptophan residues was estimated by the method of Pajot.[10] Samples were dissolved in 6 M guanidine hydrochloride solution containing 30 mM 2-mercaptoethanol, and then the fluorescence intensity at 355 nm of each sample excited at 295 nm was measured with a Shimadzu RF 502 spectrofluorometer. Tryptophan residues were also estimated by the colorimetric method of Spies *et al.*[11]

Fluorescence Spectra of NBS-Oxidized Enzymes——Fluorescence spectra of NBS-oxidized $Gluc_1$ and $Gluc_2$ were measured at pH 5.0 and 25°C with a Shimadzu RF 502 spectrofluorometer. The excitation wavelength was 295 nm and the enzyme fluorescence was measured at 345 nm. The enzyme concentration used was 1.2 μM or less.

Measurement of CD Spectrum——CD spectra were measured with a JASCO J-40 spectropolarimeter at room temperature in cells of 0.05 and 1.0 cm light path for the wavelength regions of 200—250 and 250—310 nm, respectively. The enzyme concentrations used were 8.0—20 μM.

Binding of NBS-Oxidized $Gluc_1$ and $Gluc_2$ with Maltitol——Fluorescence spectra of NBS-oxidized $Gluc_1$ and $Gluc_2$ mixed with 0.5—10 mM maltitol were measured at pH 5.0. When the fluorescence intensity of the enzyme in the absence of maltitol is F and the decrease in the fluorescence intensity caused by the addition of maltitol is ΔF, the following equation is valid:

$$F/\Delta F = F/\Delta F_{max} \times (1 + K_d/[\text{maltitol}]),$$

where ΔF_{max} is the decrease in fluorescence intensity when the enzyme is saturated with maltitol. The dissociation constant, K_d, was determined according to this equation.

Results

Solvent Perturbation Difference Spectrum

$Gluc_1$ and $Gluc_2$ contain about 13 and 11 tryptophan residues, respectively.[1] To estimate the number of tryptophan residues on the surface of the enzymes, the solvent perturbation difference spectra of $Gluc_1$ and $Gluc_2$ were measured with ethylene glycol and polyethylene glycol as perturbants according to the method proposed by Herskovitz and Laskowski.[7] The results are shown in Table I. The numbers of tryptophan residues perturbed with ethylene glycol were about 7.7 and 6.5 for $Gluc_1$ and $Gluc_2$, respectively. When polyethylene glycol was used as a perturbant, about 3.9 and 3.1 tryptophan residues were perturbed for $Gluc_1$ and $Gluc_2$, respectively. The results indicate that in $Gluc_1$ one more tryptophan residue exists on the surface than in $Gluc_2$.

TABLE I. Tryptophan and Tyrosine Residues in $Gluc_1$ and $Gluc_2$ Perturbed with Ethylene Glycol and Polyethylene Glycol at pH 5.0

Amino acid	$Gluc_1$ Perturbant			$Gluc_2$ Perturbant		
	EG	PEG	Total	EG	PEG	Total
Tryptophan	7.7	3.9	13	6.5	3.1	11
Tyrosine	20.9	13.8	37	13.0	8.8	26

EG, ethylene glycol; PEG, polyethylene glycol.

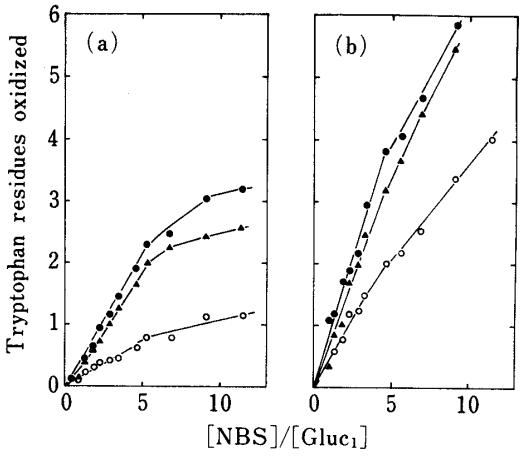

Fig. 1. NBS Oxidation of $Gluc_1$ at Various pH

The number of tryptophan residues oxidized was estimated from the decrease in absorbance at 280 nm in 5 min after mixing (a) and by colorimetry according to Spies and Chambers[11] (b). The pH of the reaction mixture was 4.5 (●), 5.0 (▲) or 6.0 (○).

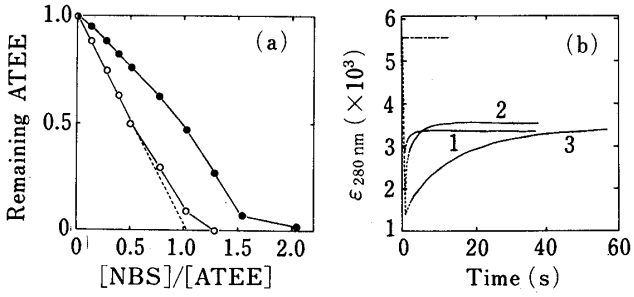

Fig. 2. NBS Oxidation of ATEE at 25 °C

(a) NBS oxidation at 25 °C. The concentration of ATEE was 0.16 mM. The rate of oxidation was measured in terms of the decrease in absorbance at 280 nm (●) and the decrease of fluorescence intensity in 6 M guanidine hydrochloride (○). (b) Time course of ATEE modification by NBS at pH 4.5, 5.0 and 6.0. ATEE (0.16 mM) dissolved in 0.1 M acetate buffer, pH 4.5 (1), pH 5.0 (2) or pH 6.0 (3), was modified by the addition of one mol eq of NBS. The change of the absorbance was measured at 280 nm. The dotted line indicates the presumed UV absorption change within 3 s after mixing of NBS. —·—, UV absorption of ATEE without addition of NBS.

NBS Oxidation of $Gluc_1$

The results of NBS oxidation of $Gluc_1$ at pH 4.5, 5.0 and 6.0 are shown in Fig. 1. The number of oxidized tryptophan residues was estimated from the absorbance at 280 nm (Fig. 1(a)). The efficiency of oxidation appeared to decrease with elevation of pH. To confirm the results, the numbers of oxidized tryptophan residues were estimated by colorimetry. The results are shown in Fig. 1(b). The numbers were much higher than those based on the absorbance decrease. The difference was most marked at pH 6.0.

NBS Oxidation of ATEE

To explain the results of NBS oxidation of $Gluc_1$, we reinvestigated the number of tryptophan residues oxidized with NBS by using ATEE as a model compound. The rate of oxidation of ATEE was calulated by two methods; from the decrease in absorbance at 280 nm and from the decrease in the fluorescence intensity. The results are shown in Fig. 2(a). The fluorescence intensity of ATEE was decreased almost to zero by reaction with about 1 mol eq of NBS. However, about 1.5—1.6 eq of NBS was apparently required for complete loss of

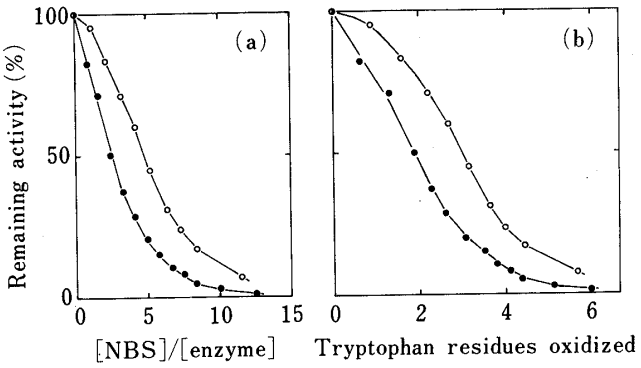

Fig. 3. Inactivation of $Gluc_2$ by Oxidation of Tryptophan Residues with NBS at pH 5.0 and 25 °C as Compared with That of $Gluc_1$

(a) Inactivation of $Gluc_1$ and $Gluc_2$ as a function of [NBS]/[enzyme] ratio. (b) Relation between the oxidation of tryptophan residues and enzymatic activity during the course of inactivation.
○, $Gluc_1$; ●, $Gluc_2$.

the indole moiety as judged from the ultraviolet (UV) absorption change at 280 nm. The different time courses of the reaction in the two methods may account for this discrepancy. When 1 eq of NBS was added to ATEE at pH 4.5, 5.0, and 6.0, a time-dependent decrease in the absorbance at 280 nm was observed. The results are shown in Fig. 2(b). The molar absorbance just after mixing of ATEE with NBS was about 1300 and subsequently rose gradually to about 3500. The increase in absorbance was slower at pH 6.0 than at pH 4.5. The results suggested that NBS oxidation of ATEE (resulting in fluorescence decrease) progressed very rapidly upon addition of equimolar NBS, *via* an intermediate having a molar absorbance of 1300, with little fluorescence. This intermediate appears to change time-dependently to a compound with a molar absorbance of 3500. Further oxidation of this compound by NBS forms a compound having ε_{280nm} of about 1300 again. The final $\Delta\varepsilon_{280nm}$ was about 4200. This value is the same as the $\Delta\varepsilon_{280nm}$ value for tryptophan reported by Spande *et al.*[6] Therefore, we concluded that the number of tryptophan residues oxidized in $Gluc_1$ or $Gluc_2$ could be more accurately estimated by fluorometry or colorimetry with DBA, because of the complex absorption changes due to the different states of tryptophan residues in the case of absorption measurement.

NBS Oxidations of $Gluc_1$ and $Gluc_2$

The results of NBS oxidation of $Gluc_1$ and $Gluc_2$ at pH 5.0 are shown in Fig. 3. $Gluc_1$ and $Gluc_2$ were inactivated by the modification of about 5 and 4 tryptophan residues, respectively. It seems that $Gluc_2$ lacks one rapidly oxidizable tryptophan residue in $Gluc_1$. The oxidation of the first two tryptophan residues in $Gluc_1$ had less effect in decreasing the enzymatic activity.

Amino Acid Compositions of NBS-Oxidized $Gluc_1$ and $Gluc_2$

The amino acid compositions of NBS-oxidized $Gluc_1$ and $Gluc_2$ were measured. The remaining activities of these samples measured with soluble starch as a substrate were 19% and 15% for NBS-oxidized $Gluc_1$ and $Gluc_2$, respectively. No decrease in tyrosine or cysteine content was found within the experimental error for either of the enzymes (data not shown). Thus, inactivation of the enzymes might be due to the modification of tryptophan residues.

CD Spectra of NBS-Oxidized $Gluc_1$ and $Gluc_2$

The CD spectra of NBS-oxidized $Gluc_1$ and $Gluc_2$ are shown in Fig. 4. The CD bands from 250 to 310 nm, which were attributed to the aromatic side chains of the protein, shifted gradually in the negative direction with the progress of NBS oxidation, but the magnitude of shift of $Gluc_2$ was greater than that of $Gluc_1$. The CD band in the region from 270 to 310 nm was nearly unchanged with the oxidation of up to about two tryptophan residues in $Gluc_1$ and therefore, the tryptophan residue which exists on the surface of the N-terminal part of $Gluc_1$ seems to be one of these two tryptophan residues and seems to contribute little to the CD band. On the other hand, the CD spectra in the short wavelength region (from 200 to 250 nm),

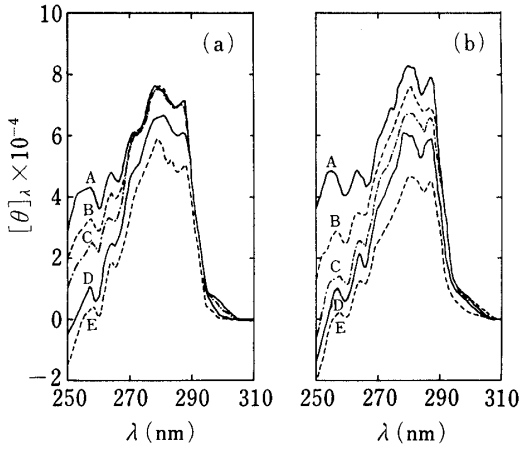

Fig. 4. CD Spectra of $Gluc_1$ and $Gluc_2$ Oxidized with NBS at pH 5.0

(a) $Gluc_1$. [NBS]/[$Gluc_1$] ratios are 0 (A), 2.1 (B), 3.1 (C), 3.2 (D) and 7.3 (E), respectively. (b) $Gluc_2$. [NBS]/[$Gluc_2$] ratios are 0 (A), 1.7 (B), 3.3 (C), 5.0 (D) and 6.7 (E), respectively.

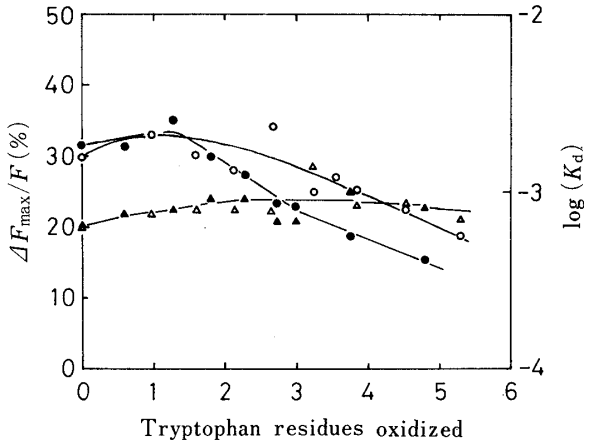

Fig. 5. Fluorescence Quenching and K_d Values of $Gluc_1$ and $Gluc_2$ on Binding with Maltitol as a Function of Tryptophan Modification

The ratio of fluorescence quenching, $\Delta F_{max}/F$, was measured for various tryptophan-modified glucoamylases.

○, $\Delta F_{max}/F$ for $Gluc_1$; ●, $\Delta F_{max}/F$ for $Gluc_2$. The K_d values of $Gluc_1$ (△) and $Gluc_2$ (▲) modified with various concentrations of NBS were calculated. The experimental conditions were as described in Materials and Methods.

which reflects the backbone conformation of the enzyme, were nearly unchanged by NBS oxidation, indicating that NBS oxidation may not produce any conformational change.

Binding of Maltitol with NBS-Oxidized $Gluc_1$ and $Gluc_2$

It has been reported that glucoamylases produce UV difference spectra[12] and a fluorescence quenching [13] attributable to tryptophan residues when substrates bind to them. Similar difference spectra were observed for $Gluc_1$ and $Gluc_2$ upon binding with maltitol, a substrate analogue. Decreases in the fluorescence intensity on binding with maltitol due to NBS oxidation are shown in Fig. 5. When about 5 tryptophan residues were oxidized and the enzymes were inactivated almost completely, the K_d values for maltitol binding with $Gluc_1$ and $Gluc_2$ were only slightly affected. The extent of the quenching was nearly constant until about 2 tryptophan residues were oxidized for both $Gluc_1$ and $Gluc_2$. When about 5 tryptophan residues were oxidized, about 54% and 49% of the fluorescence intensity of $Gluc_1$ and $Gluc_2$ remained, respectively. Therefore, the maltitol-binding ability seems to be decreased more slowly by NBS oxidation than the glucoamylase activity.

Discussion

The possible contribution of tryptophan residue(s) to the activity of glucoamylase has been reported for a glucoamylase from *Rhizopus niveus*[5] and for a glucoamylase from *A. saitoi*.[14] It was also reported that these glucoamylases had two rapidly oxidizable tryptophan residues which were little related with the enzymatic activity, and that one of the tryptophan residues oxidized following the two tryptophan residues mentioned above was important for the glucoamylase activity.

The amount of oxidized tryptophan residues was usually estimated from the decrease in absorbance at 280 nm according to the method of Spande and Witkop.[6] However, as mentioned above, the number of oxidized tryptophan residues in $Gluc_1$ estimated by this method was very much less than that estimated by the colorimetric method. Therefore, we

reinvestigated the NBS oxidation of indole derivatives using ATEE as a model compound. It appeared from the results that the decrease in absorbance at 280 nm caused by NBS oxidation did not accurately reflect the extent of the modification of tryptophan residues. The results of the present study indicate the involvement of at least two intermediates during the course of NBS oxidation. Namely, addition of 1 eq of NBS caused the absorbance at 280 nm of tryptophan residue to decrease rapidly with a concomitant decrease in tryptophan fluorescence. The second rather slow step was accompanied with an increase of UV absorbance up to a molar extinction coefficient of about 3500, but with no increase of the fluorescence, and the further addition of NBS caused a decrease to about 1300 again. The final $\Delta\varepsilon$ was 4200. The first step may be a bromination step and the latter two may be oxidation steps. If protein has several tryptophan residues which are in different states, the changes in OD_{280nm} due to NBS oxidation of individual tryptophan residues may be in different directions (decrease or increase). Thus, the estimated values of oxidized tryptophan residues are less than the actual values.

$Gluc_1$ has one more tryptophan residue on the surface of the molecule than $Gluc_2$ does. These glucoamylases seemed to be inactivated by oxidation of all tryptophan residues on the surface of the molecules (those which could be perturbed by polyethylene glycol). About 80% of $Gluc_1$ activity remained when the first two tryptophan residues were oxidized. When we compare the residual activity–tryptophan residue relations for $Gluc_1$ and $Gluc_2$ (Fig. 3), the tryptophan residue which exists on the surface of the N-terminal peptide region of $Gluc_1$ but is lacking in $Gluc_2$ appears to be the most reactive tryptophan residue towards NBS and to be essentially unrelated to the glucoamylase activity. The other reactive tryptophan residue is in the $Gluc_2$ part of the $Gluc_1$ molecule. The essential tryptophan residue(s) in $Gluc_1$ is included among the three tryptophan residues oxidized next. Since the inactivation curve of $Gluc_2$ is slightly convex, the essential tryptophan residue is thought to be the 3rd or 4th most reactive tryptophan residue in $Gluc_1$.

$Gluc_1$ and $Gluc_2$, in which about five tryptophan residues were oxidized, showed about half of the fluorescence intensity of the native enzymes. The results of fluorescence quenching by binding with maltitol may indicate that in addition to the tryptophan residue(s) that is related to the enzymatic activity and is oxidized rapidly, another tryptophan residue(s) may participate to the binding with maltitol. The latter tryptophan residue(s) is fairly resistant to NBS oxidation as compared with the former.

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