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Differentiation of Two Types of Tryptophan Residues Related to Enzymatic Activity in a Glucoamylase from a *Rhizopus* sp. by *N*-Bromosuccinimide Oxidation in the Presence of Tris(hydroxymethyl)aminomethane and Maltitol

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In order to differentiate tryptophan residues in the active site of a major glucoamylase from a *Rhizopus* sp. ($Gluc_1$), *N*-bromosuccinimide (NBS) oxidation of $Gluc_1$ in the presence of maltitol and tris(hydroxymethyl)aminomethane (Tris) was studied.

1. When $Gluc_1$ was oxidized in the presence of maltitol, the hydrolytic activities of $Gluc_1$ towards both soluble starch and *p*-nitrophenyl α-D-glucopyranoside (PNPG) were scarcely protected from NBS oxidation. On the other hand, when $Gluc_1$ was oxidized in the presence of Tris, the activity towards PNPG increased due to NBS oxidation of about 2 mol of tryptophan residues, whereas the activity towards maltose or soluble starch decreased.
2. The fluorescence quenching and the ultraviolet (UV) difference spectrum of $Gluc_1$ induced by the addition of maltitol decreased with NBS oxidation. The decreases were small in the case of $Gluc_1$ oxidized in the presence of maltitol, but were large and nearly parallel with the decrease in the activity towards maltose or soluble starch when $Gluc_1$ was oxidized in the presence of Tris.
3. It seems that at least two tryptophan residues exist in the catalytic locus of the enzyme active site, and one of them is related to the binding with a glucose moiety of substrates such as maltose but is scarcely related to the binding with the *p*-nitrophenyl moiety of PNPG. Another tryptophan residue seemed to be involved mainly in the catalytic action and to contribute little to maltose binding.

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

Three forms of glucoamylase [EC 3.2.1.3] have been purified from a *Rhizopus* species by Takahashi *et al.*[1] As regards the active site of the major glucoamylase, $Gluc_1$, the participation of carboxyl group(s) and tryptophan residue(s) was proved by chemical modification with 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide[2] and *N*-bromosuccinimide (NBS),[3] respectively. The inactivation of $Gluc_1$ proceeded with the oxidation of about five tryptophan residues. However, even when the enzymatic activity of $Gluc_1$ was destroyed by NBS oxidation, the binding ability of $Gluc_1$ with maltitol was fairly well retained, because fluorescence quenching of tryptophan residues induced by the addition of maltitol was quite well retained.[3] Therefore, we expected that $Gluc_1$ has at least two tryptophan residues in or near the enzyme active site. On the other hand, we found that aminoalcohol derivatives inhibited glucoamylases, and the mode of inhibition by aminoalcohols was different from that by substrate analogues such as maltitol.[4]

In this work, in order to differentiate two types of tryptophan residues in the active site of $Gluc_1$, NBS oxidation of $Gluc_1$ in the presence of two inhibitors, maltitol and tris(hydroxymethyl)aminomethane (Tris), was studied.

Materials and Methods

Chemicals——Soluble starch was purchased from Wako Pure Chemicals (Osaka) and used as a substrate after

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exhaustive dialysis against distilled water. *p*-Nitrophenyl α-D-glucopyranoside (PNPG) was obtained from Koch-Light Lab. Ltd. (Colnbrook, Bucks, England). Maltose was the product of Wako Pure Chemicals. Maltitol and Tris were purchased from Tokyo Kasei Kogyo (Tokyo) and Sigma (St. Louis, Mo., U.S.A), respectively. NBS was obtained from Nakarai Chemicals (Kyoto) and recrystallized from hot water. All other chemicals were of reagent grade.

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

Determination of Glucoamylase Activity——(a) The enzymatic activity with soluble starch as a substrate was measured at pH 5.0 and 37 °C as described previously.[1] The glucose released was measured by using the "Glucose-C-Test" (Wako Pure Chem.). (b) The enzymatic activity with PNPG as a substrate was measured as follows. The substrate (10 mM) in 1 ml of acetate buffer (0.1 M, pH 5.0) was hydrolyzed with 20—50 μl of enzyme solution at 37 °C. The reaction was terminated by heating the mixture at 100 °C for 1 min. *p*-Nitrophenol released was measured in terms of the increase in absorbancy at 400 nm after addition of 0.5 ml of 0.2 N NaOH to the reaction mixture.

NBS Oxidation of $Gluc_1$——$Gluc_1$ was oxidized at 25 °C and pH 5.0 according to the method reported previously.[3] In the case of NBS oxidation in the presence of inhibitors, specified concentrations of inhibitors were previously mixed with $Gluc_1$ solution, and then NBS oxidation was performed. All parameters for oxidized $Gluc_1$ were measured after dialysis of the reaction mixture. The number of tryptophan residues oxidized was estimated from the fluorescence intensity of NBS-oxidized $Gluc_1$ in 6 M guanidine hydrochloride, according to the method of Pajot.[5]

Estimation of NBS-Oxidized $Gluc_1$ Concentration——The concentration of $Gluc_1$ was estimated by assuming $A_{\mathrm{cm}}^{280\,\mathrm{nm}}$ (1%) to be 13.2.[1] NBS-oxidized $Gluc_1$ concentrations after dialysis were estimated from the ultraviolet (UV) absorption at 280 nm, which was corrected for the decrease in the UV absorbancy due to NBS oxidation before dialysis.

Fluorescence Intensity of NBS-Oxidized $Gluc_1$——Fluorescence intensities of NBS-oxidized $Gluc_1$ were measured at pH 5.0 (0.1 M acetate buffer) with a Shimadzu RF-502 spectrofluorometer. The enzyme fluorescence was measured at 345 nm with excitation at 295 nm. The enzyme concentration used was 0.6—1.2 μM. The fluorescence quenching of NBS-oxidized $Gluc_1$ induced by the addition of 0.5—10 mM maltitol was measured and the maximum quenching value, ΔF_{max}, was calculated as the value obtained when $Gluc_1$ was saturated with maltitol.

Difference Absorption Spectra of NBS-Oxidized $Gluc_1$ Induced by Addition of Maltitol——The UV difference spectra of NBS-oxidized $Gluc_1$ induced by the addition of 5 mM maltitol were measured at pH 5.0 (0.1 M acetate buffer) and 25 °C with a Shimadzu UV-240 spectrophotometer using tandem cells.

Measurement of Kinetic Parameters——The kinetic parameters (k_0, K_m) of NBS-oxidized $Gluc_1$ were determined with PNPG as a substrate at pH 5.0 and 37 °C. PNPG concentrations were 1.5—10 mM. Aliquots of 50 μl of 4—9 μM enzyme were used for assays. Other conditions were the same as those described in Determination of Glucoamylase Activity.

Results

NBS Oxidation of $Gluc_1$ in the Presence of Inhibitors

$Gluc_1$ was oxidized with various amounts of NBS in the presence and absence of maltitol (0.6 M) or Tris (10 mM) at pH 5.0 and 25 °C. As shown in Fig. 1, in which the remaining activity was measured with soluble startch, maltitol slightly prevented $Gluc_1$ inactivation by NBS oxidation. On the other hand, in the presence of Tris, $Gluc_1$ was inactivated with oxidation of

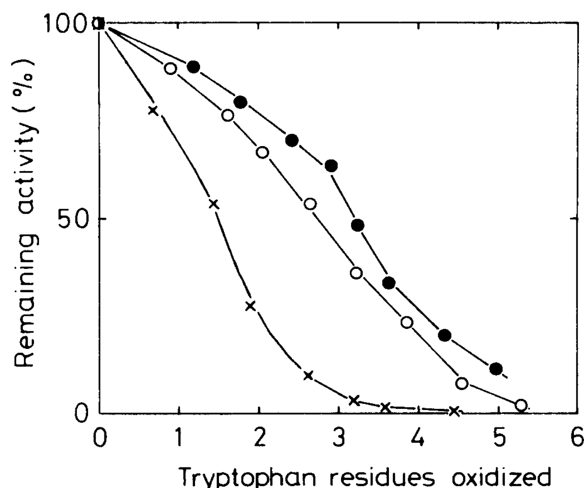

Fig. 1. NBS Oxidation of $Gluc_1$ in the Presence of 0.6 M Maltitol (●), 10 mM Tris (×), and No Inhibitor (○)

The enzyme activity was measured with soluble starch, as described in Materials and Methods.

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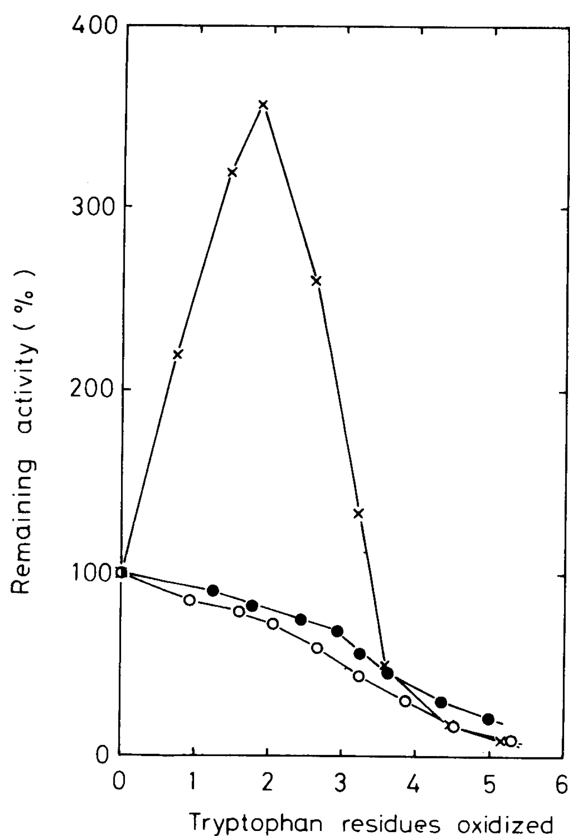

Fig. 2. The Activity of NBS-Oxidized Gluc Was Measured with 10 mM PNPG

The enzymatic activity was measured with PNPG, as described in Materials and Methods. The symbols are the same as Fig. 1.

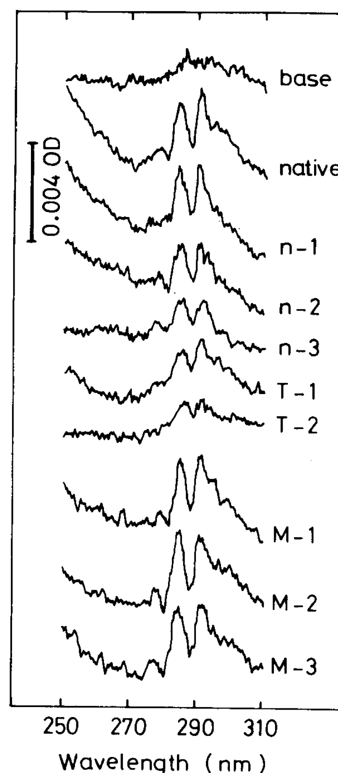

Fig. 3. The Difference UV Spectra of NBS-Oxidized $Gluc_1$ Induced by the Addition of 5 mM Maltitol

The enzyme concentration was 8.7 μM. The samples were the same as for Table I.

a smaller amount of tryptophan residues; the oxidation of about 3 tryptophan residues decreased the enzymatic activity to almost zero. These results indicated that the reactivities of tryptophan residues which were related to the glucoamylase activity seemed to be enhanced by Tris.

Enzymatic Activity of NBS-Oxidized $Gluc_1$ Measured with PNPG as a Substrate

The activity of NBS-oxidized $Gluc_1$ was measured with 10 mM PNPG (Fig. 2). The samples were the same as those used for Fig. 1. The results of NBS oxidation of $Gluc_1$ with and without maltitol were nearly the same as in Fig. 1, but the remaining activity of $Gluc_1$ oxidized in the presence of Tris was very different from that measured with soluble starch. PNPG-hydrolyzing activity was remarkably increased by oxidation of tryptophan residues up to about two, and decreased subsequently.

Fluorescence Quenching of NBS-Oxidized $Gluc_1$ on Binding of Maltitol

The fluorescence spectra of $Gluc_1$ oxidized in the presence and absence of maltitol or Tris were measured at pH 5.0. On addition of maltitol, the fluorescence intensity of oxidized $Gluc_1$ decreased, as was the case for native $Gluc_1$ (Table I). The fluorescence quenching observed on addition of maltitol to the $Gluc_1$ oxidized in the presence of maltitol, and having 10% residual activity, is about 70% of ΔF_{max} (fluorescence quenching) obtained with the native enzyme, whereas $Gluc_1$ oxidized in the absence of maltitol, showed about 50% of ΔF_{max}. In the case of $Gluc_1$ oxidized in the presence of Tris, the ΔF_{max} value decreased nearly in parallel with the remaining activity measured with soluble starch. Therefore, it could be concluded that in the

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TABLE I. Fluorescence Intensity of NBS-Oxidized $Gluc_1$

Sample[a]	Tryptophan residues oxidized	Remaining activity[b]	F (arbitrary unit)	$\Delta F_{max}/F_0$[c] (%)
Native	0	100	100	31.7
n-1	0.76	83.9	92.7	30.6
n-2	3.58	31.9	74.3	20.6
n-3	4.10	9.0	66.7	16.7
M-1	1.74	71.7	93.0	29.5
M-2	3.76	36.5	77.4	25.5
M-3	4.28	10.5	61.4	22.7
T-1	1.45	47.6	83.7	18.1
T-2	4.07	5.5	63.5	3.4
T-3	4.41	0.6	50.0	—

a) $Gluc_1$ was oxidized in the absence of inhibitor (n-1, n-2, and n-3) and in the presence of 0.6 M maltitol (M-1, M-2, and M-3) and 10 mM Tris (T-1, T-2, and T-3). *b*) Remaining activity of NBS-oxidized $Gluc_1$ was measured with soluble starch. The activity of the native enzyme was taken as 100. *c*) The fluorescence quenching induced by the addition of maltitol. $\Delta F_{max}/F_0$ is the ratio of ΔF_{max} of each sample to the fluorescence intensity of the native enzyme, F_0.

TABLE II. Kinetic Parameters of NBS-Oxidized $Gluc_1$ with PNPG as a Substrate

Sample[a]	$K_m \times 10^3$ (M)	$k_0 \times 10^2$ (s^{-1})	k_0/K_m ($s^{-1} \cdot M^{-1}$)
Native	2.50	10.4	41.4
n-1	2.59	8.74	33.8
n-2	2.86	4.57	16.0
n-3	4.00	2.16	5.4
M-1	2.45	7.92	32.4
M-2	2.75	4.85	17.7
M-3	3.00	2.46	8.2
T-1	15.2	74.3	48.9
T-2	23.3	38.3	16.5
T-3	20.4	10.7	5.2

a) The abbreviations of the enzyme samples are the same as for Table I.

presence of Tris, the rate of oxidation of tryptophan residue(s) which interacts with maltitol is markedly enhanced.

Difference Spectrum of NBS-Oxidized $Gluc_1$ Induced by Addition of Maltitol

$Gluc_1$ produces a typical difference spectrum upon binding with substrates or analogues.[2] The difference UV spectra of NBS-oxidized $Gluc_1$ induced by the addition of maltitol (5 mM) are shown in Fig. 3. The magnitude of the difference spectra decreased with NBS oxidation. In particular, the extent of the decrease was remarkable in the case of $Gluc_1$ oxidized in the presence of Tris. On the other hand, the magnitude of the difference spectra of $Gluc_1$ oxidized in the presence of maltitol was little altered. These results agreed well with those on the decrease in fluorescence quenching, as shown in Table I.

Kinetic Parameters of NBS-Oxidized $Gluc_1$

The kinetic parameters of NBS-oxidized $Gluc_1$ were measured with PNPG at pH 5.0 and 37 °C. The results are shown in Table II. When $Gluc_1$ was oxidized in the absence of inhibitor, the k_0 value decreased with NBS oxidation and the K_m value increased a little. When $Gluc_1$

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was oxidized in the presence of maltitol, the changes in the k_0 and K_m values were very similar to those in the case of $Gluc_1$ oxidized in the absence of maltitol. On the other hand, when $Gluc_1$ was oxidized in the presence of Tris, the K_m value increased greatly, and the k_0 value also increased. As compared with the results shown in Fig. 2, the k_0 value may increase with the oxidation of up to about two tryptophan residues. When the kinetic parameters of NBS-oxidized $Gluc_1$ were measured with maltose, the K_m values were nearly constant among all the samples tested and it seemed that the decreasing activity with NBS oxidation was owing to the decreasing k_0 values (data not shown). These results indicated that the PNPG-hydrolyzing activity of $Gluc_1$ was protected from NBS oxidation by Tris but not by maltitol.

Discussion

In this work, we oxidized $Gluc_1$ with NBS in the presence of two inhibitors, maltitol and Tris, whose mechanisms of inhibition are different from each other,[4] and it became clear that there were two types of tryptophan residues in $Gluc_1$ related to the glucoamylase activity.

When $Gluc_1$ was oxidized with NBS in the presence of maltitol, maltitol showed a little protective effect against the inactivation of $Gluc_1$. The fluorescence quenching and the difference UV spectrum induced by binding with maltitol indicated that the maltitol-binding ability of $Gluc_1$ oxidized in the presence of maltitol, and having about 10% activity, was fairly well retained. Because maltitol mostly binds to subsites 2 and 3 of glucoamylases,[6,7] the tryptophan residue which can be protected by maltitol from NBS oxidation probably exists in subsite 2 or 3, and may contribute to the binding of substrates such as maltose. It seems that the fluorescence quenching and the difference UV absorption induced by binding of substrates or analogues are mostly caused by this tryptophan residue. In this case, therefore, the decrease in enzymatic activity at the early stage of oxidation is due to the catalytic (=PNPG-hydrolyzing activity) site tryptophan.

On the other hand, when $Gluc_1$ was oxidized with NBS in the presence of Tris, which might interact through the amino group with the carboxyl group of $Gluc_1$ involved in PNPG-hydrolyzing activity, the catalytic site tryptophan residue was protected from oxidation. Therefore, a tryptophan residue(s) in the maltitol binding site (subsite 2 and subsite 3) which contributes to the enzymatic activity of $Gluc_1$ towards soluble starch was destroyed more markedly than in the absence of Tris. This tryptophan residue contributes to the binding with a glucose moiety, but the binding affinity of the subsite with the *p*-nitrophenyl group might be unchanged by oxidation of this tryptophan residue. Therefore, the productive binding mode of PNPG in subsites 1 and 2 might be increased relatively by NBS oxidation, and thus the PNPG-hydrolyzing activity is increased. The two tryptophan residues oxidized first in the absence of inhibitor seemed not to be related to the enzymatic activity. However, in the presence of Tris (Fig. 1), an almost linear decrease in enzymatic activity with the progress of tryptophan oxidation was observed. The results may indicate a decrease in the oxidation rates of tryptophan residues which are not related to enzymatic activity, probably induced by the interaction of Tris with subsite 1. Hiromi *et al.* reported that a tryptophan residue which induced the fluoresence quenching upon binding of substrates might exist in subsite 1 of the glucoamylase from *Rhizopus niveus*, because gluconolactone was a mixed-type inhibitor of the glucoamylase and bound with subsite 1, causing a fluorescence quenching.[8,9] We suggested that this tryptophan residue may exist in subsite 2. The discrepancy could be explained by assuming that the tryptophan residue is located in subsite 2, though very close to subsite 1, and is consequently affected by gluconolactone, which binds with subsite 1.

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