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ENZYMIC AND PHYSICOCHEMICAL PROPERTIES OF AN EXO-(1→3)-β-D-GLUCANASE FROM *Rhizoctonia solani*

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ABSTRACT

An exo-(1→3)-β-D-glucanase, isolated from a commercial lytic enzyme preparation "kitalase", had mol. wt. 74,000, isoelectric point pH 8.1, optimum pH 5.6, optimum temperature 53°, pH stability 5.0–8.0, and temperature stability up to 65°. The enzyme contained 703 amino acids, including 10 Cys, 7 Trp, 11 His, 83 Asp (Asn), and 51 Glu (Gln). The activity was inhibited on modification of the tryptophan, histidine, or carboxyl residues. Oxidation of Trp was inhibited by the addition of substrate. The enzyme released only glucose from a (1→3)-β-D-glucan, glucose and gentiobiose from a 6-branched (1→3)-β-D-glucan, and had no effect on gentiobiose and methyl α- and β-D-glucopyranosides. For the series of laminari-oligosaccharides G3–G7, the relative velocities of reaction compared to that of laminarin were G7:G6:G5:G4:G3 = 77:65:54:34:3. The enzyme acted on the glucan that had the reducing end modified but not that with the non-reducing end modified. The glucose residues liberated were α. The enzyme is suitable for use in the structural characterization of (1→3)-β-D-glucans.

INTRODUCTION

Many kinds of (1→3)-β-D-glucans have been investigated and some, such as lentinan[1] and schizophyllan[2], have been used for cancer immunotherapy. Antitumor activity resulted from the activation of the host immune system. We have isolated and characterized the antitumor (1→3)-β-D-glucans grifolan from *Grifola frondosa*[3–6] and SSG from *Sclerotinia sclerotiorum*[7–9].

(1→3)-β-D-Glucanases have various applications such as cell-wall lytic enzymes for the preparation of protoplasts and in the determination of the structure of yeast cell walls and polysaccharides. They have been isolated from various microbial sources, and their properties and mode of action have been investigated[10–14]. However, little information is available concerning the active site of these enzymes[15–19].

Identification of the amino acid residues involved in the enzyme activity and

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analysis of ultrastructure are important in the elucidation of the hydrolytic mechanisms of enzymes. The involvement of carboxyl and histidine residues in the active site of the endo-(1→3)-β-D-glucanase from *Frav. dormitator var. glucanolyticae* FA-5[15], and histidine and tryptophan residues in the catalytic site of the exo-(1→3)-β-D-glucanase from *Basidiomycete* QM806[17], *Mucor hiemalis*[18], and of a commercial (1→3)-β-D-glucanase preparation zymolyase[19], have been investigated.

Kitalase is a commercial lytic enzyme preparation obtained from the culture filtrate of *Rhizoctonia solani*[10,11], and we now report on an exo-(1→3)-β-D-glucanase isolated therefrom.

EXPERIMENTAL

Materials. — Kitalase was purchased from Keiai Kasei Co., Ltd., and laminarin (from *Laminaria digitata*) was from Sigma. Grifolan LE and SSG were purified from *Grifola frondosa*[4] and *Sclerotinia sclerotiorum*[7], respectively. Islandican [(1→6)-β-D-glucan] was prepared by alkaline hydrolysis of the luteic acid produced by *Penicillium islandicum*[20]. Low-molecular-weight (1→3)-β-D-glucans, such as small-LE (mol. wt. 20,000) and small-SSG (mol. wt. 40 000), were prepared from grifolan LE and SSG by mild acid hydrolysis. Laminari-oligosaccharides (d.p. 2–7) were purchased from Seikagaku Kogyo Co. Ltd.

Enzyme assay. — The reaction mixture containing 0.2% of laminarin, 0.1M phosphate buffer (pH 5.6), and enzyme solution was kept for 30 min at 40°, and 1 unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar as glucose. The reducing sugar produced was determined by literature methods[21,22].

Purification of (1→3)-β-D-glucanase. — A solution of kitalase (50 g) in 10mM phosphate buffer (50 mL, pH 5.0) was centrifuged (2,500 r.p.m., 10 min) and the supernatant solution was applied to a column (3.0 × 25 cm) of SP-Sephadex C-50 equilibrated with 10mM phosphate buffer (pH 5.0). After washing with the same buffer, the column was eluted with a linear gradient of 10 → 30mM phosphate buffer (pH 5.0). The fractions that contained (1→3)-β-D-glucanase activities were combined, concentrated by ultrafiltration (PM-10, Amicon), equilibrated with 10mM phosphate buffer (pH 6.8) by dialysis, added to a column (2 × 10 cm) of hydroxyapatite that had been washed with the starting buffer, and eluted with a linear gradient of 0.01→0.1M phosphate buffer (pH 6.8). Fractions containing (1→3)-β-D-glucanase were concentrated as above, and subjected to chromatofocusing using a column (1 × 50 cm) of PBE-94 (Pharmacia) equilibrated with 25mM ethanolamine–HCl (pH 9.4) and eluted by polybuffer 96-HCl (pH 7.4) (300 mL). The concentrated glucanase fraction was eluted from a column (2.5 × 90 cm) of Bio-Gel P-100 equilibrated with 0.1M phosphate buffer (pH 5.6). The glucanase activity was eluted as a single protein peak, and this fraction gave a single band in SDS–polyacrylamide gel electrophoresis.

General properties of the enzyme. — *(a) Optimum pH.* Acetate, phosphate, and Tris-HCl buffers (50mM) in the pH ranges 3.5–6.5, 6.0–7.5, and 7.0–9.0, respectively, were used.

(b) pH Stability. The enzyme was treated with each of the buffers (0.1M) in (*a*) for 1 h at 40° and the residual activity was determined under the standard conditions.

(c) Temperature stability. The residual activity of the enzyme was determined under the standard conditions after the treatment for 30 min in the temperature range 30–70°.

Amino acid analysis. — Enzyme protein (0.2–0.5 mg) was hydrolyzed with 6M HCl in evacuated, sealed tubes at 110°. The hydrolyzates were analyzed[23] after 24, 48, and 72 h with a Hitachi L-8500 amino acid analyzer. The content of tryptophan was measured[24] with a fluorescence spectrometer.

Chemical modification. — *(a) By 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodi-imide methotoluene-*p*-sulfonate* (CMC)[25,26]. — A solution of the enzyme (0.2 mg) in 0.1M 3-(*N*-morpholino)propanesulfonic acid (1 mL, pH 5.0) was treated with 0.04M CMC for 1 h at room temperature. Aliquots, withdrawn at intervals, were added to acetate buffer (1 mL, pH 5.0) in order to stop the reaction.

(b) By cyclohexane-1,2-dione[27]. A solution of the enzyme (0.2 mg) in 0.2M borate buffer (1 mL, pH 8.0) was treated with 50mM cyclohexane-1,2-dione for 1 h at 40°.

(c) By Pyrocarbonate (DEP)[28]. DEP was added (to 5mM) to a solution of the enzyme in 0.05M phosphate buffer (pH 6.0), and the mixture was kept for 1 h at room temperature.

(d) By N*-bromosuccinimide (NBS)*[29,30]. NBS was added to 0.5mM to a solution of the enzyme in 0.1M phosphate buffer (pH 5.6), and the mixture was kept for 30 min at 0°.

(e) Other chemical modifications. Treatments with succinic anhydride[31], acetic anhydride[32], and 2,4-dinitrobenzenesulfonic acid[33] for amide, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB)[34] for sulfhydryl, and *p*-toluenesulfonchloramide (chloramine T)[35] for methionine (in 0.1M Tris-HCl buffer) were performed by the literature methods.

Determination of anomeric configuration of the D*-glucose liberated by the enzyme.* — H.p.l.c. with an amide-NH_2 column (Tosoh Co., Ltd.), with a post-column reaction apparatus[36], was used.

Other methods. — Protein contents were determined either by the method of Lowry *et al.*[37] (with bovine serum albumin as a standard) or by measuring the absorbance at 280 nm.

RESULTS AND DISCUSSION

The steps for enzyme purification of the enzyme from Kitalase are summarized in Table I. The purified enzyme gave one band in SDS–polyacrylamide

TABLE I

PURIFICATION OF THE EXO-(1→3)-β-D-GLUCANASE FROM KITALASE[a]

Step	*Total protein (mg)*	*Total activity ($U \times 10^{-3}$)*	*Specific activity (U/mg)*	*Yield (%)*
Centrifugation	5110.0	396.5	77.6	100.0
SP-Sephadex C50	42.4	37.4	883.1	8.3
Hydroxyapatite	10.0	10.9	1001.0	2.0
Chromatofocusing	1.2	4.2	3576.3	0.2
Biogel P100	1.0	4.0	4135.4	0.2

[a]See Experimental.

gel electrophoresis (SDS–PAGE), and the molecular weight was estimated to be 74,000 by SDS–PAGE using the oligomeric forms of cytochrome C as molecular weight markers (Fig. 1). The isoelectric point of the enzyme was estimated to be pH ~8.1 from the elution profiles on chromatofocusing during the purification step (Fig. 2). The maximum activity was obtained at 5.6, and the enzyme was stable in the pH range 5.0–8.0 when heated for 30 min at 40° (Fig. 3). Heat inactivation of the enzyme occurred above 53° and activity was almost lost at 65°. The enzyme was inhibited moderately by Pb^{2+}, Zn^{2+}, Fe^{3+}, Sn^{2+}, Cu^{2+}, and Al^{3+} (10mM), and strongly by Hg^{+} (0.1mM (Table II).

Table III shows the action of the enzyme on various glucans or glucosides. The enzyme was specific for β-(1→3) linkages in such glucans as laminarin, curdlan, grifolan, and SSG.

Fig. 4 shows the effect of the enzyme on grifolan, a (1→3)-β-D-glucan having a β-D-glucosyl branch at position 6 of every third unit; glucose and gentiobiose (identified by t.l.c.) were produced without the accumulation of intermediates. Curdlan, a linear (1→3)-β-D-glucan gave only glucose during the hydrolysis (data

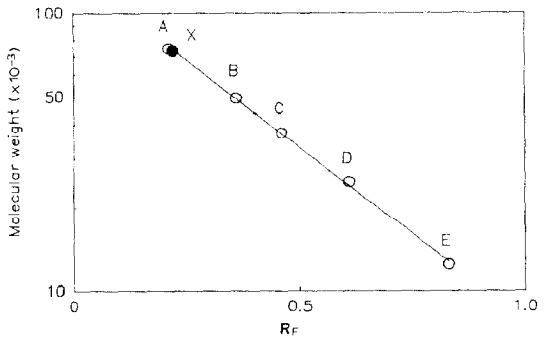

Fig. 1. Molecular weight of the (1→3)-β-D-glucanase determined by SDS–PAGE. Marker proteins were A, cytochrome C hexamer (mol. wt. 74 400); B, tetramer (49 600); C, trimer (37 200); D, dimer (24 800); E, monomer (12 400); X, (1→3)-β-D-glucanase.

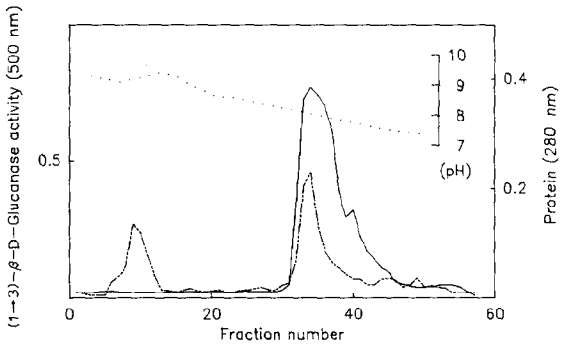

Fig. 2. Chromatofocusing of the (1→3)-β-D-glucanase (see Experimental): protein A_{280}, ··—··—; (1→3)-β-D-glucanase activity A_{500}, ——; pH ----.

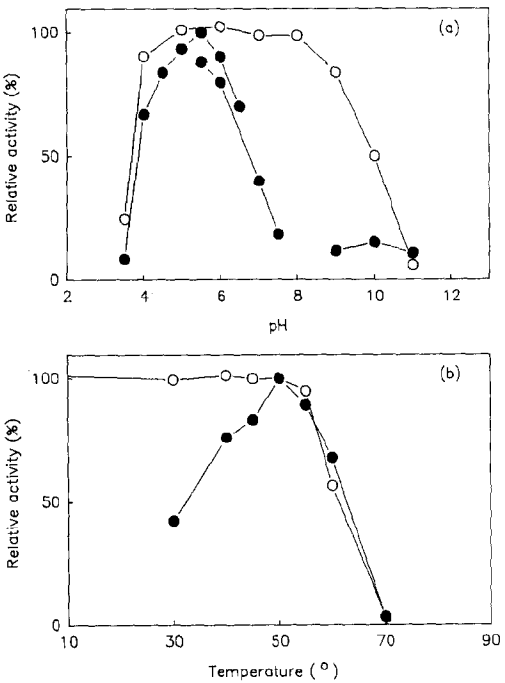

Fig. 3. Effect of temperature and pH on the activity (—●—) and stability (—○—) of the (1→3)-β-D-glucanase (see Experimental).

not shown). The enzyme hydrolyzed borohydride-reduced laminarin but not periodate-oxidized laminarin (Table III). The rates of hydrolysis of partially hydrolyzed curdlan, grifolan, and SSG were faster than those of the parent glucans. It is concluded that the enzyme hydrolyzed glucosyl groups from the (1→3)-β-D-linked glucans exclusively from the non-reducing end and that the released glucose was α (Fig. 5).

TABLE II

EFFECT OF METAL IONS ON ACTIVITY OF THE (1→3)-β-D-GLUCANASE[a]

Metal ions	*Relative activity (%)*		
	10^{-2}M	10^{-3}M	10^{-4}M
$CuCl_2$	12.2	84.5	94.1
$Pb(OAc)_2$	48.0	106.3	107.6
$ZnSO_4$	45.5	101.2	111.1
$CaCl_2$	104.4	116.1	98.1
$FeCl_2$	0	83.0	105.0
$SnCl_2$	3.0	88.7	81.0
$MgCl_2$	98.8	103.9	104.8
$MnCl_2$	105.7	92.0	89.6
$AlCl_3$	36.8	77.9	95.1
$HgCl_2$	—	0	0
None	100	—	—

[a]See Experimental.

TABLE III

SUBSTRATE SPECIFICITY OF THE (1→3)-β-D-GLUCANASE[a]

Substrate [*Linkage*]	*Relative activity (%)*	*Substrate*	*Relative activity (%)*
Laminarin [(1→3,1→6)-β]		Small-curdlan (mol. wt. 10k)	120.6
(from *Laminaria digitata*)	100.0	Small-grifolan (mol. wt. 20k)	40.3
(from *Eisenia araborea*	24.7	Small-SSG (mol. wt. 40k)	27.2
Curdlan [(1→3)-β]	6.1	IO_4-Oxidized laminarin	0.5
Grifolan [(1→3,1→6)-β]	10.0	IO_4-Oxidized and BH_4-reduced laminarin	0
SSG [(1→3,1→6)-β]	16.4	BH_4-Reduced laminarin	108.0
Islandican [(1→6)-β]	0	Laminaribiose	0
Cellulose [(1→4)-β]	0	Laminaritriose	3.3
Amylose [(1→4)-α]	0	Laminaritetraose	33.6
Starch [(1→4,1→6)-α]	0	Laminaripentaose	53.8
Dextran [(1→6)-α]	0	Laminarihexaose	65.2
Gentiobiose	0	Laminariheptaose (d.p. = 7)	76.5
Methyl β-D-glucopyranoside	0		
Methyl α-D-glucopyranoside	0		

[a]See Experimental.

Table III also shows the effect of the enzyme on laminara-oligosaccharides. The di- and tri-saccharides were not attacked, which suggests that, for enzyme action, at least four glucosyl residues must be available at the non-reducing end.

Treatment of the enzyme by NBS and DEP caused complete inactivation (Table IV), and modification of carboxyl groups decreased the activity by ~50%. Other modifications (see Experimental) did not affect the activity. These results suggested the involvement of Trp, His, and carboxyl residues at the active site of

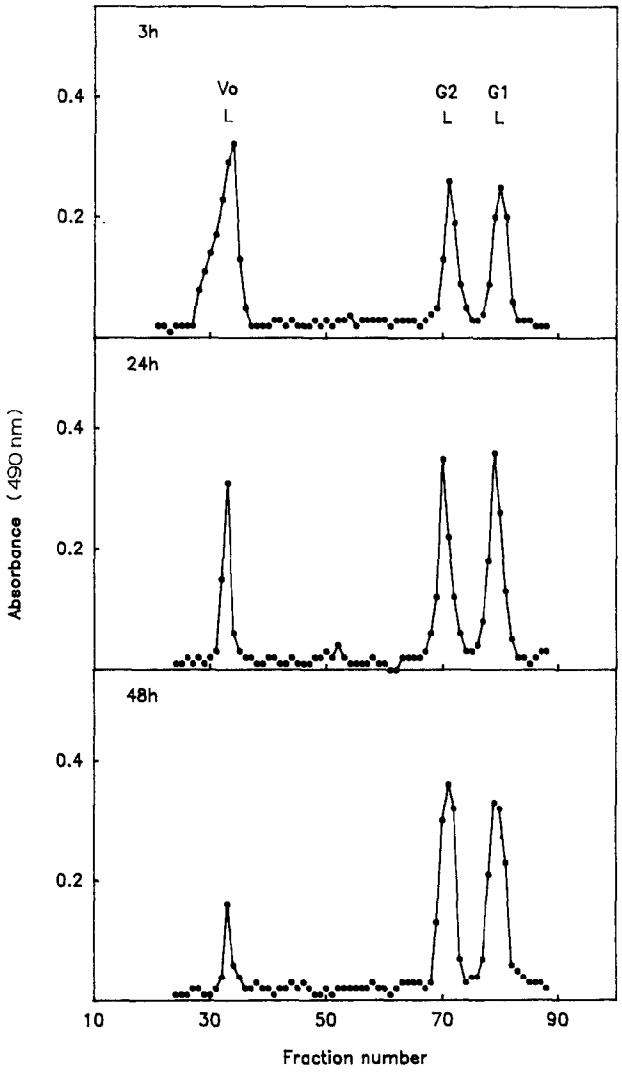

Fig. 4. Elution profiles of the enzymic digests of grifolan. Grifolan (~5 mg) was incubated with the enzyme (10 U) in 50mM phosphate buffer (pH 5.6) for 3, 24, and 48 h at 40°. The hydrolyzate was applied to a column (1.8 × 137 cm) of Biogel P-2 and eluted with water. Fraction (2.4 mL) were analyzed by the phenol–H_2SO_4 method. The elution volumes of dextran T2000 (V_0), glucose (G1), and laminaribiose (G2) were as shown.

the enzyme. In the presence of laminarin (0.05 and 0.25%), the enzyme was protected from inactivation by NBS in a dose-dependent manner (Fig. 6); soluble starch and dextran did not protect the enzyme (data not shown). However, laminarin did not protect the enzyme from inactivation by DEP, suggesting that the His residue was not involved in the binding site of the substrate (Fig. 6).

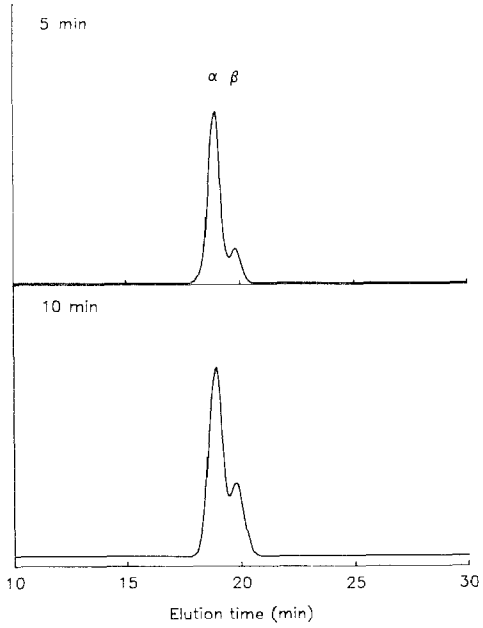

Fig. 5. Anomeric configuration of the glucose released by the (1→3)-β-D-glucanase. Laminaritriose (200 μg) was incubated with the enzyme (10 U) in 100 μL of 50mM phosphate buffer (pH 5.6) for 5 or 10 min, and aliquots were subjected to h.p.l.c. on a column of Amide-NH_2.

TABLE IV

EFFECT OF CHEMICAL MODIFICATION ON THE ACTIVITY OF THE (1→3)-β-D-GLUCANASE[a]

Reagent	*Residues*	*Remaining activity (%)*
Acetic anhydride	-NH_2 (Lys)	100.0
Succinic anhydride	-NH_2 (Lys)	80.0
DNBS	-NH_2 (Lys)	86.3
CMC	-COOH (Asp, Glu)	52.8
Diethyl pyrocarbonate	His	5.0
N-Bromosuccinimide	Trp	0
Chloramine T	Met	102.1
Cyclohexane-1,2-dione	Arg	97.0
DTNB	Cys	99.4

[a]See Experimental.

The amino acid composition of the exo-(1→3)-β-D-glucanase is shown in Table V. The enzyme contained 7 Trp and 11 His residues per molecule. The intrinsic fluorescence of Trp excited at 295 nm was not changed significantly in the presence of 6M guanidine hydrochloride, suggesting the Trp residues to be in a

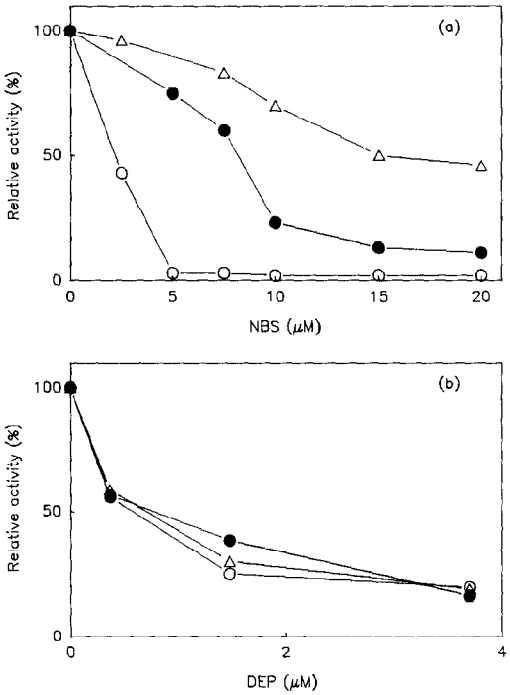

Fig. 6. Protective effect of laminarin (○, 0%; ●, 0.05%; △, 0.25%) against the inactivation of the (1→3)-β-D-glucanase by (a) NBS or (b) DEP.

TABLE V

AMINO ACID COMPOSITION OF THE (1→3)-β-D-GLUCANASE

Amino acid	*Residues per molecule (nearest integer)*	*Amino acid*	*Residues per molecule nearest integer)*
Asp(Asn)	83	Tyr	31
Thr	67	Phe	28
Ser	71	Lys	22
Glu(Gln)	51	His	11
Gly	85	Arg	21
Ala	68	Pro	32
Val	46	Cys1/2[a]	10
Ile	33	Met[a]	7
Leu	33	Trp[b]	7

[a]Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, after hydrolysis in 6M HCl of performic acid-oxidized protein. [b]Determined as in the Experimental.

relatively hydrophilic environment (data not shown). The intrinsic fluorescence of Trp was removed almost completely by oxidation with NBS (Fig. 7). Inactivation

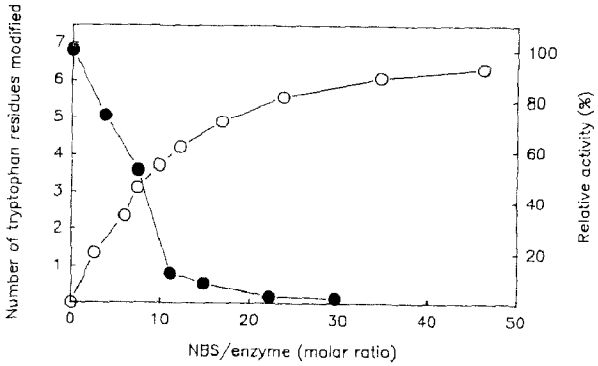

Fig. 7. Correlation of Trp oxidation and enzyme inactivation (0.2% of laminarin as substrate) on treatment with NBS (see Experimental): —○—, fluorescence intensity of the Trp residues; —●—, residual activity of the enzyme.

of the enzyme was achieved when ~50% of the Trp residues had been modified, suggesting that some participate in binding the substrate. Thus, the mechanism of action is similar to those of the (1→3)-β-D-glucanases from *Flav. dormitator* var. *glucocanolyticae* FA-5[15] and *Basidiomycete* QM806[17].

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