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AN ENDO-(1→3)-β-D-GLUCANASE FROM *Mucor hiemalis**†

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ABSTRACT

An endo-(1→3)-β-D-glucanase (EC 3.2.1.6), isolated from the culture filtrate of the fungus *Mucor hiemalis*, was purified by ammonium sulfate fractionation, gel filtration, and column chromatography on *O*-(carboxymethyl)cellulose. The optimum pH, optimum temperature, and K_m value of the enzyme were pH 5.0, 50°, and 0.048%, respectively. The enzyme was strongly inactivated by Pb^{2+}, Cu^{2+}, and Hg^{2+} ions and also inhibited by Zn^{2+} and Fe^{3+} ions. The enzyme was specific for laminaran and the action pattern of the enzyme was of the endo-type. The molecular weight of the enzyme, as determined by gel filtration, was 30,000.

INTRODUCTION

In our previous paper[1], it was shown that a (1→3)-β-D-glucanase is produced in the culture filtrate of *Mucor hiemalis*. A (1→3)-β-D-glucanase, accompanied by a (1→6)-β-D-glucanase, is present in a wide variety of fungi, such as *Penicillium* species[2], *Streptomycetes*[3], *Schizophyllum commune*[4], *Rhizopus* sp.[5], *Bacillus circulans*[6], *Saccharomyces cerevisiae*[7], *Aspergillus usami*[8], and *Rhizopus chinensis*[9]. We now describe purification methods and some properties of the enzyme.

MATERIALS AND METHODS

Enzyme assay. — One unit is defined as the amount of enzyme that yields 1 μmol of reducing sugar when a mixture of 0.5 ml of laminaran (0.5%), 0.5 ml of enzyme solution, and 1 ml of 0.1M acetate buffer (pH 5.0) is incubated for 1 h at 50°. A range of 0.2–0.4 U of enzyme was suitable for the experiments described. Reducing sugars were measured by the Somogyi–Nelson method[10,11].

Protein assay. — For the chromatographic separation, the absorbance at 280 nm, and for the quantitative analysis, the methods of Lowry *et al.*[12], with bovine serum albumin as standard, were used.

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Substrates. — Laminaran was prepared from brown marine algae by the method of Black[13]. (1→6)-β-D-Glucan (islandican) was purified from a culture filtrate of *Penicillium islandicum* IFO 4872 according to the method of Nakamura *et al.*[14,15]. Yeast mannan[16] and nigeran[17] were purified by the procedures described in the literature cited. The following substrates were obtained commercially: dextran, *O*-(carboxymethyl)cellulose (Na^+ salt), soluble starch, methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, cellobiose, gentiobiose, isomaltose, melibiose, maltose, sucrose, trehalose, turanose, melezitose, and raffinose. (1→3)-β-D-Linked gluco-oligosaccharides were prepared by partial acid hydrolysis (0.25M sulfuric acid, 1.5 h, 100°) of pachyman. The hydrolyzate was applied to a charcoal–Celite column[18] and the fraction adsorbed was separated by multiple-descent (3 times) paper chromatography with Whatman No. 3MM paper 6:1:3 1-propanol–ethyl acetate–water[19] (solvent A). Each oligosaccharide was isolated from the paper chromatogram by extraction with water.

Purification of the enzyme. — A culture of *Mucor hiemalis* (1.9 l) was incubated in a reciprocal shaker (115 times/min) for 3 days at 25°. The culture medium contained 0.2% of islandican, 0.2% of ammonium sulfate, 0.2% of potassium dihydrogenphosphate, 0.1% of magnesium sulfate heptahydrate, and 0.01% of yeast extract (Wako Pure Chemical Industries, Ltd.). The culture filtrate was lyophilized, fractionated with ammonium sulfate, and chromatographed on Sephadex G-100 by using 0.01M phosphate buffer (pH 7.2) containing 0.5M potassium chloride, as described in the previous paper[1]. The (1→3)-β-D-glucanase-active fraction was rechromatographed on Sephadex G-100 (1.5×89 cm) with 0.01M phosphate buffer (pH 7.2) in the absence of 0.5M potassium chloride. A portion (0.1 ml) of each fraction (4.3 ml) was assayed for enzyme activity. The active fraction eluted was dialyzed against distilled water at 5°, concentrated under diminished pressure at 30–34°, and chromatographed on an *O*-(carboxymethyl)cellulose column (1.5×27 cm) by using 10mM acetate buffer (pH 6.0) with a potassium chloride gradient. A portion (0.1 ml) of each fraction (2 ml) was assayed for enzyme activity.

General properties of the enzyme. — A. *Effect of pH.* Acetate, phosphate, and carbonate ($NaHCO_3$–Na_2CO_3) buffers were used in the pH ranges of 3.5–6.0, 7.0–8.0, and 9.0–11.0, respectively. The enzyme treated by each buffer (0.033M) for 20 h at 22° was assayed for activity remaining under the standard conditions. The optimum pH was determined by use of each buffer (0.1M) under the standard conditions.

B. *Effect of temperature.* To determine the heat stability of the enzyme, the activity remaining was assayed under the standard conditions after the enzyme had been kept with 0.09M acetate buffer (pH 5.0) for 10 min at each temperature (30–70°) in a thermostatically controlled incubator. The optimum temperature was established by assaying the enzyme activity at each temperature (40–60°) under the standard conditions.

C. *Effect of metal ions.* The effect of metal ions on the enzyme activity was examined for the following salts in the range of concentration 10^{-2}–10^{-4}M: $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $ZnCl_2$, $FeCl_3 \cdot 6H_2O$, $Pb(OAc)_2 \cdot 3H_2O$, $CuSO_4 \cdot$

5 H_2O, and $HgCl_2$. The relative activity was expressed as a percentage of the enzyme activity in the absence of metal ions.

D. *Effect of albumin.* The relative activity of the enzyme to which 100 or 200 μg/ml of bovine serum albumin had been added was expressed as a percentage of the activity in the absence of bovine serum albumin.

E. K_m *value.* The K_m value was obtained by from a Hofstee plot, namely, the value of substrate concentration divided by reducing power after incubation for 30 min plotted as ordinate, and the concentration of substrate as abscissa.

F. *Substrate specificity.* Measurements of substrate specificity were conducted under the standard assay-conditions with the following substrates: 0.5% of laminaran, islandican, *O*-(carboxymethyl)cellulose (Na^+ salt), nigeran, soluble starch, dextran, and yeast mannan. For methyl glucosides and oligosaccharides (methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, cellobiose, gentiobiose, isomaltose, laminarabiose, maltose, melibiose, sucrose, trehalose, turanose, melezitose, and raffinose), concentrations in the range of 0.032 (1.78)–0.056% (3.11mM) were used.

G. *Mode of action of the enzyme.* After the enzyme had acted on 2.5 mg of laminaran for 0.5, 1, 3, and 5 h, each solution was concentrated, deionized by Dowex 50-W resin, and lyophilized. The lyophilyzate was subjected to chromatography on Toyo Roshi No. 50 filter paper (solvent A). The products of enzyme action, after prolonged incubation (48 h), on 2 mg of each (1→3)-β-D-linked oligosaccharide (di- to penta-) prepared from pachyman were similarly detected by paper chromatography. The products were detected with silver nitrate–sodium hydroxide[20], and corresponding areas were extracted with water and the ratio of the products was determined by the phenol–sulfuric acid method[21], with D-glucose as the standard.

H. *Molecular weight of the enzyme.* The molecular weight of the enzyme was determined by the method of Whitaker[22], with a Sephadex G-100 column (1.9 × 90 cm) and the following proteins as standards: bovine serum albumin (mol. wt. 67,000), ovalbumin (mol. wt. 45,000), myoglobin (mol. wt. 17,800), and cytochrome C (mol. wt. 12,400).

I. *Disc electrophoresis.* Disc electrophoresis of the enzyme was performed in Tris–glycine buffer (6.0 g and 28.8 g/l, pH 8.3)[23,24] for 2.5 h, and in β-alanine–acetic acid buffer (31.2 g and 8.0 ml/l, pH 4.5)[25], for 1.8 h with a constant current of 1.5 and 1 mA for each Bio-Gel column. After electrophoresis, the column was cut into 10 slices (each of length 5 mm). Each slice was extracted with 0.5 ml of 0.1M acetate buffer (pH 5.0) and the active fraction was detected by incubation of the extracts with 0.5 ml of laminaran (0.5%), 0.5 ml of albumin (0.01%), and 0.5 ml of 0.1M acetate buffer (pH 5.0).

RESULTS AND DISCUSSION

It has been reported that (1→3)-β-D-glucanase is secreted in the culture medium of *Mucor hiemalis* and is roughly separated from an accompanying (1→6)-β-D-glucanase on a Sephadex G-100 column[1]. The (1→3)-β-D-glucanase has been further

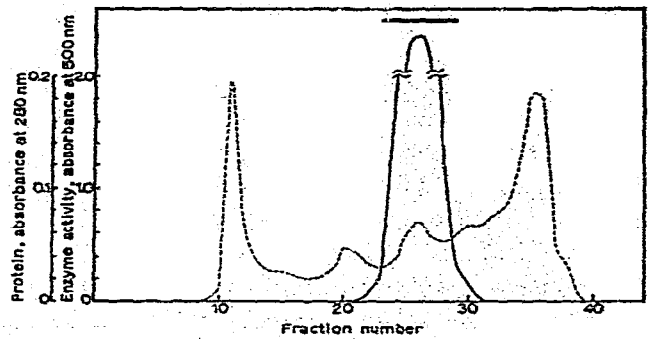


Fig. 1. Gel-filtration pattern of the enzyme on Sephadex G-100 with 0.01M phosphate buffer (pH 7.2): – – –, protein; ———, (1→3)-β-D-glucanase activity. Fractions 23–29, as indicated by the heavy bar, were collected as the (1→3)-β-D-glucanase component. One fraction contained per 4.3 ml of eluate.

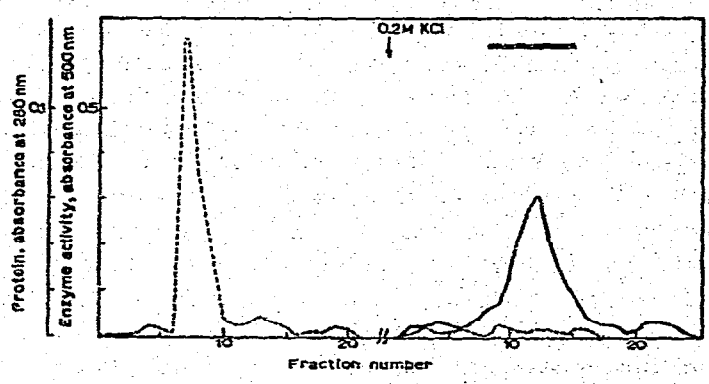


Fig. 2. Chromatography of the enzyme on a column of *O*-(carboxymethyl)cellulose: – – –, protein; ———, (1→3)-β-D-glucanase activity. Each fraction contained 2.0 ml of eluate. Starting from the point indicated by the arrow, 0.2M potassium chloride in 0.01M acetate buffer (pH 6.0) was added. Fractions indicated by the heavy bar were collected as the (1→3)-β-D-glucanase component.

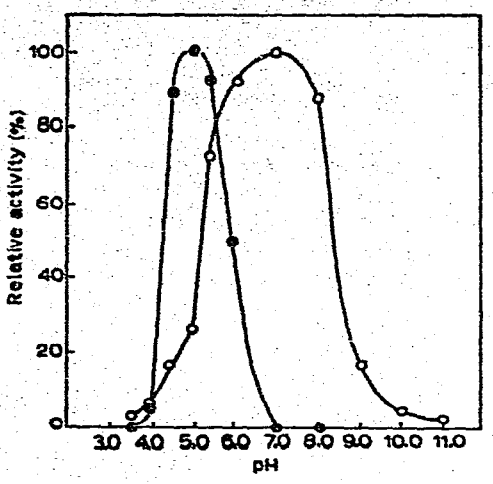
TABLE I

PURIFICATION OF (1→3)-β-D-GLUCANASE

Purification step	*Total activity (units)*	*Total protein (mg)*	*Specific activity (units/mg)*	*Yield (%)*
Culture filtrate	6223	86.6	71.9	100
Ammonium sulfate fractionation	4474	41.9	106.8	71.9
Sephadex G-100 gel filtration	619	4.99	123.8	9.9
Sephadex G-100 gel filtration without KCl	332	0.83	400.0	5.3
O-(Carboxymethyl)cellulose	112	0.14	800.0	1.8

purified by rechromatography on Sephadex G-100 (Fig. 1) and by column chromatography on *O*-(carboxymethyl)cellulose (Fig. 2). An eleven-fold purification of the enzyme was obtained after several purification steps (Table I). The enzyme was the most active at pH 5.0, however, it was the most stable at pH 7.0 (Fig. 3). The enzyme was most active at 50° and became unstable at higher temperatures (Fig. 4).

As described in the previous paper[1], the accompanying (1→6)-β-D-glucanase was the most active at 55° and pH 4.5, and was stable over wide temperature and pH



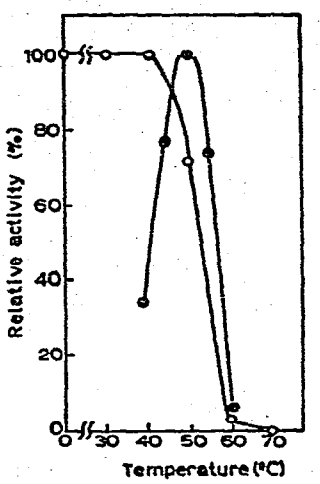


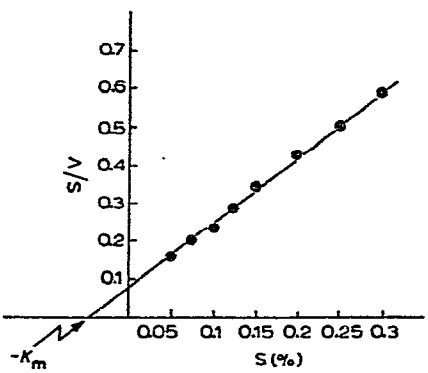
Fig. 3. Effect of pH on the enzyme activity: O—O, pH stability; ●—●, pH optimum.

Fig. 4. Effect of temperature on the enzyme activity: O—O, heat stability; ●—●, optimum temperature.

TABLE II

EFFECT OF METAL IONS ON THE ENZYME ACTIVITY

Metal ion (concentration)	*Relative activity (%)*	*Metal ion (concentration)*	*Relative activity (%)*
		Fe^{2+} (10^{-4} M)	96.7
None	100	(10^{-3} M)	96.5
		(10^{-2} M)	39.3
Mg^{2+} (10^{-4} M)	99.2	Pb^{2+} (10^{-4} M)	89.8
(10^{-3} M)	100.9	(10^{-3} M)	82.2
(10^{-2} M)	89.5	(10^{-2} M)	8.4
Ca^{2+} (10^{-4} M)	102.8	Cu^{2+} (10^{-4} M)	80.4
(10^{-3} M)	102.9	(10^{-3} M)	20.3
(10^{-2} M)	102.7	(10^{-2} M)	9.8
Zn^{2+} (10^{-4} M)	92.4	Hg^{2+} (10^{-4} M)	84.9
(10^{-3} M)	81.6	(10^{-3} M)	34.0
(10^{-2} M)	70.8	(10^{-2} M)	0.0



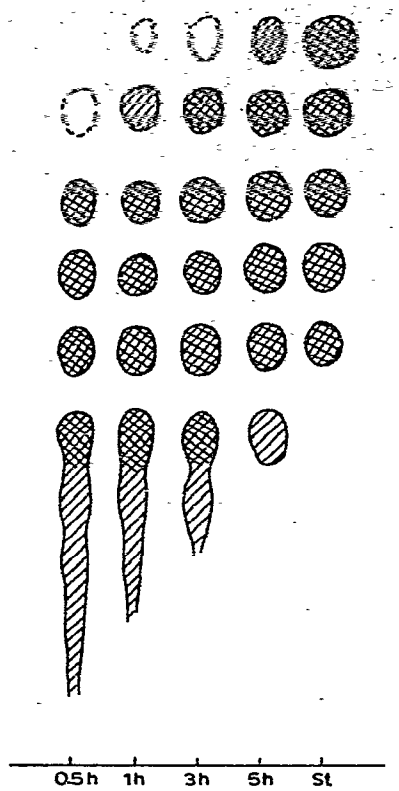


Fig. 5. Hofstee plot of the (1→3)-β-D-glucanase.

Fig. 6. Mode of action of the enzyme. Laminaran (2.5 mg) was incubated with the enzyme in 0.025M acetate buffer (pH 5.0) at 50°, for 0.5, 1, 3, or 5 h. The products of enzymolysis were detected by multiple-descent (3 times) paper chromatography (solvent A) with silver nitrate–sodium hydroxide as the spray reagent. St: glucose, laminarabiose, laminaratriose, laminaratetraose, and laminarapentaose.

TABLE III

SUBSTRATE SPECIFICITY OF THE PURIFIED ENZYME[a]

Polysaccharide	*Linkage*	*Activity (%)*[b]
Laminaran	(1→3)-β-D-, (1→6)-β-D-	100
Islandican	(1→6)-β-D-	0
O-(Carboxymethyl)cellulose (Na^+ salt)	(1→4)-β-D-	0
Nigeran	(1→3)-α-D-, (1→4)-α-D-	0
Soluble starch	(1→4)-α-D-, (1→6)-α-D-	0
Dextran	(1→6)-α-D-	0
Yeast mannan	(1→6)-α-D-, (1→3)-α-D-, (1→2)-α-D-	0

[a]No activity was observed against methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, cellobiose, gentiobiose, isomaltose, laminarabiose, sucrose, melibiose, maltose, trehalose, turanose, melezitose, and raffinose. [b]Relative activity was determined after incubation for 1 h.

ranges. At present, the biological function of these glucanases in the fungus is not known.

The activity of the enzyme was not affected by Mg^{2+} and Ca^{2+} ions, but the enzyme was moderately inhibited by Zn^{2+} and Fe^{3+}, and strongly influenced by Pb^{2+}, Cu^{2+}, and Hg^{2+} ions (Table II). The activity of the enzyme was protected and enhanced (by 134 and 151%, respectively) by the addition of 100 or 200 μg/ml of bovine serum albumin.

The K_m value of the enzyme, as obtained by a Hofstee plot, was found to be 0.048% (Fig. 5).

Taking the activity of the (1→3)-β-D-glucanase against laminaran as 100%, the enzyme was inactive against the other potential substrates listed in Table III. The fact that the enzyme acted on laminaran to produce a series of (1→3)-β-D-linked oligosaccharides suggests that it acts by an endo-type of mechanism (Fig. 6).

When the enzyme acted on the (1→3)-β-D-oligomers prepared from pachyman by partial acid hydrolysis for 48 h, the following conversions were observed: trisaccharide was hydrolyzed to glucose and disaccharide, with approximately 30% of the trisaccharide remaining; tetra- and penta-saccharides were hydrolyzed to glucose, disaccharide, and a very small proportion of trisaccharide (Fig. 7). Thus, the trisaccharide was hydrolyzed with difficulty by the enzyme, unlike the tetra- or penta-

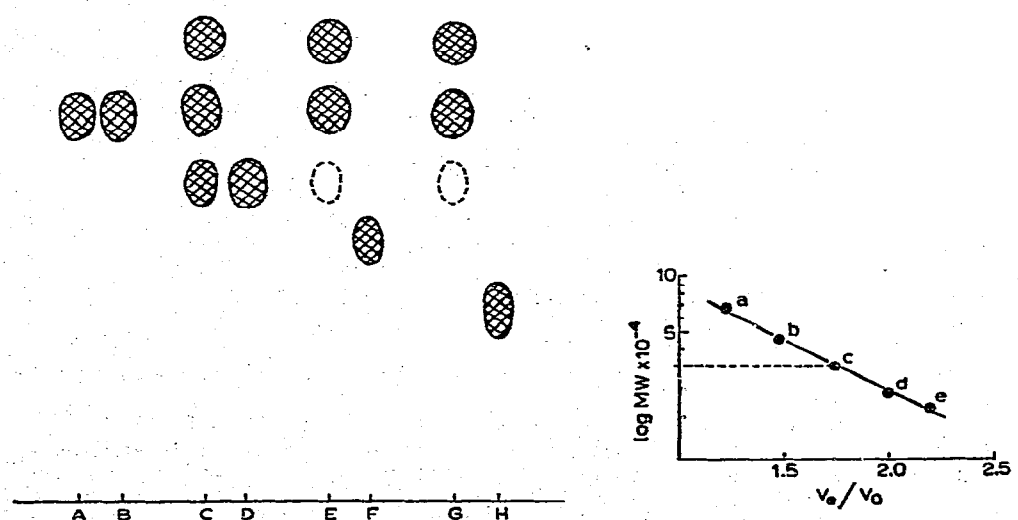


Fig. 7. Enzymic action on the (1→3)-β-D-oligosaccharides. A, hydrolyzate of disaccharide; B, disaccharide; C, hydrolyzate of trisaccharide; D, trisaccharide; E, hydrolyzate of tetrasaccharide; F, tetrasaccharide; G, hydrolyzate of pentasaccharide; H, pentasaccharide. Each oligosaccharide (2 mg) was incubated with the enzyme for 48 h at 50° in 0.025M acetate buffer (pH 5.0). The products formed by the enzyme were detected by multiple-descent (2 times) paper chromatography (solvent A) with silver nitrate–sodium hydroxide as the spray reagent.

Fig. 8. Determination of molecular weight of the enzyme by gel-filtration on Sephadex G-100: a, bovine serum albumin; b, ovalbumin; c, (1→3)-β-D-glucanase; d, myoglobin; e, cytochrome C.

saccharides. Therefore, the enzyme may be used for determining the presence of chains longer than a trisaccharide.

Nagasaki *et al.* reported that prolonged incubation of laminaratriose gave rise to laminarabiose and glucose with the (1→3)-β-D-glucanase of *Rhizopus chinensis* R-69, whereas laminarabiose did not show quantitatively significant alteration[9]. Interestingly, the action pattern of the (1→3)-β-D-glucanase and (1→6)-β-D-glucanase from *Mucor hiemalis* resemble those from *R. chinensis*, and these two organisms are representative of the Mucorales.

The molecular weight of the enzyme, as determined by the method of Whitaker, was 30,000 (Fig. 8). By disc electrophoresis, a protein band could not be detected at low concentration, but the active fraction was detected in the 5th slice from the origin when a β-alanine–acetic acid buffer (pH 4.5) was used, whereas the active fraction did not migrate when Tris–glycine buffer (pH 8.3) was employed. Therefore, it was presumed that the enzyme was essentially homogeneous and was a basic protein.

Thus, the (1→3)-β-D- and (1→6)-β-D-glucanases isolated from *Mucor hiemalis* should be of value for the structural examination of soluble glucans.

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