Separation and Characterization of the *endo*- β -(1 $\rightarrow$ 3)-D-Glucanase from *Rhizoctonia solani*

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The *endo*- β -(1 $\rightarrow$ 3)-D-glucanase from extracts of *Rhizoctonia solani* has been purified to homogeneity by chromatographies on CM Bio-Gel A and Bio-Gel P-60 and finally by affinity chromatography on short-chain pachyman-AH-Sepharose. In the affinity chromatography, the adsorption of the enzyme seemed to be brought into close with the large spacer arm by prior biospecific adsorption on an attached ligand. The isoelectric point was pH 9.8 and the molecular weight was 29,000. The optimum pH for short-chain pachyman was 5.5. The enzyme was fairly stable at pH 5 to 8.5 and at temperatures below 50°C. The enzyme favors stretches of β -D-(1 $\rightarrow$ 3)-linkages, and also depolymerize β -(1 $\rightarrow$ 3)- β -(1 $\rightarrow$ 4) mixed-linked glucan. It has transglycosylase activity.

Purification of β -(1 $\rightarrow$ 3)-D-glucanase has been of great interest for use in the structural analysis of β -D-glucan, and for the degradation of yeast and fungal cell walls. It has also been used in studies of the mode of action of β -D-glucanases. The starting material is a commercially available yeast cell wall lytic enzyme preparation "Kitalase" prepared from the culture filtrates of *Rhizoctonia solani*. It has been shown to contain a mixture of enzymes that hydrolyze β -(1 $\rightarrow$ 3)- β -(1 $\rightarrow$ 4)-linked glucans such as lichenan.¹⁾ We attempted to purify such a glucan-degrading enzyme from Kitalase.

MATERIALS AND METHODS

Enzyme and substrates. A commercially available yeast cell lytic enzyme preparation, Kitalase (Kumiai Chemicals Co.), prepared from the culture filtrates of *Rhizoctonia solani*, was used as the source of *endo*- β -(1 $\rightarrow$ 3)-D-glucanase. Thus Kitalase (12 g) was suspended in 60 ml of 50 mM acetate buffer at pH 5.5 with stirring for 1 hr, and insoluble material was removed by centrifugation. To the solution, solid ammonium sulfate was added to give 80% saturation. After centrifugation of the precipitate formed, it was dissolved in 50 ml of 10 mM acetate buffer at pH 4.5 and then dialyzed against 10 liters of the same buffer. The

crude enzyme solution was used for purification of β -(1 $\rightarrow$ 3)-D-glucanase.

Pachyman was prepared from air-dried Bukuryo (*Poria cocos*) by the method of Saito *et al.*²⁾ Short-chain pachyman ($\overline{DP}$ 20) was prepared by formic acid treatment of pachyman.³⁾ Barley β -glucan prepared by the method of Morris was kindly provided by the Asahi Beer Co. Laminara-oligosaccharides (DP 2~7) were purchased from Seikagaku Kogyo Co. All other substrates were obtained from commercial sources.

Enzyme assays. β -(1 $\rightarrow$ 3)-D-glucanase was tested as follows. A mixture containing 1.0 mg of short-chain pachyman in 0.1 ml, 50 mM acetate buffer (pH 5.5) and an appropriate amount of enzyme in a total volume of 1.0 ml was incubated at 40°C for 15 min. Reducing sugar produced per 1.0 ml of the reaction mixture was estimated by the method of Somogyi and Nelson.^{5,6)} One unit of activity was defined as that amount of enzyme releasing one μ mol of reducing sugar equivalents, expressed as glucose, per min under the standard assay conditions.

Yeast cell lytic activity was assayed with a lyophilized preparation of baker's viable yeast (Oriental Yeast Co.) by the method of Kobayashi *et al.*¹⁾ One unit was defined as the amount which causes a 20% decrease of the optical density at 660 nm of the cell suspension, per hr under the standard assay conditions. Other assays those of β -(1 $\rightarrow$ 6)-D-glucanase, cellulase, and β -glucosidase activities were estimated by pustulan (from *P. papulosa*, Calbiochem), Avicel and carboxymethyl-cellulose, and salicine as sub-

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strates, respectively. Protease activity using casein was measured by the method of Kobayashi *et al.*¹⁾

Measurement of protein. The protein content of enzyme solutions at each step of the purification were measured by the extinction at 280 nm. When necessary, protein content was also measured by the method of Lowry *et al.*⁷⁾ with bovine serum albumin as a standard.

Affinity adsorbent. Short-chain pachyman was coupled to ω -aminoethyl (AH)- or aminoethyl (AE)-Sephacrose CL 6B by our method.⁸⁾ The gel was packed to give an affinity column.

Gel electrophoresis on sodium dodecyl sulfate (SDS). To examine the homogeneity of the purified enzyme preparation, SDS polyacrylamide gel electrophoresis was done by the procedure of Laemmli with a discontinuous buffer system.⁹⁾

The molecular weight of the purified enzyme was measured by SDS polyacrylamide slab gel electrophoresis by the procedure of Weber and Osborn with a continuous buffer system.¹⁰⁾ MW-Marker (Oriental Yeast Co.) containing a oligomer series of cytochrome *c* was used as molecular weight standards: hexamer (74,400); tetramer (49,600); trimer (37,200); dimer (24,800); monomer (12,400). In both systems, bromophenol blue (Nakarai Chemicals Co.) was used as a tracking dye and protein bands were detected with Coomassie Brilliant Blue R-250 (Nakarai Chemical Co.).

Isoelectric focusing. Isoelectric focusing experiments were done by running preparative flat-bed electrofocusing in a granulated gel with the LKB 2117 Multiphore with Ampholine specified for the pH range 7.8 ~ 10.5 according to directions supplied to manufacture.

General properties of the purified endo- β -(1 $\rightarrow$ 3)-D-glucanase

1) *Effects of pH on activity and stability.* Buffer solutions were prepared to cover the pH range 3.5 ~ 10; 50 mM acetate buffer (pH 3.5 ~ 6.4), 30 mM phosphate buffer (pH 6.0 ~ 8.8) and 0.1 M glycine buffer (pH 9.0 ~ 10.0). The activity was measured at various pHs under the standard assay conditions described above. The effects of pH on stability of the enzyme were observed by assaying the enzyme activity after incubation at 25°C for 24 hr in the absence of substrate at each of the pHs described above.

2) *Effects of temperature on activity and stability.* The activity was measured at various temperatures (20 ~ 70°C) under the standard assay conditions. The heat stability of the enzyme was observed by assaying enzyme activity after incubation of the enzyme for 30 min at various temperatures in the absence of substrate.

3) *Effects of metal ions.* The enzyme was incubated at 40°C for 15 min in the presence of 1×10^{-4} M of each of the following salts: $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, CaCl_2 , ZnCl_2 , $\text{FeSO}_4 \cdot$

$7\text{H}_2\text{O}$, $\text{Pb}(\text{OAc})_2 \cdot 3\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, HgCl_2 and the residual activity was measured by the standard assay procedures.

4) *Km value.* The reaction velocity (*v*) was measured for a series of concentrations of short-chain pachyman ranging from 0.001 ~ 0.1% (w/v) in the final incubation mixture. The *Km* value was calculated from a Hofstee plot. A mixture of 1.0 ml of substrate solution and 2.0 ml of 50 mM acetate buffer (pH 5.5) was equilibrated at 40°C. The reaction was stated by the addition of 1.0 ml of a solution of purified enzyme (0.05 ~ 0.1 units) warmed to the same temperature. After incubation (for 5, 10, or 15 min), 1.0-ml portions were removed and assayed as for the standard assay procedures.

5) *Stereochemistry of hydrolysis products.* Short-chain pachyman was dissolved in 50 mM acetate buffer (pH 5.5) to a concentration of 20 mg/ml. The solution was left overnight at room temperature to ensure mutarotation and put into a 3-ml cell that was placed at 25°C in the sample chamber of a Union Automatic Digital Polarimeter PM 101 equipped with sodium D line rays. The enzyme (10 units) in 200 μ l of acetate buffer was added to a 1-dm polarimeter cell. Then changes in the rotation were measured after intervals of 2 min. After incubation for 25 min, one drop of concentrated ammonium hydroxide was added to the polarimeter tube, and the rotation was again measured after thorough mixing.

6) *Transferase activity.* The action of the enzyme on oligosaccharides substrates of laminarabiose through laminarapentaose was examined by paper chromatography (PC). The oligosaccharide concentration was 10 mg/ml and the enzyme concentration was 0.5 units/ml in 50 mM acetate buffer (pH 5.5) at 40°C. Portions were removed at 1 hr and 48 hr. After inactivation of the enzyme by heating 10 min at 100°C, the incubation mixture was analyzed by PC on Toyo No. 50 filter paper by the ascending method with the following solvent system (v/v): (A) 1-butanol-pyridine-water (6:4:3), and (B) 1-propanol-ethyl acetate-water (7:1:2). Sugar spots on chromatograms were detected by alkaline silver nitrate reagent. High-performance liquid chromatography (HPLC) was done with a PNH₂-10/s 2504 column (4 mm i.d. $\times$ 25 cm) in a Shimadzu liquid chromatograph equipped with a RID-4 differential refractometer. The solvent was 3:7 water-acetonitrile pumped at 0.8 ml/min.

RESULTS

1. Purification of the enzyme

All operations were at 4°C unless otherwise stated.

1) *CM Bio-Gel A ion-exchange chromatography.* The desalted enzyme solution obtained by solid ammonium sulfate precipitation was put on a CM Bio-Gel A column (2.6 $\times$ 19 cm)

FIG. 1. Bio-Gel P-60 Chromatography of E-I and E-II Fractions Obtained from CM Bio-Gel A.

Enzyme solution (2 ml) was put on a column (1.5 $\times$ 85 cm) of Bio-Gel P-60 at a flow rate of 10 ml/hr. A and B are elution profiles of fractions E-I (500 units) and E-II (130 units), respectively. The void volume was measured with Blue Dextran 2000. Fractions were analyzed for protein A_{280} (—) and for β -(1 $\rightarrow$ 3)-D-glucanase (—●—) on short-chain pachyman and for lysis (—○—) of lyophilized yeast cells.

equilibrated with 10 mM acetate buffer (pH 4.5). The column was washed with 1 liter of equilibration buffer at a flow rate of 20 ml/hr. Elution was done with a linear gradient from 0 (500 ml) to 0.1 M NaCl (500 ml) in equilibration buffer, and the effluent was collected in 10-ml fractions. Two main peaks with short-chain pachyman-degrading activity were eluted at the beginning of the salt gradient (tube numbers 30~38) and a higher concentration of the salt (tube numbers 50~60). The former peak was designated E-I and the latter E-II. Eluates of the corresponding enzyme fractions were combined in each case, concentrated to 4~5 ml using an Amicon Diaflo unit with YM-5 membrane operating at 50 psi pressure, and then dialyzed overnight against 20 liters of 50 mM acetate buffer, pH 5.5 (buffer A).

2) Bio-Gel P-60 gel chromatography. A

FIG. 2. Short-chain Pachyman-AH-Sepharose CL 6B Chromatography of E-I and E-II Fractions Partially Purified by Gel Filtration.

The column was washed with several volumes of buffer A containing 1 M NaCl at a flow rate of 10 ml/hr. Elution solutions were (1) buffer A; (2) 0.2% short-chain pachyman in buffer A. A and B are elution profiles of E-I (24 ml, 420 units) and E-II (18 ml, 100 units), respectively. Fractions were analyzed for protease activity (—△—) on casein.

portion (2 ml) of both enzyme fractions obtained from the CM Bio-Gel A column was put on a 1.5-by-85-cm column of Bio-Gel P-60 equilibrated with buffer A. Elution was done with the buffer of Fig. 1. The enzyme activity of E-II was eluted from the gel as a peak overlapping with a low level of lytic enzyme contaminant, while that of E-I, which was devoid of lytic enzyme activity, was eluted as a peak very near the solvent front.

3) *Short-chain pachyman-AH-Sepharose CL 6B affinity chromatography.* Enzyme solution partially purified by Bio-Gel P-60 was directly loaded onto a column of short-chain pachyman-AH-Sepharose CL 6B equilibrated with buffer A containing 1 M NaCl. Elution was as shown in Fig. 2. In neither enzyme fraction was the enzyme activity eluted when the column was washed with the same buffer containing NaCl. When the eluant was changed to buffer A not containing NaCl, the enzyme activity of E-II was eluted from the column as in Fig. 2 (B); that of E-I was still adsorbed on the column as shown in Fig. 2 (A), but could

FIG. 3. Course of the Hydrolysis of Short-chain Pachyman by E-II Enzyme Preparation.

Short-chain pachyman (30 mg) was incubated with the enzyme (3 units) in 3 ml of buffer A at 40°C, and samples were removed at 1 and 48 hr for gel filtration and PC. (A) Gel filtration on a Bio-Gel P-2 column (1.5 × 95 cm). The chromatographic patterns of the reaction mixture after incubation for 0 (—△—), 1 (—○—), and 48 (—●—) hr are shown. Each fraction was assayed for the total sugar content by the phenol sulfuric acid method. The elution positions of Blue Dextran (*V*₀), laminaraoligosaccharides (*G*₂~*G*₇) and glucose (*G*₁) are shown in the figure. (B) PC with solvent A. It was run three times at 60°C. R, authentic glucose (1) and laminaraoligosaccharides (2~7).

TABLE I. PURIFICATION PROCEDURES OF THE *endo*-β-(1→3)-D-GLUCANASE

The enzyme was prepared from 12 g of kitalase.

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)
0~80% AmSO ₄	884	1581	1.8	1.0
CM Bio-Gel A	96.1	394	4.1	2.4
Bio-Gel P-60	8.4	211	25.0	14
Affinity chromatography	2.6	170	66.0	37

be eluted with substrate solution using biospecific counterligands. The eluates forming the corresponding enzyme peak were combined and concentrated to 1~2 ml using Amicon Diaflo as mentioned above. The resulting solution were lyophilized and then stored over calcium sulfate at 4°C.

E-I and E-II were formed to be *endo* and *exo* in nature, respectively, based on their action toward short-chain pachyman. Thus, the hydrolysis of E-II yielded a mixture of products ranging from biose to hexaose with possibly heptaose and glucose within 1 hr, and these products were almost completely hydrolyzed

to glucose and biose within 48 hr (Fig. 3). The *endo*-glucanase designated E-II was purified 37-fold with a 10.7% yield (Table I) and was completely free from yeast cell lytic enzyme, β-(1→6)-D-glucanase, cellulase, β-glucosidase, and protease activities. It was homogenous on Bio-Gel P-60 gel filtration, and gave a single protein band on SDS polyacrylamide gel electrophoresis (Fig. 4) and on polyacrylamide gel isoelectronic focusing (Fig. 5). The molecular weights of E-I and E-II were estimated to be 70,000 and 29,000, respectively, as judged by SDS-electrophoresis, and the isoelectric points of E-I and E-II were pH 9.5 and 9.8, re-

FIG. 4. SDS Polyacrylamide Gel Electrophoresis of the *endo*- β -(1 $\rightarrow$ 3)-D-Glucanase (E-II).

Lanes 1 and 2 are enzyme preparations of E-I and E-II, respectively. Samples were electrophoresed in 12.5% polyacrylamide gel in the presence of 0.1% SDS.

FIG. 5. Isoelectric Focusing of the *endo*- β -(1 $\rightarrow$ 3)-D-Glucanase.

Samples were focused in 5% polyacrylamide gel (60 $\times$ 115 $\times$ 2 mm) containing 2.5% Ampholine (LKB, pH 7.6~10.7) at 4°C for 3 hr at 1,360 V. Gels were stained with Coomassie Brilliant Blue. Marker proteins and their pIs (Oriental Yeast Co.,) were: cytochrome *c* (10.6), acetylated cytochrome *c* (9.7 and 8.3).

FIG. 6. Adsorption Characteristics of *endo*- β -(1 $\rightarrow$ 3)-D-Glucanase on Affinity Chromatography.

The enzyme preparation (1 ml, 5 units) was put on a column (1.0 $\times$ 3.0 cm). Elution conditions other than A were as in Fig. 2. Elution solutions were (1) buffer A; (2) 0.2% short-chain pachyman in buffer A. In A, the column was first washed with buffer A, then equilibrated with the same buffer, and elution was initiated with (2). A and B are elution profiles of E-II enzyme on AH-Sepharose CL 6B under different conditions. C and D are elution profiles of E-II enzyme on short-chain pachyman-AH-Sepharose CL 6B, and short-chain pachyman-AE-Sepharose CL 6B, respectively. E is an elution profile of E-I enzyme on short-chain pachyman-AE-Sepharose CL 6B as reference.

spectively, as judged by isoelectric focusing. These properties of E-I regarded as *exo*-glucanase were analogous to those observed by Tsujisaka *et al.*¹¹⁾

2. *Adsorption characteristics of the purified endo-glucanase (E-II) to affinity adsorbent*
Elution profiles of the purified E-II enzyme on some affinity adsorbents are given in Fig. 6

(A)~(D). E-II enzyme activity had no detectable affinity for the controlled unsubstituted AH-Sepharose column when it was chromatographed with a low-concentration salt solution as in Fig. 6 (A). However, significant retardation of its activity was observed when the column was washed with a high-concentration salt solution as in Fig. 6 (B). Two glucan-coated agarose preparations that differed only in the length of hydrocarbon chains bridging the ligand and the bead had very different retention of the enzyme at high ionic strength. Thus the enzyme was tightly bound to the column with 6-carbon-atom (–AH–) bridges as in Fig. 6 (C), but it did not bind firmly and was retarded to a slight extent on that with 2-carbon-atom (–AE–) bridges as in Fig. 6 (D). However, the enzyme activity of E-I was strongly adsorbed even on the column with 2-carbon-atom bridges, so that it could be easily eluted with substrate solution using biospecific counter-ligand as in Fig. 6 (E).

3. Properties of the purified *endo*-glucanase

The pH optimum of the enzyme was 5.5, and it was stable in the range of 5~8.5. The maximum activity of the enzyme was at 60°C, and activity was stable in the range 20~50°C. The enzyme incubated in the presence 1×10^{-4} M of metal ions was little affected by Mg^{2+} , Ca^{2+} , Zn^{2+} , Fe^{2+} , Pb^{2+} , and Cu^{2+} , but was strongly inhibited by Hg^{2+} . The loss of activity with Hg^{2+} was 87%. The K_m value of the enzyme, obtained by a Hofstee plot, was 0.013%. Since the short-chain pachyman had $\overline{DP}$ 20, the molecular weight was 3,240, and the K_m therefore 4.0×10^{-6} M.

The stereochemistry of the enzymic hydrolysis was investigated by monitoring the mutarotation of the hydrolysis products. The rate of change of optical rotation with time precisely followed the kinetics expected of a mutarotation process, and the observed change in the rotator was upward. We think that the hydrolysis mechanism proceeds with retention of configuration.

The initial rate of hydrolysis of glucans compared with that of short-chain pachyman

TABLE II. RELATIVE RATE OF HYDROLYSIS OF THE *endo*- β -(1 $\rightarrow$ 3)-D-GLUCANASE ON SOME GLUCANS

Substrates, 1 mg/ml, were incubated at 40°C for 10 min in the presence of the enzyme (1 unit), then the reducing sugar formed was measured by the Somogyi-Nelson method.

Substrate	Main linkage type	Relative rate ^a (%)
Short-chain pachyman	β -1,3	100
Pachyman	β -1,3	21
Barley glucan	β -1,3 : β -1,4 (3 : 7)	31
Lichenan	β -1,3 : β -1,4 (3 : 7)	34
Laminaran (<i>Laminaria japonica</i>)	β -1,3 : β -1,6 (7 : 1)	15
Laminaran (<i>Eisenia bicyclis</i>)	β -1,3 : β -1,6 (3 : 2)	0.8
β -Glucan from <i>Ganoderma applanatum</i>	β -1,3 : β -1,6 (3 : 1)	2.4
Pustulan	β -1,6	0
Avicel	β -1,4	0
Carboxymethyl cellulose	β -1,4	0
Soluble starch	α -1,4	0
Glycogen	α -1,4, α -1,6	0
Dextran	α -1,6	0

^a The rate on short-chain pachyman was arbitrarily set at 100.

^b It shows the ratio of linkage type.

(100) are shown in Table II. The enzyme had a preference for stretches of β -(1 $\rightarrow$ 3)-linked D-glucosyl residues, but increased frequency of 6-glucosyl substitution decreased the rate of hydrolysis. The enzyme had more effect on barley glucan and lichenan than on *Eisenia* glucan¹²⁾ or *Ganoderma* glucan,¹³⁾ despite of the high proportions of β -D-(1 $\rightarrow$ 4) linkage into those chains. It had no effect on (1 $\rightarrow$ 4)- β -D-glucan.

4. Transglycosylase activity of the purified *endo*-glucanase

We have further investigated the products formed by the enzyme when it acts on all members of the homologous series from laminarabiose to laminarapentaose (Fig. 7). For example, when the enzyme acts on laminara-

FIG. 7. PC Showing Transglycosylase Activity by the endo- β -(1 $\rightarrow$ 3)-D-Glucanase.

The method is described in MATERIALS AND METHODS. Chromatography was run three times with solvent system A at 60°C. R, authentic glucose (Glc) and laminaraoligosaccharides (L_2 to L_8); 0, 1, 48, digests of L_2 to L_5 after incubation for 0, 1, and 48 hr, respectively.

pentaose, it produces, in addition to the expected products of lower molecular weight, the initial formation of oligosaccharides that appear upon PC in the positions for hexa-, hepta-, and octa-saccharides, with possibly some nona-saccharide as well. The resulting oligomers were also found to correspond to DP 2~8 of the standard laminaraoligosaccharides by HPLC analysis. The linkages produced by the transglycosylation process are probably all β -D-(1 $\rightarrow$ 3).

DISCUSSION

We have previously reported the separation of endo- and exo- β -(1 $\rightarrow$ 3)-D-glucanases from *Rhizoctonia solani* by use of short-chain pachyman-AH-Sepharose column affinity chromatography.⁸⁾ Here, for purification, better results were obtained if the enzyme fractions obtained by CM Bio-Gel A were partially purified by gel filtration before being put on the affinity column. The endo- β -(1 $\rightarrow$ 3)-D-glucanase (E-II) was purified to a state of homogeneity, as judged by gel filtration, SDS polyacrylamide gel electrophoresis, and isoelectric focusing. When the E-II fraction obtained from Bio-Gel P-60 was put on the affinity column, the enzyme activity with β -(1 $\rightarrow$ 3)-D-glucanase was recovered by elution

with a low-concentration salt solution. Lytic enzyme or protease contaminant inseparable on CM Bio-Gel A and Bio-Gel P-60 chromatography could be removed completely from the E-II fraction by this step. In this affinity method, lytic enzyme contaminant was still adsorbed on the column, but could be eluted with biospecific counterligand. Such a yeast cell lytic enzyme also possesses β -(1 $\rightarrow$ 3)-D-glucanase activity and its separation can be effected by this affinity method.¹⁴⁾ The adsorption characteristics of endo-glucanase (E-II) to short-chain pachyman-AH-Sepharose adsorbent were further examined in detail (Fig. 6). A significant retardation of the enzyme was observed when the controlled unsubstituted AH-Sepharose was washed with a high-concentration salt solution. It seems to dominate the interactions with the hydrophobic arm of this derivatives, since hydrophobic interaction should be strengthened rather than weakened by raising the ionic strength of the medium.¹⁵⁾ When the short-chain pachyman ligand was coupled to AH-Sepharose, the enzyme was completely adsorbed to the gel at high ionic strength. On the contrary, a short-chain pachyman-AE-Sepharose column with 2-carbon-atom bridges only slightly retarded the enzyme. From these results, it is supposed that the adsorption of the enzyme on short-

chain pachyman-AH-Sepharose is brought into close with the large spacer arm by prior biospecific adsorption on an attached ligand, derivative with shorter arm being ineffective.

The *endo*- β -(1 $\rightarrow$ 3)-D-glucanase favors stretches of β -D-(1 $\rightarrow$ 3) linkages, and is capable of hydrolyzing glucans containing both (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 4)- β -glucosidic linkages. On the other hand, hydrolysis of β -(1 $\rightarrow$ 3)-glucan by the enzyme was greatly reduced by the frequency of 6-*O*-glucosyl substitution, such as that present in *Eisenia* glucan or *Ganoderma* glucan. The enzyme hydrolyzes glucan with retention of its configuration and has significant transglycosylase activity. These properties are very similar to those of the *Rhizopus arrhizus* enzyme,¹⁶⁾ which hydrolyze (1 $\rightarrow$ 4)-D linkages adjacent to (1 $\rightarrow$ 3)- β -D linkages in cereal D-glucans, thus belonging to the class (EC 3.2.1.6.)^{17,18,19)} The action patterns of this *endo*-glucanase on barley glucan and lichenan will be reported in detail in the near future.

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