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# DEGRADATION OF A GLUCAN CONTAINING β-(1→3) AND β-(1→6) LINKAGES BY EXO-(1→3)-β-D-GLUCANASE*

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ABSTRACT

A β-D-glucan isolated from a Japanese marine alga known as "Arame" *(Eisenia bicyclis)* was shown by methylation analysis to be composed of (1→3) and (1→6) linkages and (1→3),(1→6) branch points. The $^{13}$C-n.m.r. spectrum confirmed these linkages and also verified the absence of β-(1→4) linkages. The endo-(1→3)-β-D-glucanase of *Rhizopus* liberates from the native glucan oligosaccharides having an odd number of glucosyl residues, such as 3, 5, 7, and 9. The exo-(1→3)-β-D-glucanase of *Basidiomyete* QM 806 produces β-glucose, gentiobiose, and 6-*O*-β-laminarabiosyl-D-glucose, and a tetrasaccharide, β-D-Glc-(1→6)-β-D-Glc-(1→3)-β-D-Glc-(1→6)-D-Glc. Thus, the exo-enzyme appears to bypass glucosyl substituents at O-6 of 3-O-linked glucose in this polymer and, because it liberates oligosaccharides, it behaves as if it mediates an endo-type hydrolysis of the glucan.

## INTRODUCTION

Most β-D-glucans isolated from marine algae of the genus *Laminaria* are believed to be composed mainly of β-(1→3)-linked D-glucose. However, some glucans from other genera of Laminariaceae have structures that differ from that of *Laminaria* laminaran. For example, Maeda and Nisizawa reported that a soluble laminaran from a marine alga, Arame *(Eisenia bicyclis)*, consists of a linear β-(1→3) and β-(1→6)-linked D-glucosyl sequence[1,2].

Exo-(1→3)-β-D-glucanases are expected to produce D-glucose upon sequential hydrolysis of laminaran from the non-reducing end[3]. In addition, some exo-(1→3)-β-D-glucanases have been reported that liberate gentiobiose from scleroglucan, a (1→3)-β-D-glucan having additional D-glucosyl groups at O-6 of every third glucose residue in the main chain[4,5]. This degradation pattern suggests that the enzyme circumvents the β-(1→6) linkages, as they appear at the terminal end, and at-

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tacks the adjacent β-(1→3) linkages. As exo-(1→3)-β-D-glucanases may ignore the presence of a monosaccharide group at O-6, the possibility exists for hydrolysis of more-extensive oligosaccharides in certain linkage-sequences. The products of such exoenzyme-mediated degradation would resemble those of an endoenzyme. As it has been reported that the laminaran from the marine alga Arame has a linear sequence of β-(1→3) and β-(1→6) linkages[2], this substrate was used to explore the potential capacity of the exo-(1→3)-β-D-glucanase derived from *Basidiomycete* QM 806 for hydrolysis of D-glucosyl linkages at positions other than at the non-reducing terminal.

We first attempted to confirm the structure of the laminaran, which had been reported previously[2], and unexpectedly found that the glucan isolated from Arame has 3,6-di-*O*-substituted D-glucose residues in addition to (1→3)- and (1→6)-linked β-D-glucose residues.

In this paper, additional information of the structure of the glucan is reported and an endo-type action of exo-(1→3)-β-D-glucanase on the glucan is proposed.

## RESULTS AND DISCUSSION

The glucan was isolated as described in the Experimental section, hydrolyzed, and analyzed by g.l.c. as the alditol acetate; the product was exclusively hexa-*O*-acetylglucitol (>99%).

The glucan powder (3 mg) was methylated according to Hakomori's method[6], and the partially methylated sugars liberated by hydrolysis were analyzed by g.l.c. as the alditol acetates. Four components were detected whose chromatographic retention-times corresponded to those of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-, and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-glucitol, respectively (Table I). These components were then subjected to g.l.c.–m.s. (ionization potential, 70 eV) and the individual fragmentation-patterns matched with the spectra of authentic compounds (Fig. 1). The molar ratio of these components was ~0.14:0.46:0.28:0.11 (Table I). Relative molar-response factors were assumed to be 39:36:33:30 for mono-, di-, tri-, and tetra-methylhexitols[7]. Methylation analysis

TABLE I

LINKAGE ANALYSIS OF THE PURIFIED NATIVE GLUCAN FROM *Eisenia bicyclis*

| *Component* | *Relative retention time* | *Molar ratio* | *Indicated linkage* |
|---|---|---|---|
| 1 | 1.00[a] | 0.14 | Terminal |
| 2 | 1.97 | 0.46 | 3 |
| 3 | 2.46 | 0.28 | 6 |
| 4 | 5.05 | 0.12 | 3 and 6 |

[a]Retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

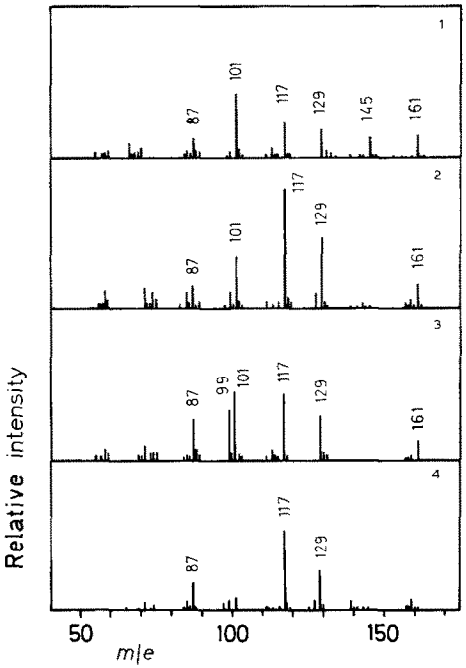


Fig. 1. Mass spectrum of components separated by g.l.c. The methylated glucan was successively hydrolyzed, acetylated, and separated on g.l.c. Each component on the chromatogram was subjected to mass-fragmentation spectrography. 1, 2, 3, and 4 constitute the corresponding components listed in Table I.

clearly revealed the presence of 2,4-di-*O*-methylglucose in the methylated glucan, in ~1:1 molar ratio with terminal glucose, indicating the glucan to be (1→3), (1→6) branched. In a previous report, the laminaran from *Eisenia bicyclis* was described as being linear and unbranched. Thus, the glucan isolated here is either structurally different or the branch points were not resolved by the techniques used to examine the polysaccharides in the previous[2] study.

To elucidate the linkage sequence within the glucan, 3 mg was successively oxidized with periodic acid, reduced, and hydrolyzed with weak acid at room temperature. The hydrolyzate was resolved on a column of Bio-Gel P-2. Sugars were eluted in the void-volume fractions and in fractions corresponding to oligosaccharides of various molecular sizes (d.p. 2.5–9) (Fig. 2). Thus, the distribution of (1→3)-linked sequences in the glucan is considered to be heterogenous. However, two rather significant components were eluted at fractions corresponding to d.p. 2.5 and 3.5. These are probably 2-*O*-β-laminarabiosyl-D-erythritol and 2-*O*-laminaratriosyl-D-erythritol (Fig. 2). Laminaratriosyl and laminaratetraosyl blocks appeared to be dominant. Apparently no single, isolated (1→3)-β-D-glucosyl residues are located between β-(1→6) linkages in the glucan.

The glucan (150 mg) was dissolved in 0.6 mL of deuterium oxide and the $^{13}C$-n.m.r. spectrum recorded at 80°. The spectrum showed one glycosidic carbon signal at ~100 p.p.m. (103–104 p.p.m. Fig. 3), indicating that the glucan is β-linked[8].

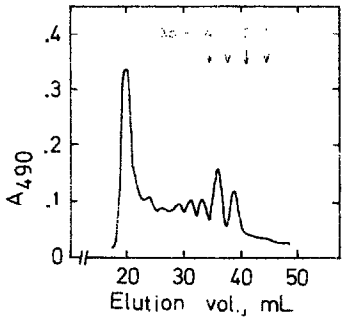


Fig. 2. Elution profile on Bio-Gel P-2 of the products from periodate oxidation of the glucan and successive hydrolysis with weak acid. The column was calibrated with stachyose, raffinose, cellobiose, and glucose.

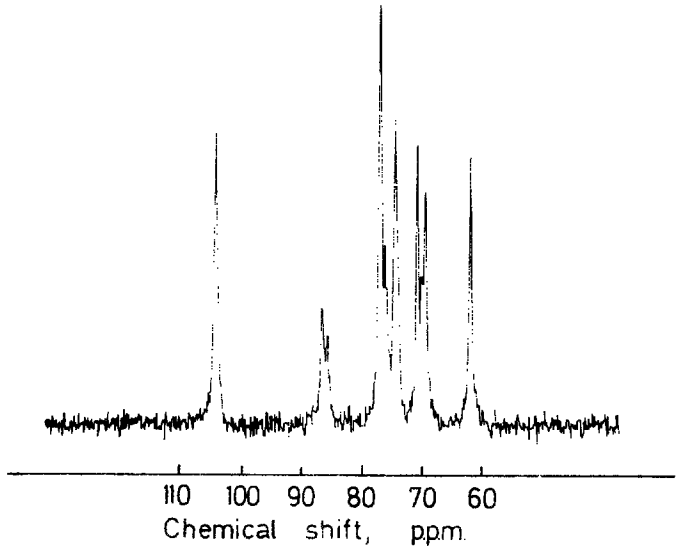


Fig. 3. $^{13}$C-N.m.r. spectrum of the glucan. See text for details.

The C-4 signal in a β-(1→4)-linked glucan would have been expected at ~80 p.p.m.[9]. Other features of the spectrum were also in agreement with the results obtained by methylation and mass-spectrographic analysis of the glucan.

The action of endo-(1→3)-β-D-glucanase on the glucan was examined. The *Rhizopus* glucanase catalyzes hydrolysis of a β-D-glucosyl linkage at C-1 of a 3-substituted glucosyl residue[10]. The authors have used this enzyme extensively to study the structure of another (1→3)-β-D-glucan[11,12]. The major products released upon enzyme treatment of *Eisenia* glucan were oligosaccharides having odd-numbered d.p. values (3, 5, 7, and 9, Fig. 4). Small amounts of oligosaccharides having even d.p. values were also detected.

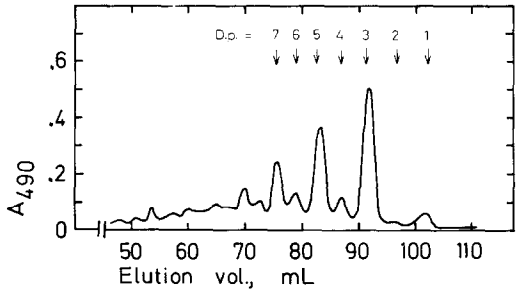


Fig. 4. Elution profile on Bio-Gel P-4 of the hydrolysis products upon treatment of the glucan with endo-(1→3)-β-D-glucanase. The column was calibrated with laminaran hydrolyzed with 0.2M sulfuric acid for 2 h at 100°.

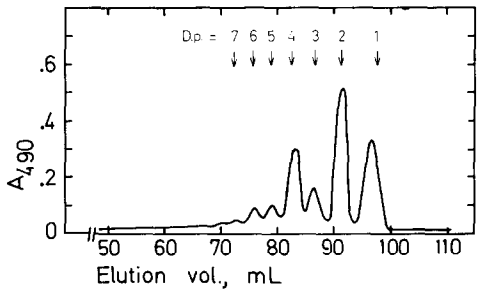


Fig. 5. Elution profile on Bio-Gel P-4 of the hydrolysis products upon treatment of the glucan with exo-(1→3)-β-D-glucanase. The column was calibrated with laminaran hydrolyzed with 0.2M sulfuric acid for 2 h at 100°.

TABLE II

LINKAGE RATIOS OF COMPONENTS ISOLATED BY PAPER CHROMATOGRAPHY GENERATED BY ACTION OF EXO-(1→3)-β-D-GLUCANASE ON *Eisenia bicyclis* GLUCAN

| *Component* | $R_{Glc}$ | *Linkage composition* | | |
|---|---|---|---|---|
| | | *Terminal* | *3* | *6* |
| 1 | 1.00 | | | |
| 2 | 0.51 | 1 | 0 | 1 |
| 3 | 0.35 | 1 | 1 | 1 |
| 4 | 0.14 | 1 | 1 | 2 |
| 5 | 0.09 | | | |
| 6 | 0.04 | | | |

Hydrolysis of the glucan by exo-(1→3)-β-D-glucanase gave mono-, di-, tri-, and tetra-saccharides (Fig. 5). The mono- and di-saccharides were chromatographed on Whatman No. 1 paper and found to be glucose and gentiobiose, respectively.

The remaining subsample of the mixture was deionized with a mixture of Dowex 1 ($OH^-$ form) and 50 ($H^+$ form) and chromatographed on Whatman No. 1 paper. The $R_{Glc}$ values for major components are listed in Table II. Components 5 and 6 appear to be pentasaccharides or larger oligosaccharides. In addition, a trace of gentiotriose was detected. There was no evidence that transglucosylase activity accounted for the formation of gentiotriose from gentiobiose. Degradation of the glucan by the exo-(1→3)-β-D-glucanase produced di-, tri- and tetra-saccharides containing at least one β-(1→6) linkage. This suggests that the oligosaccharide-producing action of the exo-enzyme requires that a β-(1→6)-linked glucosyl group be terminal at the recognition site.

The results given in Table II indicate ~36% of β-(1→6) linkages in the components shown in Fig. 5. This figure agrees with the proportion of β-(1→6)- plus β-(1→3)- and β-(1→6)-linked D-glucose (40%) in the glucan (Table I), indicating that the enzyme hydrolyzed only β-(1→3) linkages in the glucan. The enzyme preparation was free of endo-(1→3)-β-D-glucanase activity, as periodate-oxidized laminaran was not hydrolyzed by the enzyme[13]. Thus, the production of tri- and tetra-saccharides is considered to be a unique property of the exo-(1→3)-β-D-glucanase. The glucotrisaccharide component (3 in Table II) contained one (1→6)

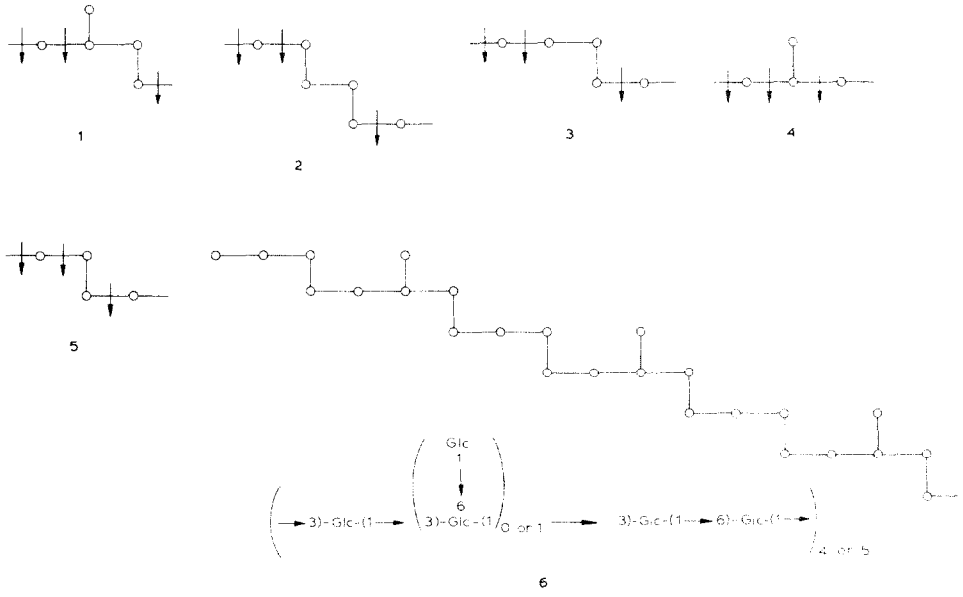


Fig. 6. Structural components of the glucan. ○: glucose, |: β-(1→6) linkage, —: β-(1→3) linkage, ↓: hydrolyzing site of the exo-(1→3)-β-D-glucanase.

and one (1→3) linkage and thus it was either 3-*O*-β-gentiobiosyl-D-glucose or 6-*O*-β-laminarabiosyl-D-glucose; borohydride reduction verified that it was the latter. Thus, the linkage at C-1 of the 6-linked glucose residue is considered to be attacked by the exo-(1→3)-β-D-glucanase to produce the trisaccharide. The release of a tetrasaccharide by the enzyme may be explained similarly. These facts indicate that the *Basidiomycete* exo-(1→3)-β-D-glucanase circumvents substituents at O-6 of 3-*O*-linked D-glucose when the enzyme attacks the β-(1→3)-linkage. Those β-(1→3)-linkages susceptible to the enzyme are thus at terminal residues or adjacent to β-(1→6)-linked D-glucose.

If this is the case, a tetrasaccharide containing two β-(1→6) linkages and a β-(1→3) linkage may be produced from **1** or **2** (Fig. 6) by the exo-(1→3)-β-D-glucanase. As already mentioned, few if any isolated β-(1→3) linkages are expected to occur in the glucan. Thus, **2** is an unlikely component in the glucan, suggesting that the tetrasaccharide was derived from **1** and is β-D-Glc-(1→6)-β-D-Glc(1→3)-β-D-Glc-(1→6)-D-Glc. The occurrence of a β-(1→3) linkage in the tetrasaccharide may be explained as follows. Generally, long-chain oligosaccharides are rapidly hydrolyzed by exoglycanases and disaccharides are resistant[14–16]. Laminarabiose is, for this reason, rather resistant to an exo-(1→3)-β-D-glucanase[14,16]. Analogous reasoning indicates that the isolated laminarabiose residue in the tetrasaccharide may also resist degradation, as additional β-(1→3) sequences constituting a recognition site are not present in this oligosaccharide. The trisaccharide, 6-*O*-β-laminarabiosylglucose is considered to be derived from **3** and gentiobiose from **4**.

We therefore conclude that the glucan is comprised of subunits of **1**, **3**, and **4** interconnected by (1→3)-β-D-glucosyl linkages. The number of interconnecting (1→3)-β-D-glucosyl residues appears to be 2 or 3, as laminaratriose and laminaratetraose components are dominant in the glucan (Fig. 2). Interconnection of **4** by (1→3)-β-D-glucosyl residues results in a longer β-(1→3) sequence. This accounts for the production of oligosaccharides of various sizes by Smith degradation of the glucan (Fig. 2).

## EXPERIMENTAL

*Isolation of the glucan.* — A Japanese marine alga "Arame" *(Eisenia bicyclis)*, purchased in dried form, from a local market in Ise, Japan was ground in a Wiley mill. The ground frond of the alga (10 g) was extracted with 300 mL of 0.09M hydrochloric acid for 2 h at room temperature. The extract was then centrifuged and 3 volumes of ethanol added. The precipitate was collected, dissolved in 50 mL of distilled water, and the solution centrifuged. Polysaccharides in the supernatant solution were then precipitated by adding 150 mL of ethanol. This procedure was repeated three times to remove water-insoluble materials. The precipitate was dissolved in 50 mL of distilled water, and 50 mL of a saturated calcium chloride solution was added to precipitate acidic polysaccharides. The solution was kept over-

night at 4° and then the soluble portion was collected and 300 mL of ethanol added. The resultant precipitate was dissolved in 50 mL of water and reprecipitated by adding 150 mL of ethanol. This procedure was repeated three times to remove calcium chloride. The precipitated polysaccharide was dissolved in water and the solution lyophilized. The resultant powder (1 g) was dissolved in 5 mL of water and applied to a column (2 × 14 cm) of DEAE-2 cellulose (Whatman DE 52) preequilibrated with 0.1M sodium phosphate, pH 7.0 and washed with water. The column was eluted with water and the elution profile monitored by a flow-cell refractometer. Fractions from the first peak from the column were combined and lyophilized to yield a white powder.

*Sugar analysis.* — The glucan powder (1 mg) was hydrolyzed with 2M trifluoroacetic acid for 1 h at 121° and the hydrolyzate reduced with 20 mg/mL of sodium borohydride. Subsequent acetylation with acetic anhydride–sodium acetate for 3 h at 121° was followed by chromatography on a column (0.2 × 100 cm) packed with 0.2% of ethylene glycol adipate, 0.2% ethyleneglycol succinate and 0.4% XF-1150 on Gas-Chrom P that was programmed from 110–170 at 1°/min[17]. *myo*-Inositol was used as the internal standard.

*Methylation analysis.* — The glucan powder (3 mg) was dissolved in 2 mL of dimethyl sulfoxide and methylated according to the method of Hakomori[6,18]. Methylated glucan was subjected to formolysis with 90% formic acid for 1 h at 100° and hydrolyzed[19] with 0.2M sulfuric acid for 18 h at 100°. The hydrolyzate was reduced in the presence of 20 mg/mL of sodium borohydride for 3 h at room temperature. The excess of sodium borohydride was decomposed by adding Dowex 50 ($H^+$ form) and borate was removed as methyl borate. Reduced sugars were then acetylated with acetic anhydride and pyridine (1:1, v/v) for 1 h at 100° and chromatographed[18] on a column (0.3 × 150 cm) packed with 3% ECNSS-M on Gas-Chrom Q at 180°.

*Periodic acid degradation.* — A sample of the glucan (3 mg) was treated with 0.1M periodic acid for 16 days at room temperature in the dark. Acid was removed by adding barium carbonate. The oxidized glucan was reduced in the presence of 30 mg/mL of sodium borohydride for 20 h at room temperature. The excess of borohydride was decomposed by adding Dowex 50 ($H^+$form) and borate was removed as methyl borate. Oxidized–reduced glucan was then hydrolyzed with 0.05M sulfuric acid at room temperature for 18 h and acid was made neutral by adding barium carbonate. The hydrolyzate was applied to a column (1.2 × 60 cm) packed with Bio-Gel P-2 (400 mesh, Bio-Rad Lab) maintained at 50°. The column was eluted with water and the elution profile was determined by the phenol–sulfuric acid method[20].

*Enzymic degradation of the glucan.* — The glucan (3 mg) was incubated for 20 h at 30° in 1 mL of McIlvaine buffer[21] (0.1 ionic strength), pH 4.6 containing 15 μg of an endo-(1→3)-β-D-glucanase of *Rhizopus* sp. After incubation, the mixture was heated for 5 min in boiling water to inactivate the enzyme and then applied to a column (1.2 × 100 cm) packed with Bio-Gel P-4. The column was

eluted with water and 1-mL fractions were collected. The sugar content in each fraction was determined by the phenol–sulfuric acid method[20].

The glucan (10 mg) was incubated for 20 h at 30° in 5 mL of McIlvain buffer[21] (0.1 ionic strength), pH 4.6 containing of the exo-(1→3)-β-D-glucanase[14] of *Basidiomycete* QM 806. The enzyme used was sufficient to liberate 0.1 μmol of D-glucose per sec from 1 mg of laminaran *(Laminaria hyperborea)* at 30°, as measured by the Nelson–Somogyi method[22]. The enzyme activity and reaction conditions employed here appeared to be adequate for complete hydrolysis of the susceptible linkages in the glucan. The mixture was boiled to inactivate the enzyme and applied to a column of Bio-Gel P-4.

*Paper chromatography.* — Components of the chromatography on Bio-Gel P-2 and products from the glucan upon *Basidiomycete* exo-(1→3)-β-D-glucanase treatment were examined by descending paper chromatography on Whatman No. 1 paper in 6:4:3 (v/v/v) butanol–pyridine–water; the development time was 48 h, and alkaline silver nitrate was used for detection[23]. Identification was based on reference to the following standards: D-glucose, laminarabiose, cellobiose, laminara-oligosaccharides, and cellooligosaccharides.

*Preparation of exo-(1→3)-β-D-glucanase.* — A culture of *Basidiomycete* QM 806 was generously supplied by Dr. E. T. Reese of the Food Science Lab., U.S. Department of the Army, Natick Laboratories. An 8-day-old culture was filtered, concentrated, and chromatographed on DEAE-cellulose (Whatman DE 52) according to the method of Huotari *et al.*[14]. Fractions corresponding to the exo-(1→3)-β-D-glucanase were combined, concentrated with an Amicon filter membrane (type PM-10), and dialyzed against distilled water. Whereas Huotari *et al.*[14] found two exo-(1→3)-β-D-glucanase peaks upon chromatography, we found only one glucanase, corresponding to the first one eluted in their chromatographic profile[14]. The concentrated preparation was then fractionated by gel chromatography (Bio-Gel P-200). McIlvain buffer[21] (0.1 strength), pH 4.6, was used to elute proteins from the gel bed of 2 × 65 cm. The protein profile was composed of one symmetrical peak, which coeluted with the enzyme activity. The protein fractions comprising the enzyme were combined, dialyzed against distilled water, and lyophilized.

## REFERENCES

1 N. HANDA AND K. NISIZAWA, *Nature*, 192 (1961) 1078–1080.
2 N. MAEDA AND K. NISIZAWA, *J. Biochem. (Tokyo)*, 63 (1968) 199–206.
3 T. E. NELSON, J. V. SCALETTI, F. SMITH, AND S. KIRKWOOD, *Can. J. Chem.*, 41 (1963) 1671–1678.
4 J. EBATA AND Y. SATOMURA, *Agric. Biol. Chem.*, 27 (1963) 478–483.
5 J. JOHNSON, S. KIRKWOOD, A. MISAKI, T. E. NELSON, J. V. SCALETTI, AND F. SMITH, *Chem. Ind. (London)*, (1963) 820–822.
6 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
7 R. YAMAMOTO, N. SAKURAI, K. SHIBATA, AND Y. MASUDA, *Plant Cell Physiol.*, 21 (1980) 373–381.
8 T. USUI, N. YAMAOKA, K. MATSUDA, K. TUZIMURA, H. SUGIYAMA, AND S. SETO, *Agric. Biol. Chem.*, 39 (1975) 1071–1076.
9 Y. INOUE AND R. CHUJO, *Carbohydr. Res.*, 60 (1978) 367–370.

10 J. J. MARSHALL, *Carbohydr. Res.*, 34 (1974) 289–305.
11 R. YAMAMOTO AND D. J. NEVINS, *Carbohydr. Res.*, 67 (1978) 275–280.
12 D. J. NEVINS, R. YAMAMOTO, AND D. J. HUBER, *Phytochemistry*, 17 (1978) 1503–1505.
13 D. J. NEVINS AND R. YAMAMOTO, unpublished data.
14 F. I. HUOTARI, T. E. NELSON, F. SMITH, AND S. KIRKWOOD, *J. Biol. Chem.*, 243 (1968) 952–956.
15 E. T. REESE, F. W. PARRISH AND M. ETTLINGER, *Carbohydr. Res.*, 18 (1971) 381–388.
16 E. T. REESE, *Recent Adv. Phytochem.*, 11 (1977) 311–367.
17 P. ALBERSHEIM, D. J. NEVINS, P. D. ENGLISH, AND A. KARR, *Carbohydr. Res.*, 5 (1967) 340–345.
18 H. BJÖRNDAL, C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem., Int. Ed. Engl.*, 9 (1970) 610–619.
19 H. O. BOUVENG, H. KIESSLING, B. LINDBERG, AND J. MCKAY, *Acta Chem. Scand.*, 16 (1962) 615–622.
20 J. E. HODGE AND B. T. HOFREITER, *Methods Carbohydr. Chem.*, 1 (1962) 388–389.
21 T. C. MCILVAINE, *J. Biol. Chem.*, 49 (1921) 183–186.
22 M. SOMOGYI, *J. Biol. Chem.*, 195 (1952) 19–23.
23 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature*, 166 (1950) 444-445.