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Studies on the cell-wall mannan of the siphonous green algae, *Codium latum* *

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ABSTRACT

Cell-wall mannan from *Codium latum* was completely solubilized as methylol mannan from their microfibrils by treatment with paraformaldehyde–Me_2SO systems at 150 °C, and it was purified by gel-permeation chromatography with Toyopearl gel under Me_2SO elution. Mannan was regenerated by dilution of the purified methylol mannan with water or methanol. The elution profile of regenerated mannan on gel permeation chromatography under Me_2SO or the IR spectrum of the deuterium-exchanged, fractionated mannan revealed that the cell-wall mannan was aggregated as higher molecular weight material through intermolecular hydrogen bonding to form a difficultly soluble material. The results of structural determination on the purified mannan, including periodate oxidation and permethylation analysis, revealed a (1 → 4)-β-linked linear polysaccharide, as well as other cell-wall microfibril polysaccharides.

INTRODUCTION

The cell-wall polysaccharides of the microfibrils obtained from siphonous green algae were not cellulosic, but were xylan or mannan, and these characteristics of the algae have been discussed by some authors as being useful because of the taxonomic significance in the phylogenetic evaluation of these algae[2]. Structural investigations of purified cell-wall xylan obtained after successive chromatographic procedures under alkaline conditions were carried out on the microfibrils from *Bryopsis maxima*[3–5]. Since the cell-wall mannan from *Codium*, *Derbesia* and *Halicoryne* were known to be remarkably insoluble in commonly used solvents,

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they were difficult to purify. In the previous investigations, cell-wall mannan was partially solubilized with 50% zinc chloride from algal microfibriles, and the structure of the precipitated mannan after addition of acetone to the extract was tentatively proposed as a (1 → 4)-β-mannan on the basis of its negative rotation and from its periodate oxidation data[4]. On the other hand, however, it was pointed out that the sample obtained by this extraction method was contaminated with non-carbohydrate material; therefore, the residual weed was treated by another procedure[6] in which it was stirred with 20% NaOH for 5 h at 80°C under nitrogen, and the cell-wall polysaccharide from this extract was isolated as its copper complex. By identification of oligosaccharides from the sample thus obtained, partial structural determination indicated a (1 → 4)-β-mannan.

As well as the mannan, many polysaccharides, including those having strong inter- and intra-molecular hydrogen bonding, are insoluble in commonly used solvents, especially water. Some of these compounds may be dissolved only under extreme conditions that require high temperature, highly acidic or basic solvents, or a high concentration of chaotropic anion solution, which leads to degradation of the compound. Moreover, solubilization is often achieved only after a long treatment time. By using such extreme conditions that lead to degradation of the compounds, almost no information is available regarding the whole structure of the mannan. Moreover, such drastic and extreme solvents were usually difficult to incorporate into liquid chromatographic procedures. Therefore, the application of an inert and expeditious solvent to dissolve these compounds, and the use these same solvents in chromatographic systems are desired for the preparation or analysis of the polysaccharide. Unless the cell-wall mannan could be dissolved in an inert and expeditious solvent, purification procedures were difficult, and elucidation of its chemical properties was also impossible. Recently, two procedures have been realized as excellent, nondegrading solubilization techniques for cellulose. One is a procedure in which cellulose is dissolved in a cyclic amine oxide such as *N*-methylmorpholine-*N*-oxide (MMNO) or *N*-methylpiperidine-*N*-oxide, etc.[7]. The other consists of a solubilization technique in which cellulose is dissolved in dimethyl sulfoxide (Me_2SO) containing an equivalent of paraformaldehyde (PF)[8]. The procedures outlined herein were applied to cellulosic materials, including cell-wall mannan, which were successfully dissolved into solution. The chromatographic purification and structural determinations of mannan were then performed. The results obtained will be described in this paper.

RESULTS AND DISCUSSION

When the cell-wall fibrous materials from *Codium latum* were treated according to the previously described procedures, solubilization of 12.4% for zinc chloride or 11.2% for hot alkaline extraction resulted. Among the potential polar solvents mentioned above for the *Codium* cell-wall mannan, the PF–Me_2SO system proved to be better than the MMNO system because the reaction products obtained from

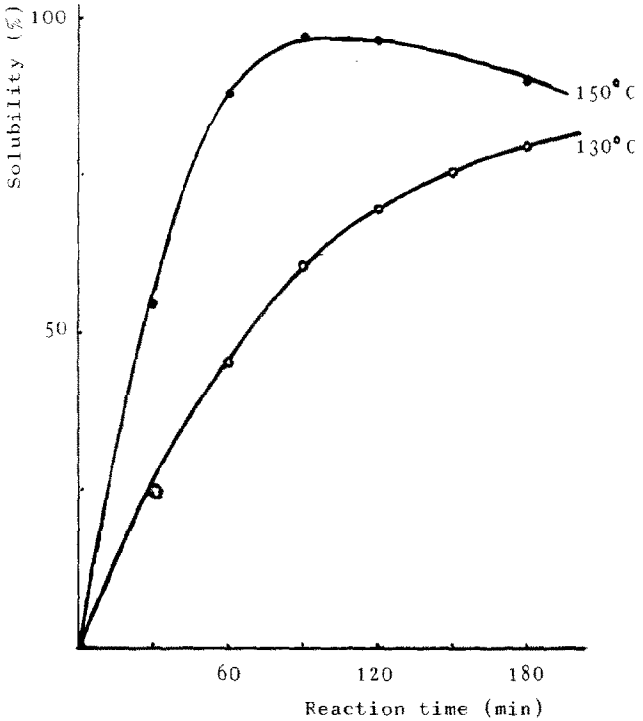

Fig. 1. Time course of solubilized reaction of *Codium* microfibriles by PF–Me_2SO system at different temperatures.

the MMNO procedure resulted in a very viscous solution that was impossible to directly apply to gel-permeation chromatography using Me_2SO for elution. Me_2SO has been known and used as a good, aprotic polar solvent for the effective extraction of hemicellulose[9]. More recent developments include the effecting of a more rapid dissolution of cellulose by heating the materials with batches of PF (which decompose readily to formaldehyde) instead of the introduction of formaldehyde gas, which is bubbled into the mixture from an external thermal decomposition of PF[8]. During this process, formation of methylol cellulose was recognized, and the regeneration of dissolved cellulose was accomplished by dilution with water or methanol. This finding showed that the PF–Me_2SO system might be capable of dissolving the *Codium* cell-wall mannan.

Although the optimum temperature of the solvent for dissolution of cellulose was shown to be 100–130°C, in the case of cell-wall mannan, such temperatures often lead to the formation of insoluble polyoxymethylene, and it is impossible to complete the dissolution of the sample. Therefore, a much higher temperature is desired. As shown in Fig. 1, when the solubilizations were compared at 130 or 150 °C for the microfibrill of *Codium*, the amount of soluble polysaccharide that was found in the clear supernatant reached a maximum value that was estimated to be

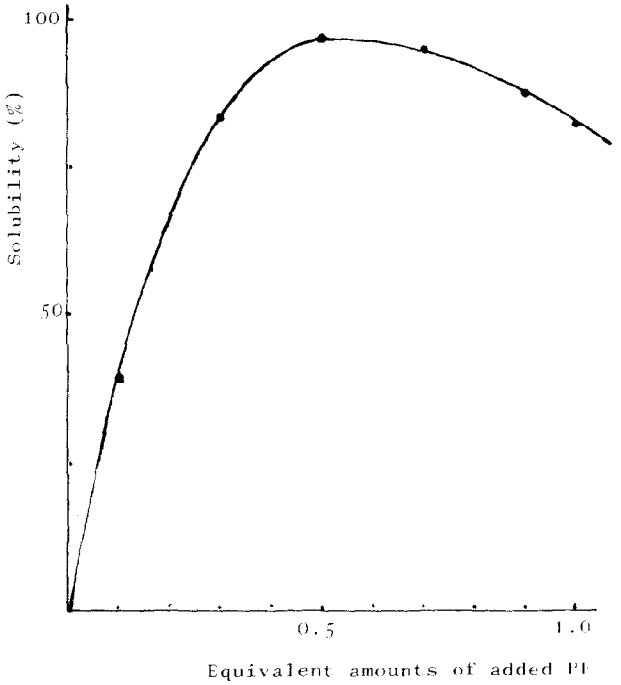

Fig. 2. Relationship between added amounts of PF and solubility of *Codium* microfibriles at 150 °C.

~ 96%, and it appeared that the mixture achieved complete dissolution of cell-wall mannan after 1.5 h of reaction time at 150°C. However, a slight decrease of soluble polysaccharide was detected after further, prolonged treatment. This decrease, as indicated by a decrease in optical density in a phenol–H_2SO_4 assay, is probably due to the partial thermal decomposition of the polysaccharides. The effect of various ratios of PF to microfibrils in Me_2SO was determined (Fig. 2). The maximum solubility was obtained when 0.5 equiv of PF was added to the sample. Thermal decomposition might have been accelerated by addition of additional PF, since the larger the amount of PF added, the greater was the decrease in the amount of soluble mannan that was determined by the phenol–H_2SO_4 assay.

When completely solubilized cellulose was lyophilized, its IR spectrum (KBr disk) had a characteristic absorption band for methylol groups (1380 cm^{-1})[8]. Since the quite similar IR absorption bands were also found at 1380 cm^{-1} for the solubilized and lyophilized mannan, solubilization of cell-wall mannan by this method must have also occurred by means of formation of methylol mannan. Cell-wall mannan was recovered by precipitation upon addition of methanol or water to the mixtures. The recovery of mannan from the fibrous materials was estimated to be about 88%.

GLC analysis of the hydrolysates of the fibrous materials in the presence of an internal standard indicated 97% of D-mannose, ~ 1.2% of D-glucose and a trace of D-xylose. Since the fibrous materials from the algae gave a faint blue-black color with I_2 reagent, appreciable amounts of D-glucose presumably should be derived from amylose in the starch grains contaminated in the fibrous materials, and D-xylose could be from hemicellulose contamination during the cell-wall preparation. However, the hydrolysates of the recovered, lyophilized sample, yielded nearly 100% of the expected D-mannose.

On the other hand, the recovery of mannan from fibrous materials by the treatment with MMNO was estimated at 84%. Noticeable degradations that were observed by the weight loss upon recovery of the mannan by this method demonstrate that the solvent action does not involve degradation from the reducing end by a recurrent, stepwise elimination in a "peeling" type of mechanism[7], but works from cleavage of internal bonds.

In our previous work[3], a semi-rigid hydrophilic porous vinyl polymer used for packing material (commercially available as Toyopearl) in gel filtration chromatography was useful under dilute alkaline conditions for long development times. Furthermore, the Toyopearl gel demonstrated that rather hard materials were more resistant to pressure than other gel types such as dextran gel, and it seemed to be suitable for incorporation into the high pressure columns of HPLC systems under various elution conditions, including the use of Me_2SO[1]. In fact, Me_2SO has been used as the mobile phase of gel permeation chromatography (GPC) for proving the helical conformation of "Pestalotan" by elution profiles[10]. With dynamic packing methods[11], a well-packed column with the gel settling into a uniform bed free of voids or channels was obtained within a shorter time than with the usual gravitational methods. The column pressure reached a constant value of 4.5 kg/cm^2 within 30 min, and the column showed no change in bed volume or flow rate throughout continuous and long periods of flow. The height equivalent to theoretical plate (HETP) was estimated as 520 for the Toyopearl HW-65 column when D-glucose as the totally permeating peak was eluted at a flow rate of 0.5 mL/min. The HETP value obtained at this flow rate was a minimum one, and it increased at both lower and higher flow rates. Me_2SO solutions of Pullulan P-82 (commercially available molecular weight marker, Showa Denko) under the same elution conditions were eluted in the order of their molecular size, and the linear calibrations for the retention volume and the logarithm of the apparent molecular weights (M_r) were obtained for the eight samples of pullulans whose M_r range was 9000 to 980000. When the cell-wall mannan regenerated from the PF–Me_2SO procedures was eluted in preparative gel-permeation chromatography, a sharp, symmetrical single peak was obtained, and the results revealed size homogeneity. By adding either water or methanol to the elute and washing the precipitates several times with water, followed by lyophilization, purified cell-wall mannan was obtained. Since the previously detected contamination by trace amounts of D-glucose and D-xylose in the hydrolysates were completely eliminated from the hydro-

lysate of purified mannan through gel-permeation chromatography, the fibrous materials thus obtained seemed to be composed, not of glucomannan as in a previous report for *C. digitata*[6], but of pure mannan. Elemental analysis was as follows: Found, C, 44.12; H, 6.36%; calcd for $C_6H_{10}O_5$: C, 44.44; H, 6.22%. In the IR spectrum of purified cell-wall mannan, splitting of absorption bands such as 3530, 3490, and 3380 cm^{-1} were observed in the range of the intra- and inter-molecular hydrogen bonds of the OH stretching region, indicating that the sample is in a highly crystalline state[12].

The regenerated purified cell-wall mannan thus obtained was easily soluble in hot anhydrous Me_2SO. When the Me_2SO solution of the purified cell-wall mannan was subjected to analytical Toyopearl gel-permeation chromatography using the Me_2SO elution system, it emerged as two peaks as shown in Fig. 3. These peaks were separated by large amounts of higher molecular weight materials that showed a partition coefficient, K, in the range of 0.43–0.52. [Note: $K = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the compound, V_0 is the void volume determined using Blue Dextran-2000 (Pharmacia), and V_t is the totally permeating bed-volume as determined by D-glucose]. On the other hand, small amounts of lower molecular weight mannan eluted in the $K = 0.9–0.8$ region. Their M_r were estimated to be ~ 84 000 for the former and to be ~ 28 000 for the latter by calibration of the Me_2SO solution of molecular weight markers obtained under the same elution conditions. These were separately fractionated, and after freeze-drying white, fluffy, fibrous solids were obtained. When the higher molecular weight mannan was dissolved again in the anhydrous hot Me_2SO and subjected to gel-permeation chromatography in the same manner, it emerged as two peaks under the same elution profiles as before. Both fractions showed quite similar elution volumes to those previously obtained, with large amounts of the higher molecular weight mannan and the small amounts of the lower molecular weight mannan, respectively. On the other hand, when the lower molecular weight mannan was dissolved into hot Me_2SO and subjected to gel-permeation chromatography, it was eluted as a single peak in the same elution volume as before.

In the IR spectrum of the lower molecular weight mannan, a decrease of splittings and broadening of the absorption bands in the OH stretching region between 3800 and 2800 cm^{-1} due to the reduction in the degree of crystallinity were observed. However, the IR spectrum of the freeze-dried, higher molecular weight mannan still remained, indicating the crystalline state in the OH regions. These facts suggested that the higher molecular weight mannan was aggregated through intermolecular hydrogen bonding and part of it was dissociated by hot Me_2SO treatments into lower molecular weight mannan. The degrees of the inter- or intra-molecular hydrogen bonds in the lower and higher molecular weight eluted mannan were compared by determining the rate of hydrogen–deuterium exchange by IR spectroscopy[13]. After each of the fractionated mannans were lyophyilized and exposed to D_2O for 4 h, a slow exchange reaction process was observed for the higher molecular weight mannan, and it still showed characteristic

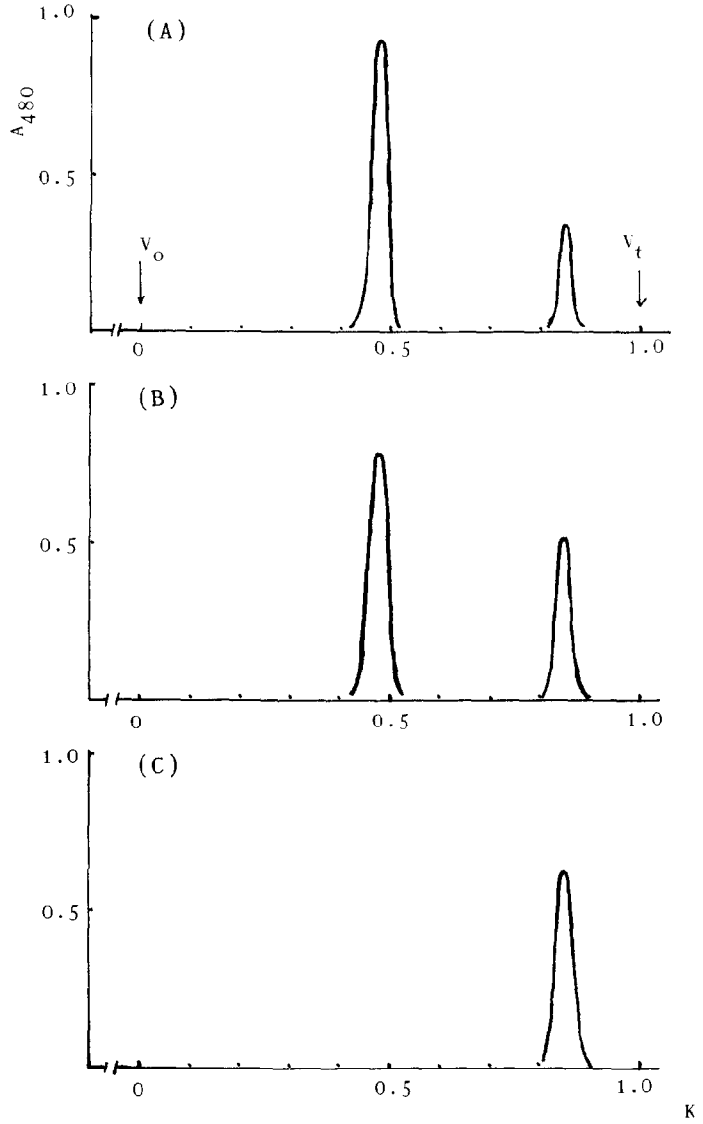

Fig. 3. Elution profiles of *Codium* cell-wall mannan through the Toyopearl gel-permeation chromatography under the Me_2SO elution systems. (A) Me_2SO solution of regenerated cell-wall mannan from *Codium* cell wall microfibriles. (B) Hot Me_2SO solution of higher molecular weight mannan from (A). (C) Hot Me_2SO solution of lower molecular weight mannan from (A).

OH stretching bands in the IR spectrum. Deuterium exchange reaction in the lower molecular weight mannan that eluted, however, proceeded rapidly based on the complete disappearance of the OH stretch band and the appearance of new OD stretch band within 30 min. The results clearly demonstrate that the lower molecular weight mannan that eluted has a more open amorphous structure

accessible to D_2O. However, the higher molecular weight mannan that eluted was more associated through intermolecular hydrogen bonds so as to form stable strand structures involving tight bundles. Since *Codium* cell-wall mannan was so difficult to solubilize by ordinary solvents, the degree of association depends on the hydrogen bonds between the neighbouring mannan chains, which should be oriented to yield maximum stability.

Since the purified mannan showed $[\alpha]_D - 45.6°$ (*c*, 1.2, Me_2SO) and 890 cm^{-1} (type 2b absorption in the IR spectrum, a β-configuration is indicated. The chemical shift for an axial anomeric proton in D-mannose residues in the $^4C_1$(D) conformation resonates at δ 4.783 ppm also indicates the β anomer of mannan. This signal appeared in the spectrum as a relatively broad singlet due to the small value of $J_{1,2}$ (0.7 Hz; H-1*a*, 2*e*).

After 96 h of periodate oxidation, the cell-wall mannan consumed 0.97 mol (nearly 1 mole) of periodate per mannose unit; the dp of the cell-wall mannan was estimated as ~ 190 by the detection of formaldehyde released from the reducing end with a chromotropic acid reagent[14]. GLC of the per-*O*-trimethylsilyl oxime derivatives derived from the complete acid hydrolyzate of the product of Smith degradation yielded two peaks. The first one was the glycolaldehyde oxime derivative (t_R 4.0) resulting from the oxidation of the C-1 to C-2 of 1 → 4 linked D-mannose residues, and the second peak was identified as the erythritol derivative from the C-3 to C-6 of 1 → 4 linked D-mannose residues, respectively. A similar result was also obtained from the permethylation analysis. The GLC of the partially methylated mannitol acetates revealed two peaks of t_R (relative to 1,5-$(Ac)_2$-2,3,4,6-$(Me)_4$-D-glucitol) 1.01 and 0.88 in a molar ratio of 1 : 180. These components were identified by GLC–MS as 2,3,4,6-tetra-*O*-methyl-D-, and 2,3,6-tri-*O*-methyl-D-mannose, respectively. Since no di-*O*-methyl mannose was found in the hydrolyzates of permethylated mannan, the cell-wall mannan is composed of solely linear β-linked polysaccharides, as are the cell-wall microfibrils of cellulose, the chitin from fungal cell wall, and (1 → 3)-β-xylan from *Bryopsis maxima*[5]. Similarly they aggregate with the neighbouring linear polysaccharide molecules to stabilize by intermolecular hydrogen bonding to form fine strands of microfibrils.

EXPERIMENTAL

General methods. — All evaporations were conducted at reduced pressure at a bath temperature not exceeding 40°C. Specific rotations were determined at 20°C with a JASCO DIP-360 digital polarimeter. Infrared (IR) spectra were recorded on a JASCO IR-700 infrared spectrophotometer using KBr disks for free sugars and a liquid film for permethylated sugars.

Materials. — *Codium latum* was collected in June, 1989 at Shirahama, Shizuoka Prefecture, along the Pacific coast near Tokyo. Macro-epiphyta were carefully removed by washing in sea water and air-drying. Anhydrous Me_2SO was obtained after distillation and was stored over 4A molecular sieves.

Acid hydrolysis and gas–liquid chromatography (GLC). — A known amount of material was well mixed in 72% H_2SO_4 and kept for 30 min at room temperature. The mixture was diluted with water to a final concentration of 2 N H_2SO_4, and hydrolysis of mannan was achieved by heating for 2 h in a boiling water bath. The sugar constituents produced were converted to the alditol trifluoroacetates[15] and determined by GLC. GLC was performed on a JEOL-20K FP gas–liquid chromatograph equipped with a flame-ionization detector and a packed column (2 mm i.d. × 100 cm) of 1.5% DC QF-1 (fluorinated silicone) on Chromosorb W or a glass capillary column (0.28 mm i.d. ×30 m) of DIASCOTT SV (OV-17). Nitrogen was used as the carrier gas with a flow rate of 30 mL/min. The temperature of the injection and detector ports was 180°C and that of the column was 120°C. The sugar constituent was determined by comparison of the peak areas relative to an internal standard of alditol trifluoroacetate prepared from 2-deoxy-D-*arabino*-hexose.

High-performance gel-permeation chromatography using Toyopearl gel under the Me_2SO elution system. — A liquid chromatograph, HLC-803 (Tosoh), was used at room temperature with a flow rate of 0.5 mL/min.

In order to swell and to remove any contaminated fine particles, the wet gel of Toyopearl HW-65 and -75 (super fine grade) were treated by repeated suspension, settling, and decantation in Me_2SO, and the gel was finally suspended in Me_2SO as a slurry after deaeration. The column was prepared by dynamic packing methods[11]. An analytical-scale stainless steel column (4 mm i.d. × 60 cm) that was equipped with Swagelok fittings and a 2-μm SS filter (Altex) was attached to a slurry reservoir of larger diameter, and the whole system was filled with Me_2SO before packing. The reservoir was filled with a thick slurry of Toyopearl gel in Me_2SO, and it was displaced into the column by pumping until the pressure reached a constant value. After the column was equilibrated by elution with Me_2SO, the sample was loaded directly onto the column through the sample introduction system. A series of PF–Me_2SO solutions of pullulan (Shodex P-82) was used for calibration of the molecular weight against the elution volumes, and the fractionated eluent was colorimetrically monitored by the phenol–H_2SO_4 method[16]. The preparative-scale column (18 mm i.d. × 40 cm) was packed in the same way.

Preparation of cell-wall fibrous materials. — The air-dried algal sample was soaked in ten volumes of distilled water for 1 h, homogenized and centrifuged. Residual algae was successively treated with 1.25% H_2SO_4 for 30 min at 100°C and 1.25% NaOH for 30 min at 100°C. The fibrous material thus obtained, after thorough water washing and chlorite bleaching, was lyophilized.

Me_2SO–PF treatments of fibrous material. — All mixing and handling operations were performed in confined, dry environments. The mixture of fibrous materials (50 mg) and Me_2SO (5 mL) in a Teflon-lined screw vessel was deaerated, then PF powder (0.25 g) was added to the mixed solution at room temperature. The mixture was then heated with rapid stirring from 120 to 150°C over a period of

2–3 h. During the reaction procedures, an aliquot of solution from the reaction vessel was removed and centrifuged, and the sugar content in the supernatant was colorimetrically estimated by the phenol–H_2SO_4 method. Me_2SO–PF solutions without fibrous materials were treated in the same way and used as controls.

One-half of the mannan solution was directly subjected to gel-filtration chromatography using the Me_2SO elution systems, and the other half of the solution was exhaustively dialyzed against distilled water. The mannan that precipitated in the dialysis bag was gravimetrically estimated after thorough washing with water and lyophilization.

Solubilization of fibrous material in 4-methylmorpholine N-oxide (MMNO). — The fibrous materials (0.1 g) in MMNO monohydrate (2.5 g) were heated for 30 min at 120°C in a tightly stoppered tube under nitrogen[18]. After solubilization of the samples, Me_2SO (10 mL) was added, and the mixtures were cooled to room temperature. The solution was exhaustively dialyzed against distilled water, and the precipitated mannan in the dialysis bag was lyophilized.

Purification of cell-wall mannan through preparative HPLC of GFC. — After solubilization of the sample by PF–Me_2SO treatment and centrifugation, a Me_2SO solution of the methylol derivative of cell-wall mannan in the supernatant was utilized in preparative gel filtration column chromatography using the Me_2SO elution systems. For the Me_2SO solution of pullulan P-10 (M_r, 10000), K was estimated as 0.68 for the HW-65 gels and 0.42 for the HW-75 gel. The results showed that both of the gels have molecular sieve effects for a wide range of polymers under the present conditions.

^1H NMR spectroscopy. — The ^1H NMR spectra of the methylol or regenerated mannan were recorded with a Bruker A-400 spectrometer operating at 400 MHz in the pulsed, FT mode, at ambient temperature for solutions in Me_2SO-d_6 at a concentration of 1%. The chemical shifts are expressed downfield from the signal of internal tetramethylsilane.

Periodate oxidation. – Samples of regenerated mannan (10 mg) were suspended in 0.1 M acetate buffer (2 mL, pH 5.6) and a 0.1 M $NaIO_4$ solution (2 mL) was added to make a final concentration of 0.05 M. Portions (20 μL) of the mixture were removed at time intervals and diluted with water to 10 mL. The amount of periodate consumed per anhydrous mannose residue was determined[18] from the extinction of the resulting solution at 223 nm. Cellulose powder (Whatman) was also oxidized under the same conditions as the controls.

After the periodate consumption had reached a constant value, an aliquot of the reaction mixture was diluted with water, the excess periodate was reduced with dil H_2SO_4 and $Na_2As_2O_3$, and formaldehyde was determined at A_{570} after addition of a chromotropic acid reagent[14].

Smith degradation[19]. — After the periodate oxidation was completed, the mixture of oxidized products was reduced with $NaBH_4$, acidified with acetic acid, dialyzed and concentrated. The reduced polyhydroxy compound was hydrolyzed with 0.25 M HCl for 1 h at 80°C. Portions (2 mL) of the hydrolyzate were treated

with hydroxylamine hydrochloride, pertrimethylsilylated, and analyzed by GLC using a capillary column of OV-17 at a temperature of 140°C.

Comparative determination of hydrogen–deuterium exchange in fractionated mannan. — The lower molecular weight and higher molecular weight mannans that eluted from gel-permeation chromatography were suspended in deuterium oxide, and the disappearance of the OH stretching bands during the hydrogen–deuterium exchange reactions of polysaccharide were measured by IR spectrophotometry over 4 h[20]. A matched pair of CaF_2 liquid cells of 0.05 mm path length were used, with one cell serving as the D_2O reference. Frequent short scans of the spectrum starting from 4000 to 2000 cm^{-1} were performed after appropriate periods of exchange, and the cells were removed from the light beams in the spectrophotometer between the scans to minimize heating effects.

Permethylation and GLC–MS. — Mannan (~ 10 mg) was dissolved in Me_2SO and permethylated three times using Hakomori's method[21]. The extent of permethylation was monitored bt IR spectroscopy for OH bands and methoxy content[22].

The acid hydrolyzates of permethylated mannan were converted to partially methylated mannitol acetates and analyzed by GLC using a packed glass column (2 mm i.d. × 1 m) containing 3% ECNSS-M on Gaschrom Q (80–100 mesh) with an isothermal column temperature of 180°C. The peaks on the chromatogram were identified by their retention times (t_R), relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in comparison with reported values. The EIMS spectrum of partially methylated mannitol acetates were obtained using a Hitachi M-80A mass spectrometer and a GLC inlet system. The packed column of 3% OV-17 (2 mm i.d. × 1 m) was used at an oven temperature of 190 °C. The mass spectra were recorded at an inlet temperature of 230°C, an ionizing potential of 20 eV, ionizing current of 110 μA, and an ion source temperature of 270 °C. Identification of fragmentation patterns was made by comparing major mass-spectral ions with data in the literature[23].

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