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GLUCOMANNAN SYNTHASE FROM SUSPENSION CULTURES OF *PINUS SYLVESTRIS*

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Key Word Index—*Pinus sylvestris*; Pinaceae; Scots pine; glucomannan synthesis; cell walls.

Abstract—The activity of a glucomannan synthase was measured in particulate preparations obtained from suspension cultured cells of *Pinus sylvestris*. The product was characterized and found to correspond to the β 1 → 4 linked glucomannan present in the pine secondary wall. The specific activity of the synthase was found to increase in culture conditions favouring tracheid formation.

INTRODUCTION

The β 1 → 4 linked linear glucomannans are a major component of the hemicellulose fraction of the secondary cell wall in gymnosperms but are only present in minor amounts in the cell walls of angiosperms [1] and in the primary walls of pine [2]. The *in vitro* synthesis of glucomannans has been extensively examined using particulate preparations from various angiosperm species but little attention has been given to the synthesis in gymnosperms [3–5].

It has been demonstrated that the cell wall of suspension cultured pine cells contains a glucomannan the deposition of which can be increased by culture in the induction medium favouring tracheid formation [2]. An essential step in the further investigation of the manner in which glucomannan deposition is regulated is the identification of the particular stage during synthesis where control could be effected. Previous studies of the enzymes involved in the pathways for the supply of precursors to the polysaccharide synthase reactions have demonstrated little variation in these activities which was related to the alterations in deposition of polysaccharides during differentiation. However the synthases in angiosperms were found to exhibit changes in activities that were consistent with the changes in wall composition during development [6]. It is possible that similar mechanisms would operate for glucomannan synthesis in gymnosperms and an examination of the glucomannan synthase represents an appropriate approach to this aspect of polysaccharide synthesis in pine.

RESULTS

Synthesis of ^{14}C-*glucomannan*

Particulate preparations from suspension cultured cells of *P. sylvestris* grown in induction medium incorporated radioactivity from GDP-[U^{14}C]-mannose into material which was immobile on electrophoresis at pH 3.5 and during chromatography in solvent A. The radioactivity was not soluble in chloroform–methanol (2:1), water or 3% sodium hydroxide. But 71% of the radioactivity present could be solubilized in 17% sodium hydroxide/4% boric acid. This demonstrated that the product was not a neutral monosaccharide, small oligosaccharide or a lipid-linked saccharide. All the radioactivity was retained during dialysis for 12 hr against water so that the product was polymeric. Resistance to degradation by pronase indicated that the material was not a glycoprotein.

Characterization of the synthesized ^{14}C-*polysaccharide*

Total hydrolysis of the radioactive polysaccharide synthesized from GDP-[U^{14}C]mannose gave principally ^{14}C-mannose. A small amount of the radioactivity (8%) was present as ^{14}C-glucose which suggested that a membrane bound epimerase activity was present.

Oligosaccharides were prepared from the radioactive glucomannan by acetolysis, mild acid hydrolysis and enzymic hydrolysis with cellulase. Hydrolysis by cellulase released three fractions which were separated by chromatography in solvent B. The first two peaks had mobilities corresponding to mannose and mannobiose and the third peak represented mannotriose. The radioactivity from each peak gave a ratio of mannotriose to mannobiose to mannose of roughly 2:2:1. The cellulase preparation used did not have any appreciable mannanase activity and did not hydrolyse the $\beta(1 \rightarrow 4)$ mannan from ivory nut. The separation of the hydrolysates produced by acetolysis and 0.1 M HCl revealed a series of disaccharides which had $R_{\text{cellobiose}}$ values corresponding to glucosyl-mannopyranoside, mannobiose, cellobiose and mannosyl-glucopyranoside [4]. Further peaks closer to the origin of the chromatogram probably contained mixtures of trisaccharides and higher oligosaccharides. The relative amounts of radioactivity present in each of the peaks are shown in Table 1. The disaccharides were eluted from the chromatogram, hydrolysed, and the amount of radioactivity present as ^{14}C-glucose was established (Table 1). The presence of both glucose and mannose in a single disaccharide confirmed that a glucomannan was synthesized.

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Table 1. Separation of products of acetolysis in ^{14}C polysaccharide synthesized from GDP-[U^{14}C]-mannose and GDP-[U^{14}C]-glucose using chromatography in solvent C

Peak	R Cellobiose	%Total radioactivity	Molar ratio Man:Glu
Oligosaccharides	0.25	11.6	—
Trisaccharides	0.50	25.5	—
Mannosylglucose	0.84	4.5	—
Cellobiose	1.00	8.9	—
Mannobiose	1.16	38.9	1:0
Glucosylmannose	1.45	10.4	1.3:1

Glucomannan linkage

Periodate oxidation of the glucomannan for six days followed by reduction and total hydrolysis yielded principally erythritol (81%) and a small amount of mannose (19%).The principal linkage in the glucomannan was therefore 1 → 4. Further oxidation for 10 days reduced the amount of radioactivity present in mannose to 13% so that the presence of this undegraded mannose was probably due to incomplete oxidation rather than the presence of 1 → 3 linkages.

Analysis of the partially methylated sugars produced from the ^{14}C-glucomannan showed that the only tri-*O*-methyl sugars formed were 2,3,6 tri-*O*-methylmannose (58%) and 2,3,6 tri-*O*-methylglucose (14.3%) which demonstrated that only 1 → 4 links were present. The small amount of tetramethyl sugar (2.3%) obtained corresponded to the terminal residues and suggested a degree of polymerization of approximately 50. The radioactivity recovered in dimethyl sugars (24.2%) included both mannose and glucose derivatives and could arise from substitution by side residues or incomplete methylation.

Factors influencing glucomannan synthesis

The variation in glucomannan synthesis with the amount of particulate preparation added was examined over the range of protein concentrations used in the assay. A linear relationship was demonstrated between incorporation of radioactivity and the amount of protein up to 100 μg.

The incorporation of radioactivity was measured over a range of pH values from 5.0 to 8.0 using K-Pi buffer and a broad optimum in the region of pH 7 was shown. Glucomannan synthesis was measured at various temperatures between 20 and 50° and the highest levels of synthesis were observed around 30°. A range of Mg^{2+} concentrations from 0.2 to 50 mM gave increasing rates of glucomannan synthesis with appreciable stimulation following from increased concentrations up to 20 mM (Fig. 1). Other divalent cations tested, Mn^{2+}, Ca^{2+} and Co^{2+} had a lesser effect.

A number of other substances were added to the standard assay to determine whether they had any effects on glucomannan synthesis (Table 2). No effect was apparent on addition of AMP, ATP, UDP-glucose, or a mixture of disaccharides from partial hydrolysis of glucomannan. Some slight stimulation was observed on addition of UDP-galactose.

The addition of 5 μl of the supernatant from the 100 000 *g* centrifugation which was used to pellet the particulate fraction caused significant stimulation of the glucomannan synthase activity though no synthesis of glucomannan was observed when the supernatant alone was assayed. There was a slight increase in the amount of ^{14}C-glucose obtained (from 8% to 23%) from the synthesized polysaccharide when supernatant was added suggesting that epimerase activity was present in the supernatant. This was not sufficient to account for the increase in polysaccharide synthesis observed. A small amount (14%) of the radioactivity that was incorporated could also be solubilized by incubation with pronase so that possibly some of the polysaccharide was associated

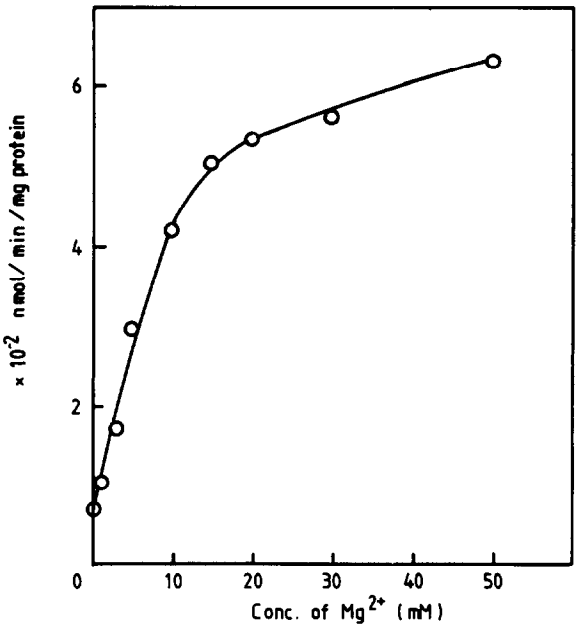

Fig. 1. Effect of Mg^{2+} concentration on incorporation of radioactivity from GDP-[U^{14}C]mannose.

Table 2. The effect of various substances on the activity of glucomannan synthase in particulate preparations

Addition to incubation as specified in text	Glucomannan Synthase Activity n mol /min/mg × 10^{-2}		
Supernatant	8.89	s.d.	1.01**
Hydrolysate	5.45	s.d.	0.11
UDP-galactose (0.17 mM)	6.47	s.d.	0.31*
UDP-glucose (0.17 mM)	5.03	s.d.	0.40
ATP (1.7 mM)	4.97	s.d.	0.41
AMP (1 mM)	5.84	s.d.	0.36
Dolichol monophosphate	4.15	s.d.	0.24**
Bacitracin	2.55	s.d.	0.41**
Amphomycin	4.78	s.d.	0.21*
Without additions	5.61	s.d.	0.26

Student's *t* test: *$P < 0.02$, **$P < 0.005$ for statistical comparison with activity in absence of additions.

The values are means of three samples measured after incubation for 15, 30 and 45 min. The synthesis of glucomannan was linear over this period in all the experiments.

with protein. Glucomannan synthesis was slightly inhibited in the presence of dolichol monophosphate and inhibition was observed when bacitracin was added; amphomycin did not inhibit the synthase. In all the above conditions the amount of glucomannan synthesized increased in a linear fashion with respect to time in incubations of up to 60 min.

Kinetics of glucomannan synthesis

The effect of different concentrations of GDP-mannose on the glucomannan synthase activity was observed and an apparent K_m of 13.8 μM (s.d. 2.02) and a V_{max} of 0.113 nmol/min/mg (s.d. 0.004) were determined according to ref. [7] (Fig. 2). When GDP-glucose was present in the presence of the radioactive GDP mannose the synthesis of glucomannan remained linear with respect to time though a slight inhibition was apparent. The kinetics of the inhibition were determined at two concentrations of GDP-glucose and non-competitive inhibition was indicated with an approximate K_i of 1.9 μM.

Induction of glucomannan synthesis

The activity of glucomannan synthase was measured in particulate preparations obtained from suspension cultures grown in induction and maintenance media at three different times after subculture (Fig. 3). There was an increase in the activity of glucomannan synthase in cells cultured in induction medium which was associated with secondary wall deposition.

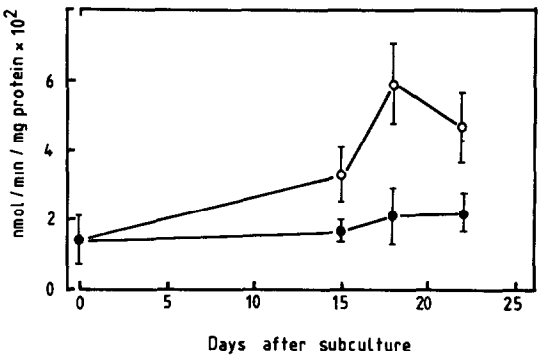

Fig. 3. The induction of glucomannan synthase activity in cell suspension cultured in, ●: maintenance medium and –O–: induction medium. The values are means of activity in preparations from three different cultures and the vertical bars represent s.d.

DISCUSSION

The particulate preparation from pine cell suspension culture has been shown to be capable of catalysing the synthesis of a β 1 → 4 linked glucomannan from GDP-mannose. The activity of the glucomannan synthase showed a similar dependance of pH and Mg^{2+} ions to the activity isolated from mung bean [3] but did not require the presence of Co^{2+} which was necessary for the glucomannan synthase from *Orchis morio* [8]. The synthesis of glucomannan observed was linear over periods of up to one hr in contrast to the activity from *Pisum sativum* [5] and *Phaseolus aureus* [9] where the initial rapid rate of synthesis decreased considerably after 10 min. With the enzymes obtained from the dicotyledons two K_m values were identified, from a low concentration component (below 3 μM GDP-mannose) and from a high concentration component of the activity above 50 μM GDP-mannose. With the enzyme from pine cell culture only a single value for K_m was identified with a value intermediate between the two previously reported values [9]. The lowest concentration of GDP-mannose used in the assays was 14 μM and it is possible that a low

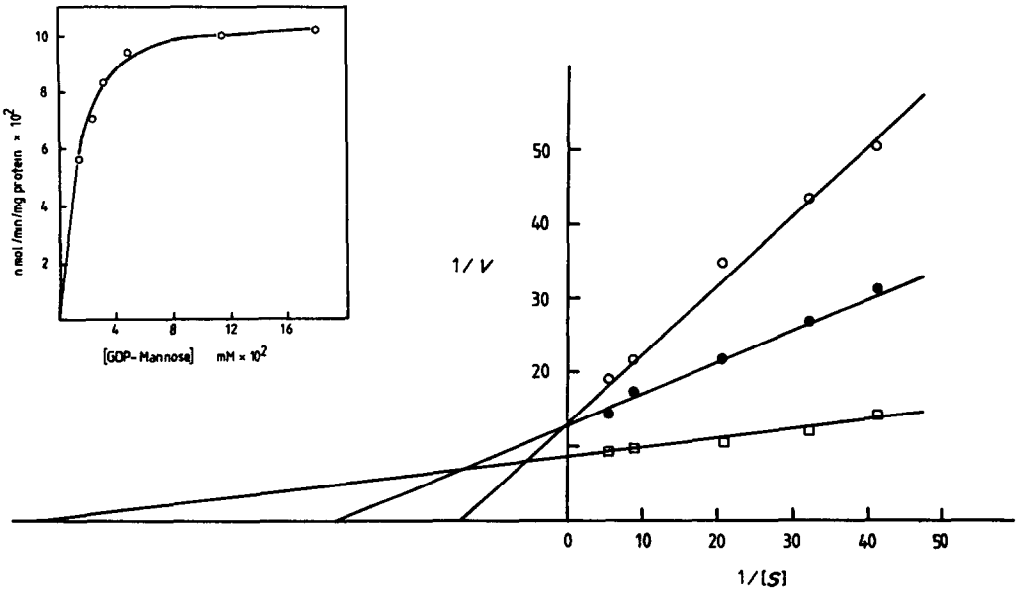

Fig. 2. Double reciprocal plot of effect of GDP-mannose concentration on the synthesis of glucomannan from GDP-[U^{14}C]-mannose; □: with GDP-mannose alone; ●: with 3.3 μM GDP-glucose and –O–: with 16.7 μM GDP-glucose. V is in units of nmol/min/mg protein x 10^2. S is the concentration of GDP-mannose in mM.

concentration component was not detected, but the double reciprocal plot showed no deviation from linearity in the low concentration region. GDP-glucose appeared to act as a competitive inhibitor of glucomannan synthesis from GDP-mannose but the inhibition was not measured with concentrations of GDP-glucose greater than the concentration of GDP-mannose where it has been suggested that uncompetitive inhibition can occur [9].

The stimulation of glucomannan synthase activity by the 100 000 *g* supernatant suggests that for full activity the enzyme requires some soluble component which is not present in the assay system. A limited number of other factors were tested for their influence on the synthetic activity but none of these gave a comparable stimulation.

The synthesis of manno-lipids and oligosaccharide-lipids has been observed from GDP-[$U^{14}C$]-mannose in membrane preparations from several species [10, 11]. These lipids are associated with the glycosylation of asparagine-linked glycoproteins, which have mannose rich chains and use dolichol phosphate derivative intermediates. The possibility of lipid intermediates participating in wall polysaccharide synthesis has been discussed [3], but no evidence exists for the involvement of lipid intermediates in hemicellulose synthesis. The antibiotics amphomycin and bacitracin are established inhibitors of dolichol-P-mannose formation [13], but could have additional effects on polysaccharide synthesis. While amphomycin had no effect on the glucomannan synthase the activity was inhibited by bacitracin. Bacitracin has been shown to be an inhibitor of β-glucan synthesis in cotton [14], but was found to stimulate β-glucan synthesis in tobacco where amphomycin was an effective inhibitor [15]. It was suggested that bacitracin could also influence the activity of membrane bound enzymes through its detergent properties. The glucomannan synthase was not stimulated by the addition of exogenous dolichol monophosphate and it is unlikely that this is involved as an intermediate in the polysaccharide synthesis.

The glucomannan synthesized gave a similar distribution of oligosaccharides on hydrolysis to those observed from the glucomannan obtained from *Phaseolus aureus* preparations [3, 9]. The proportion of mannose oligosaccharides obtained from cellulase hydrolysis differed slightly from the distribution of oligosaccharides obtained from *Picea abies* where mannotetraose was also observed in equal proportion to mannotriose [16]. The distribution of hexose residues in the glucomannan chain is thought to be due to random incorporation and as the glucomannan from the pine cell culture had a higher proportion of glucose than the corresponding pine polysaccharide produced in the intact plant a lower proportion of the longer mannose oligosaccharides would be expected. The pine glucomannan is known to include galactose side residues linked to the six position on mannose [17] and in view of the stimulation of the glucomannan synthase afforded by UDP-galactose it is possible that the particulate preparation could also catalyse the incorporation of galactose which could facilitate continued extension of the glucomannan chain. The variation of glucomannan synthesis activity from cells cultured in induction medium is in agreement with the observed changes in cell wall mannose contents and secondary wall formation found in cells grown in induction medium [2], and this suggests that the increased deposition of glucomannan in the secondary walls of differentiating cells is a consequence of the increased activity of the glucomannan synthase. A similar variation in the activity of the enzymes for hemicellulose synthesis has been found with glucomannan synthase in pine trees [18]. The precursors for glucomannan synthesis are GDP-mannose and GDP-glucose. GDP-mannose is formed from mannosyl-phosphate by a GDP-mannose pyrophosphorylase but there is no information on any variation in the activity of this enzyme during differentiation. The two precursors can be interconverted by the action of GDP-glucose-2-epimerase. This enzyme is known to be present in the particulate preparations [3, 18] and could epimerise sufficient of the exogenous GDP-mannose to maintain normal elongation of the glucomannan chain, since the synthesis of glucomannan could take place in the absence of added GDP-glucose. Variation in the activity of the 2-epimerase could alter the proportion of either sugar available for incorporation into the glucomannan. This could affect the overall level of synthesis if the synthase binds more readily to extending chains with a particular ratio of residues. Such indirect control of glucomannan synthesis may be possible particularly as GDP-glucose can act as an inhibitor of GDP-mannose incorporation. The increase in glucomannan synthase observed was sufficient to account for the increase in mannose content of the walls of cells cultured in the induction medium [2], and even if regulation of glucomannan synthesis can be effected by control of precursor levels the synthase remains a site of major control of glucomannan deposition during differentiation. It would also be expected that the actual increase in synthase activity in the cells undergoing differentiation would be considerably higher than that observed with the preparations from a culture containing a mixture of cells the majority of which did not subsequently form tracheids.

EXPERIMENTAL

Materials. GDP-glucose, GDP-mannose, UDP-galactose and UDP-glucose were all from Sigma. GDP-[$U^{14}C$]-mannose (specific activity 10.73×10^{12} Bq/mol) and GDP-[$U^{14}C$]-glucose (specific activity 10.25×10^{12} Bq/mol) were from Amersham International. Cellulase, pronase, amphomycin, bacitracin, and dolichol monophosphate were from Sigma.

Cell cultures. Preparations were obtained from suspension cultured cells of *P. sylvestris* grown in induction or maintenance media as described in ref. [2].

Particulate preparations. Cells were harvested by filtration and washed with ice cold 0.1 M K-Pi buffer pH 7.2. Cells were then ground in a cooled mortar together with a little sand and 1 ml per mg cell fr. wt of a homogenisation buffer containing 0.1 M K-Pi pH 7.2, 1 mM dithiothreitol, 0.4 M sucrose and 1 mM $MgCl_2$ followed by sonication for 10 sec at 4°. The homogenate was squeezed through four layers of muslin and was centrifuged at 1000 *g* for 20 min to remove whole cells and wall debris. The supernatant was removed and membranes pelleted by centrifugation at 100 000 *g* for 60 min at 2°. The pellet was resuspended in 0.5 ml of the homogenisation medium and stored in liquid N_2.

Assay of glucomannan synthase. Incubations were carried out for 30 min at 28° using 10 μl of particulate preparation in 0.1 M K-Pi pH 7.2 together with 0.43 nmol GDP-D-[U-^{14}C]mannose (277 000 dpm), 2 nmol cold GDP-mannose, 0.5 nmol GDP-glucose and $MgCl_2$ to a final concentration of 30 mM in a total volume of 30 μl. For some experiments these quantities were

varied as specified in the text. The reaction was stopped by heating at 100° for 3 min and then stored at −20°.

To separate synthesized polymer from other labelled constituents of the reaction mixture 5 μl was spotted onto Whatman No. 1 paper and electrophorized for 20 min at pH 3.5 and 5 kV in 0.5% pyridine, 5% HOAc buffer. After drying the paper was chromatographed for 16 hr in solvent A. Finally the dried paper was cut into strips 10 × 40 mm and placed in plastic vials together with 2 ml of scintillant containing 4 g PPO and 50 mg POPOP in 1 l toluene for counting of radioactivity. For characterization of the reaction product polysaccharide was precipitated from the reaction mixture by addition of 0.5 ml of 95% ethanol and centrifuged at 10000 *g*, followed by 5 further washes with 75% ethanol.

Total acid hydrolysis. Synthesized polysaccharides were hydrolysed by 72% (w/w) H_2SO_4 diluted to 3% (w/w) and heated at 120°, 103.4 kPa for 60 min. The solution was neutralized by 3 washes with an equal volume of 15% *N*, *N*-dioctylmethylamine in $CHCl_3$, and the dioctylmethylamine was removed by 3 further washes with $CHCl_3$. Sugars produced were separated by PC for 30 hr in solvent A.

Partial acid hydrolysis. To produce oligosaccharides the synthesized polysaccharide was hydrolysed with 0.1 M HCl for 10 min at 95°. The acid was removed by evaporation under reduced pressure over NaOH. The oligosaccharides were separated by chromatography for 36 hr in solvent B.

Acetolysis A sample of polysaccharide was stirred overnight at 22° with 0.5 ml of a mixture of 1 ml Ac_2O, 1 ml HOAc and 0.1 ml H_2SO_4. The hydrolysis was terminated by dilution to 4 ml with H_2O and neutralization with 10 M NaOH. The solution was extracted × 5 with $CHCl_3$ and the extract was washed with 1 M $NaHCO_3$, dried with KCl, filtered and evaporated to dryness. The residue was dissolved in 2 ml MeOH and 0.5 ml barium methoxide was added and allowed to react for 30 min. The barium was precipitated by addition of a pellet of solid CO_2 and the supernatant was removed and dried. The disaccharides formed were separated in solvent C for 96 hr.

Enzyme digestions. For degradation of the polysaccharide by cellulase, samples were incubated for 12 hr at 22° with 0.5 ml of cellulase solution (1 mg/5 ml 0.1 M NaOAc pH 5.0). The reaction was terminated by freezing at −20° and drying under red. pres. To test for the presence of glycoprotein strips of chromatogram from the origin after electrophoresis were incubated for 4 hr at 40° with 5 ml of pronase solution (1 mg/20 ml). After incubation the strips were dried and recounted to determine if any radioactivity had been solubilized.

Periodate oxidation. Synthesized polysaccharide was reacted with 2 ml of 0.05 M $NaIO_4$ for six days at 4° in the dark. Excess periodate was removed by addition of 0.1 ml of ethanediol and the samples were reduced by two additions of 0.5 ml of 0.1 M $NaBH_4$ at 30 min intervals. The reaction was terminated by addition of 72% (w/w) H_2SO_4 to a final concentration of 3% (w/w) and then hydrolysed for 1 hr at 120°, 103 kPa. Neutralization was effected by washing with *N*,*N*-dioctylmethylamine as described previously. The sugar alcohols were separated by PC in solvent A for 16 hr.

Methylation. The synthesized polysaccharide was methylated as described in ref. [18]. The methylated sugars were identified by GC on a 3 m column packed with 3% SP2340 at 190° with argon at 30 ml/min. Material was collected at the exit of the column in a 0° trap and this was used to measure the radioactivity present in fractions corresponding to the methylated sugars.

Chromatography. Descending paper chromatography was performed on Whatman No. 1 paper with the following solvent systems. A: EtOAc–pyridine–H_2O, 8:2:1. B: *n*-BuOH–pyridine–H_2O, 4:3:4. C: PrOH–EtOAc–H_2O, 7:1:2. Sugar markers were detected by the alkaline $AgNO_3$ method of ref. [19].

Protein determination. Protein present in the particulate fractions was determined by a modification of the Folin–Lowry assay. Samples were dissolved in 5%, SDS, 0.5 M NaOH before application of the Lowry method [20].

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