Synthesis of (1→2)-β-D-Glucan by Cell-free Extracts of *Agrobacterium radiobacter* IFO 12665b1 and *Rhizobium phaseoli* AHU 1133

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Particulate preparations from *Agrobacterium radiobacter* IFO 12665b1 and *Rhizobium phaseoli* AHU 1133 have been shown to catalyze the synthesis of (1→2)-β-D-glucan from UDP-D-[¹⁴C]glucose. The (1→2)-β-D-glucans synthesized are suggested to be in a cyclic form without other glycosidic linkages and to consist of a mixture of several components with degrees of polymerization of 17 and more. The enzyme systems from *A. radiobacter* IFO 12665b1 and *R. phaseoli* AHU 1133 both required Mn²⁺ and had optimum activities at pH 7.5~8, and their *K_m* values for UDP-D-[¹⁴C]glucose were 5×10^{-5} M and 3.3×10^{-5} M, respectively.

(1→2)-β-D-Glucan is a polysaccharide produced by strains of *Agrobacterium* and *Rhizobium* and found in both the culture filtrates^{1~11)} and the cells.^{6,12,13)} In 1964, Dedonder and Hassid³⁾ studied the *in vitro* synthesis of (1→2)-β-D-glucans. They showed that a particulate preparation obtained from two strains of *Rhizobium japonicum* catalyzed the incorporation of glucose of UDP-D-[¹⁴C]glucose into (1→2)-β-D-glucan. This glucan had mainly β-(1→2)-linkages with some minor β-(1→3)- and β-(1→6)-linkages. In 1979, Sandermann and Dekker¹⁴⁾ reported that a particulate fraction of *Acetobacter xylinum* synthesized an alkali-soluble material that was probably (1→2)-β-D-glucan from UDP-D-[¹⁴C]glucose, although this glucan has not been isolated from cultures of *Acetobacter*.

Recently^{7,8,10~13,15,16)} the (1→2)-β-D-glucans produced by cultures of *Agrobacterium* and *Rhizobium* were shown to have a cyclic structure composed exclusively of β-(1→2)-linked D-glucosyl residues, and to consist of a mixture of components with different degrees of polymerization (DPs) the distribution patterns of which were characteristic for different strains. The DPs of the cyclic (1→2)-β-D-glucans produced by

Agrobacterium radiobacter IFO 12665b1 were distributed between 17 and 24 (mainly 19 to 22), while that of cyclic (1→2)-β-D-glucan from *Rhizobium phaseoli* AHU 1133 was mainly 17 with a minor proportion of molecules of 18 to 20.^{15,16)} The smallest DP of these cyclic glucans found so far is 17.

These findings prompted us to study the *in vitro* synthesis of this glucan further to elucidate how linear glucans are circularized and how the molecular sizes of the glucans are controlled. This paper reports the synthesis of cyclic (1→2)-β-D-glucan by cell-free particulate fractions obtained from strains of *A. radiobacter* and *R. phaseoli*.

MATERIALS AND METHODS

Chemicals. UDP-D-[¹⁴C]glucose (2.92 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, U.K.). Cyclic (1→2)-β-D-glucan was prepared from culture filtrates of *Agrobacterium radiobacter* IFO 12665b1 and *Rhizobium phaseoli* AHU 1133 as described previously.⁷⁾ Partially hydrolyzed (1→2)-β-D-glucan was obtained by hydrolyzing cyclic (1→2)-β-D-glucan of *A. radiobacter* IFO 12665b1 with 0.1 M trifluoroacetic acid for 1 hr at 100°C and then removing the acid with Amberlite IR-45 (OH⁻) resin. Sophorotriose, sophorotetraose, laminaritetraose and gentiotetraose were prepared by partial hydrolysis of cyclic (1→2)-β-D-glucan,

curdlan, and luteose, respectively, and by fractionation of their partial hydrolysates on charcoal columns. The other chemicals used were commercial products of the highest purity available.

Organisms. *A. radiobacter* IFO 12665b1 was isolated as an organism forming large amounts of curdlan ((1→3)- β -D-glucan) from a stock culture of *A. radiobacter* IFO 12665.¹⁷⁾ The strain IFO 12665 was obtained from the Institute for Fermentation, Osaka (IFO). *R. phaseoli* AHU 1133 was obtained from Prof. S. Higashi, Kagoshima University, Japan.

Media. The bacteria were grown in a chemically-defined medium (100 ml) containing 1 g of glucose, 0.15 g of $(\text{NH}_4)_2\text{HPO}_4$, 0.1 g of KH_2PO_4 , 0.05 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g of CaCO_3 and 0.1 ml of mixed salt solution (containing NaCl 10 g, CaCl_2 10 g, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 10 g, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 10 g, $\text{ZnCl}_2 \cdot 7\text{H}_2\text{O}$ 70 mg, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 50 mg, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 20 mg and H_3BO_3 10 mg per liter). The pH of the medium was 7.0. For the culture of *R. phaseoli* AHU 1133, 20 μg of thiamine and 2 μg of biotin per 100 ml were added to the above medium.

Culture conditions. The medium (95 ml) in 500 ml conical flasks was inoculated with a seed culture (5 ml) that had been grown in the same medium. The culture was shaken reciprocally at 120 strokes per min for 24 hr at 30°C.

Preparation of cell-free extracts. Cells obtained from 15 ml of culture by centrifugation were washed with 30 ml of 0.05 M Tris-HCl buffer (pH 7.4) with centrifugation. Then they were suspended in 3 ml of the same buffer and sonicated in a 200W Kaijo Denki sonic oscillator (Tokyo, Japan) for 5 min. The crude extract was centrifuged for 30 min at $15,000 \times g$ and the supernatant was then centrifuged for 1 hr at $105,000 \times g$. The pellet thus obtained was suspended in 3–6 ml of the same buffer and used as the enzyme preparation.

Enzyme assay. Standard assays are essentially as described by Dedonder and Hassid.³⁾ A mixture of 5 μl of the extract in 0.05 M Tris-HCl buffer, pH 7.4, 3 μl of UDP-D-[^{14}C]glucose (0.257 nmol, 0.075 μCi), 1 μl of 0.01 M ATP (10 nmol), 2 μl of 0.02 M NAD (20 nmol), 5 μl of 0.1 M MnCl_2 (0.5 μmol) and 5 μl of 0.02 M glycine-NaOH buffer, pH 9.0 (1 μmol) in a total volume of 30 μl at a final pH of 8.0 was incubated for 30 min at 30°C. Then the mixture was heated for 1 min at 100°C and centrifuged. The supernatant (5 μl) was applied to Toyo-Roshi No. 50 paper and developed twice with solvent A (see below) for 16 hr each. The amount of enzymatically-synthesized polysaccharide was determined by counting the radioactivity of the material remaining at the origin of the chromatogram.

Chromatography. Paper chromatography was performed with solvent A (1-butanol–pyridine–water; 6:4:3, by vol.). Reducing compounds (standard samples) on the paper were detected by the alkaline silver nitrate method and radioactive compounds were located by radioautography. Spots of radioactivity were cut from the paper and immersed in scintillation fluid.

Thin layer chromatography was carried out on a Silica Gel Plate (Wako, Osaka, Japan) with solvent B (1-butanol–ethanol–water; 5:5:4, by vol.) and solvent C (benzene–acetone–water–ammonium hydroxide; 100:400:6:3, by vol.).¹⁸⁾ Radioactive compounds were located by radioautography and sugars (standard samples) by spraying the chromatograms with 5% conc. H_2SO_4 in ethanol and heating them at 100°C.

High performance liquid chromatography was conducted with a model 6000A solvent-delivery system, a model U6K injector, and a column of $\mu\text{Bondapak CH}$ (all from Waters Associates, Milford, Mass., U.S.A.), and a model SE-41 differential refractometer (Showa Denko, Tokyo, Japan) with acetonitrile–water (29:21, by vol.) as the solvent system at a flow rate of 1.4 ml/min, and pressure of 9.6 MPa, as described by Hisamatsu *et al.*¹⁵⁾ Enzymatically synthesized, labeled polysaccharide was mixed with a solution of standard cyclic (1→2)- β -D-glucan of *A. radiobacter* IFO 12665b1 and injected into the instrument. The effluent from the refractometer was collected at 20 sec intervals and the radioactivity of each fraction was counted.

Measurement of radioactivity. Radioactive label was determined by standard techniques of liquid scintillation spectrometry in a Beckman LS9000 instrument (Calif., U.S.A.).

Acid hydrolysis. The labeled compound synthesized enzymatically was partially hydrolyzed with 0.2 M trifluoroacetic acid for 0.5–6 hr at 100°C. After hydrolysis, the acid was removed by treatment with Amberlite IR-45 (OH^-) resin.

Enzymatic hydrolysis. The labeled compound was digested with appropriate amounts of endo-(1→2)- β -D-glucanase and β -D-glucosidase. These enzymes were partially purified from cells of *Cytophaga arvensicola* A21L as described previously.¹⁹⁾

Methylation. Methylation was performed as described by Hakomori²⁰⁾ with sodium methyl sulfoxide. The methylated samples were hydrolyzed with acid (4 hr, 100°C, 2 M trifluoroacetic acid) and analyzed by thin layer chromatography.

Purification of the compound synthesized enzymatically. For preparation of a large quantity of the labeled product, the standard assay mixture scaled up to 600 μl , containing 1.5 μCi of UDP-[^{14}C]glucose, and 320 μg , as

protein, of the cell-free extract of *A. radiobacter* IFO 12665b1 or 360 μg, as protein, of the cell-free extract of *R. phaseoli* AHU 1133 was incubated for 2 hr at 30°C. Then the mixture was diluted with 1 ml of water and heated for 1 min and the resulting precipitate was removed by centrifugation. The supernatant was applied to a column (1.5 × 48 cm) of Sephadex G-50 and the labeled compound was eluted with water. Fractions of 1 ml were collected and the radioactivities of 20 μl portions of each were counted. The results are shown in Fig. 2(a) and Fig. 2(b). The labeled material corresponding to the main peak (indicated by a solid bar) was pooled and concentrated to a small volume by vacuum evaporation. This compound was applied to a column (0.7 × 8 cm) of DEAE-cellulose equilibrated with 1 mM KCl and eluted with 18 ml of 1 mM KCl and then with 30 ml of 100 mM KCl. Fractions of 2 ml were collected and the radioactivities of 20 μl portions of each were counted (Fig. 2(c), Fig. 2(d)). The radioactivity passing unadsorbed through the column (indicated by a solid bar) was pooled, concentrated to a small volume by vacuum evaporation and desalted by passage through a column (1.5 × 28 cm) of Sephadex G-10. Fractions containing radioactivity were collected and concentrated to dryness by vacuum evaporation.

RESULTS AND DISCUSSION

Synthesis of labeled (1→2)-β-D-glucan by cell-free extracts

When the cell-free extracts from *Agrobacterium radiobacter* IFO 12665b1 and *Rhizobium phaseoli* AHU 1133 were incubated with UDP-D-[¹⁴C]glucose, labeled compounds that remained at the origin of the chromatogram on paper chromatography were formed with a high yield (Fig. 1).

For chemical characterization, larger quantities of the labeled compounds were prepared as described in MATERIALS AND METHODS (see Fig. 2). By these procedures, 79 and 82% of the initial radioactivities were recovered in the purified labeled compounds from *A. radiobacter* IFO 12665b1 and *R. phaseoli* AHU 1133, respectively, showing that most of the glucose moiety of UDP-D-[¹⁴C]glucose was incorporated into high-molecular-weight products under these conditions. The compound corresponding to the small peak in the elution profiles from Sephadex G-50 (Fig. 2(a), (b)) was found to be the remaining UDP-D-[¹⁴C]glucose, judging from paper chromatography.

FIG. 1. Enzymatic Synthesis of ¹⁴C-Labeled Compounds from UDP-D-[¹⁴C]Glucose.

Test tubes containing the standard assay mixture with 16.0 μg, as protein, of the cell-free extract of *A. radiobacter* IFO 12665b1 or 9.0 μg, as protein, of the cell-free extract of *R. phaseoli* AHU 1133 were incubated for the indicated periods. (●), *A. radiobacter* IFO 12665b1; (○), *R. phaseoli* AHU 1133.

FIG. 2. Elution Patterns from Columns of Sephadex G-50 (a, b) and DEAE-Cellulose (c, d) of the ¹⁴C-Labeled Compounds Synthesized by the Cell-free Extracts of *A. radiobacter* IFO 12665b1 (a, c) and *R. phaseoli* AHU 1133 (b, d).

Arrows 1, 2 and 3 indicate the elution volumes of blue dextran, standard cyclic (1→2)-β-D-glucan from *A. radiobacter* IFO 12665b1 and glucose, respectively, determined in different runs. Solid bars indicate pooled fractions.

Acid hydrolysis (4 hr, 100°C, 2 M trifluoroacetic acid) of the compounds synthesized by the cell-free extracts of *A. radiobacter* IFO

FIG. 3. Paper Chromatogram of a Partial Acid Hydrolysate of the ^{14}C -Labeled Compounds Synthesized by a Cell-free Extract of *A. radiobacter* IFO 12665b1.

G1, G2, G3, G4 and G5 indicate the positions of glucose, sophorose, sophorotriose, sophorotetraose, and sophoropentaose, respectively. Std; partially-hydrolyzed standard cyclic (1 $\rightarrow$ 2)- β -D-glucan of *A. radiobacter* IFO 12665b1.

12665b1 and *R. phaseoli* AHU 1133 yielded only radioactive glucose. Partial hydrolysis of the compounds with 0.2 M trifluoroacetic acid for 0.5, 2, 4 and 6 hr at 100°C resulted in the formation of a series of oligosaccharides whose *Rf* values coincided with those of standard sophoro-oligosaccharides (Fig. 3).

The labeled compounds were not hydrolyzed by α -amylase (*B. subtilis*, Seikagaku), glucoamylase (*R. niveus*, Seikagaku), or β -D-glucosidase (almond, Sigma), but were hydrolyzed by endo-(1 $\rightarrow$ 2)- β -D-glucanase prepared from the cells of *Cytophaga arvensicola*. As reported in our previous paper¹⁵⁾ the latter enzyme preparation has high specificity for β -(1 $\rightarrow$ 2)-glucosidic linkages and readily hydrolyzes the cyclic (1 $\rightarrow$ 2)- β -D-glucan of *A. radiobacter* IFO 12665b1 and sophoro-oligosaccharides higher than sophoropentaose in a random manner. The hydrolysis products from the labeled compounds by the (1 $\rightarrow$ 2)- β -D-glucanase were examined by paper chromatography and found to be glucose and oligosaccharides corresponding to sophorose to

FIG. 4. Paper Chromatogram of the Digests with Endo-(1 $\rightarrow$ 2)- β -D-glucanase and β -D-Glucosidase of the ^{14}C -Labeled Compounds Synthesized by Cell-free Extracts of *A. radiobacter* IFO 12665b1 and *R. phaseoli* AHU 133.

A, B; ^{14}C -labeled compounds of *A. radiobacter* IFO 12665b1 digested with endo-(1 $\rightarrow$ 2)- β -D-glucanase and with a mixture of endo-(1 $\rightarrow$ 2)- β -D-glucanase and β -D-glucosidase, respectively. C, D; ^{14}C -labeled compounds of *R. phaseoli* AHU 1133 digested with endo-(1 $\rightarrow$ 2)- β -D-glucanase and with a mixture of endo-(1 $\rightarrow$ 2)- β -D-glucanase and β -D-glucosidase, respectively. G1, G2, G3, G4, G5 and Std; cf., Fig. 3.

sophoro-pentaose (Fig. 4). These oligosaccharides were hydrolyzed completely by the β -D-glucosidase of *Cytophaga arvensicola* (Fig. 4) which hydrolyzes β -(1 $\rightarrow$ 2)- and β -(1 $\rightarrow$ 3)-glucosidic linkages. These results strongly support the hypothesis that the compounds synthesized were (1 $\rightarrow$ 2)- β -D-glucans.

The labeled glucans from *A. radiobacter* IFO 12665b1 and *R. phaseoli* AHU 1133 were fully methylated and then hydrolyzed and the products were subjected to thin layer chromatography. The only detectable spot on the chromatogram was that of 3,4,6-tri-*O*-methyl-D-glucose; there was no spot of tetra-*O*-methyl-D-glucose or di-*O*-methyl-D-glucose, indicating the absence of appreciable amounts of non-reducing terminal and branching residues and linkages other than (1 $\rightarrow$ 2)-linkages (Fig. 5(a)).

FIG. 5. Thin Layer Chromatograms of Methylated Products of ^{14}C -Labeled Polysaccharides (a) and Reduced ^{14}C -Labeled Polysaccharides (b).

The following polysaccharides and oligosaccharides were methylated, hydrolyzed and subjected to thin layer chromatography. A, F; ^{14}C -labeled polysaccharides of *A. radiobacter* IFO 12665b1 and *R. phaseoli* AHU 1133, respectively. G, H; reduced ^{14}C -labeled polysaccharides of *A. radiobacter* IFO 12665b1 and *R. phaseoli* AHU 1133, respectively. B, C, D, E; laminaritetraose, maltose, gentiotetraose and sophorotetraose, respectively. Numbers 1, 2, 3, 4, 5 and 6 represent the positions of 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-glucose (2,4,6-tri-*O*-methyl-D-glucose), 3,4,6-tri-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-glucose and 1,3,4,5,6-penta-*O*-methyl-D-glucitol, respectively.

The labeled (1→2)-β-D-glucans were reduced with sodium borohydride and then methylated to determine the reducing terminal of the glucans. Thin layer chromatographic examination revealed that only a spot of 3,4,6-tri-*O*-methyl-D-glucose and no spot of 1,3,4,5,6-penta-*O*-methyl-D-glucitol was detected (Fig. 5(b)).

These results suggested that there are no free non-reducing and reducing terminal residues, showing a cyclic structure, or else that the degrees of polymerization (DPs) of the synthesized glucans are too high to detect these terminals by chromatography.

Therefore, the DPs of the labeled glucans

were examined by liquid chromatography. Cyclic (1→2)-β-D-glucan obtained from the culture fluid of *A. radiobacter* IFO 12665b1 has been shown to be separated by liquid chromatography into materials of DP 17 to 24, while cyclic (1→2)-β-D-glucan from the culture fluid of *R. phaseoli* AHU 1133 has been found by this method to be composed mainly of material of DP 17 with small amounts of materials of DP 18 to 20.^{15,16)} Samples of the labeled glucans were injected into a column of μBondapak CH with standard cyclic (1→2)-β-D-glucan of *A. radiobacter* IFO 12665b1. As shown in Fig. 6, labeled glucans were separated into several components and the retention times of these components coincided with those of the components of the standard cyclic (1→2)-β-D-glucan. Thus, if the labeled glucans are cyclic, the DPs of those from *A. radiobacter* IFO 12665b1 are mainly 18, 19 and 20 and the DP of the glucan from *R. phaseoli* AHU 1133 is mainly 17. If the labeled glucans were linear, the DPs of the glucans would be less than these values, because the cyclic form of (1→2)-β-D-glucan is always eluted earlier than the linear form with the same DP from this type of column.¹⁶⁾ Non-reducing and reducing terminal residues of these sizes of linear glucans, if present, should be detectable by methylation analysis and since they were not detected, the labeled glucans are probably cyclic.

The distribution patterns of each component in the labeled glucan from *A. radiobacter* IFO 12665b1 and the standard glucan were different, as seen in Fig. 6. However, the distribution pattern of each component of the labeled glucan from *R. phaseoli* AHU 1133 was similar to that of each component of the glucan synthesized *in vivo* by this organism; *i.e.*, the labeled glucan was composed mainly of a component of DP 17, although it also contained small amounts of components of higher DP (>21) which are not detected in the glucan synthesized *in vivo*.

The lowest DP of the glucan components in the glucans synthesized both *in vivo* and *in vitro* was 17. It seemed possible that lower-

FIG. 6. Liquid Chromatograms of the (1→2)- β -D-Glucans Synthesized by Cell-free Extracts of *A. radiobacter* IFO 12665b1 (a) and *R. phaseoli* AHU 1133 (b).

The labeled glucans were eluted together with the standard cyclic (1→2)- β -D-glucan of *A. radiobacter* IFO 12665b1. Histograms and solid lines show the elution patterns of the labeled glucans and standard cyclic glucans, respectively. Numbers 17 to 24 represent degrees of polymerization of the components of the standard cyclic glucan.

FIG. 7. Thin Layer Chromatograms of the (1→2)- β -D-Glucans Synthesized in Various Incubation Times by Cell-free Extracts of *A. radiobacter* IFO 12665b1 (a) and *R. phaseoli* AHU 1133 (b).

At incubation times of 5, 10, 20 and 60 min as indicated, 11, 22, 30 and 80%, respectively, of the initial radioactivity of UDP-D-[14 C]glucose was incorporated into the glucans. Samples of the glucans with approximately the same amounts of radioactivity were applied to the plates. A; partially hydrolyzed standard cyclic (1→2)- β -D-glucan. B, C; standard cyclic (1→2)- β -D-glucans of *R. phaseoli* AHU 1133 and *A. radiobacter* IFO 12665b1, respectively. Numbers in the figure indicate degrees of polymerization.

molecular-weight glucans or oligosaccharides might be present in a linear form at an early stage of incubation. To examine this possibility, the reaction mixture was incubated for different periods (5, 10, 20 or 60 min), heated and centrifuged and a neutral fraction obtained from the supernatant by passage through a mixed bed of Amberlite IR-120 (H⁺) resin and Amberlite IR-45 (OH⁻) resin was subjected to thin layer chromatography. The chromatographic patterns (Fig. 7) of the labeled glucans from the two strains were the same regardless of the incubation time; the predominant DP of the glucan synthesized was 18 with *A. radiobacter* IFO 12665b1 and 17 with *R. phaseoli* AHU 1133. No lower-molecular-weight glucan or oligosaccharide was synthesized even at an early stage of incubation, but rather higher glucans (DP > 24) seemed to be present. Thus in the reaction mixtures, matured glucans in a cyclic form seem to be released from the particulate fractions in a soluble form. Elongation of glucosyl residues and cyclization of the chain seem to be carried out on the particulate fractions.

Properties of the enzyme system

The activity of the (1→2)-β-D-glucan-synthesizing enzyme system was assayed by counting the radioactivity found at the origin after paper chromatography. By eluting it

from the paper with water and digesting it with endo-(1→2)-β-D-glucanase, the non-mobile compound was found to be (1→2)-β-D-glucan uncontaminated by other compounds. The amount of radioactivity incorporated into the polysaccharide was linearly proportional to the protein concentration (Fig. 8).

Table I shows that omission of ATP or NAD from the reaction mixture had no effect on the activity, but that omission of both compounds reduced the activity slightly with the *A. radiobacter* IFO 12665b1 enzyme and more extensively with the *R. phaseoli* AHU 1133 enzyme, and that Mn²⁺ ion was required

FIG. 8. Effects of Protein Concentration on the Synthesis of (1→2)-β-D-Glucan by Cell-free Extracts of *A. radiobacter* IFO 12665b1 and *R. phaseoli* AHU 1133.

The standard assay mixture was used but with various amounts of protein. (●), *A. radiobacter* IFO 12665b1; (○), *R. phaseoli* AHU 1133.

TABLE I. EFFECTS OF ATP, NAD AND CATIONS ON THE SYNTHESIS OF (1→2)-β-D-GLUCAN BY CELL-FREE EXTRACTS OF *A. radiobacter* IFO 12665b1 AND *R. phaseoli* AHU 1133

The standard assay mixture containing 2.51 μg, as protein, of the cell-free extract of *A. radiobacter* IFO 12665b1 or 4.5 μg, as protein, of the cell-free extract of *R. phaseoli* AHU 1133 was incubated with the omission and/or addition of the component listed.

Omission	Addition	[¹⁴ C]Glucose incorporated into (1→2)-β-D-glucan (nmol/min/mg protein)	
		<i>A. radiobacter</i>	<i>R. phaseoli</i>
None		1.10	0.60
ATP		1.16	0.67
NAD		1.04	0.69
ATP, NAD		0.93	0.20
MnCl ₂		0.009	0.005
MnCl ₂	MgCl ₂ (0.5 μmol)	0.99	0.088

FIG. 9. Effects of pH on the Activities of the (1→2)-β-D-Glucan-synthesizing Enzymes of *A. radiobacter* IFO 12665b1 (a) and *R. phaseoli* AHU 1133 (b).

Assay mixtures contained 0.257 nmol UDP-D-[¹⁴C]glucose, 10 nmol ATP, 20 nmol NAD, 0.5 μmol MnCl₂, 0.1 μmol Tris-HCl buffer (pH 7.4), 1.85 μg, as protein, of the cell-free extract of *A. radiobacter* IFO 12665b1 or 3.45 μg, as protein, of the cell-free extract of *R. phaseoli* AHU 1133, and 2 μmol buffer in a total volume of 30 μl. (●), glycine-NaOH buffer; (○), Tris-HCl buffer; (■), Tris-maleate buffer.

FIG. 10. Reciprocal Plot of Velocity (v) of (1→2)-β-D-Glucan Synthesis against Concentration ($[S]$) of UDP-D-[¹⁴C]glucose.

The standard assay mixture containing 2.05 μg, as protein, of the cell-free extract of *A. radiobacter* IFO 12665b1 or 1.20 μg, as protein, of the cell-free extract of *R. phaseoli* AHU 1133 was used except that the amount of UDP-D-[¹⁴C]glucose was varied. (●), *A. radiobacter* IFO 12665b1; (○), *R. phaseoli* AHU 1133. v is expressed as the amount (nmol) of [¹⁴C]glucose incorporated into (1→2)-β-D-glucan per min per mg of protein.

for the reaction. Mn²⁺ ion could be replaced by the same concentration of Mg²⁺ ion with *A. radiobacter* IFO 12665b1 enzyme but not with the *R. phaseoli* AHU 1133 enzyme. A requirement for divalent cations has been reported for the (1→2)-β-D-glucosyl transfer reaction by membrane fractions of *R. japonicum*³⁾ and *A. xylinum*.¹⁴⁾

The effect of pH on the activity is shown in Fig. 9. The activity was very different with Tris-maleate, Tris-HCl and glycine-NaOH buffers, being highest with glycine-NaOH buffer. The pH optimum was 7.5 in Tris-maleate and Tris-HCl buffers, but higher in glycine-NaOH buffer.

The K_m values for UDP-D-[¹⁴C]glucose of the *A. radiobacter* IFO 12665b1 enzyme and *R. phaseoli* AHU 1133 enzyme were 5×10^{-5} M and 3.3×10^{-5} M, respectively, measured in the standard assay mixture (pH 8, glycine-NaOH buffer) (Fig. 10). Because of this high affinity for the substrate, the synthesis of this glucan *in vitro* is very efficient. The V_{max} values for the *A. radiobacter* IFO 12665b1 and *R. phaseoli* AHU 1133 enzymes were 7.65 and 4.55 nmol glucose incorporated/min/mg protein, respectively. Although these organisms produce acidic heteropolysaccharides and *A. radiobacter* IFO 12665b1 also produces larger amounts of (1→3)-β-D-glucan, only (1→2)-D-glucan was synthesized *in vitro* from UDP-D-[¹⁴C]glucose under the conditions employed.

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