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Microbial preparation of α,α'-[1,1'-^{13}C]trehalose

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ABSTRACT

The time-course of the biosynthesis of α,α'-trehalose by the phytopathogenic fungus, *Rhizoctonia solani*, in the presence and absence of the trehalase inhibitor, validamycin A, is described. In addition, a mg-scale synthesis of α,α'-[1,1'-^{13}C]trehalose from D-[1-^{13}C]glucose is demonstrated in which the C-1 and/or C-1' sites were 76% labeled with carbon-13.

INTRODUCTION

α,α'-Trehalose is a commonly occurring disaccharide which is widespread in Nature. This sugar is of importance to lower animals and plants[1] as a means of sugar transport and as a readily available supply of energy[2]. Trehalose is now recognized as the characteristic blood sugar of insects, and haemolymph trehalose functions as the energy source to sustain insect flight. In fungi, this sugar serves as an endogenous source of stored energy, especially during phases of spore germination and hyphal extension. Trehalase is the only enzyme capable of mobilizing this sugar by its hydrolysis to D-glucose. An understanding of the mechanism of action of glycosyl hydrolases obviously requires knowledge of the anomeric configuration of the glycosyl unit released. Clifford[3] analysed derivatives of the glucose released by trehalase of the flesh fly by gas chromatography for anomeric composition and found that equimolar amounts of α- and β-D-glucopyranose were produced. Hehre et al.[4], using either a rabbit renal-cortical trehalase or the enzyme from *Candida tropicalis* and ^{1}H NMR spectroscopy, obtained the same result. Defaye et al.[5] gained the fast acquisition time for spectral data measurements by using 1,1'-^{13}C-labeled α,α'-trehalose and ^{13}C NMR spectroscopy to overcome the competition with the expected mutarotation of the monosaccharide unit released. This

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attractive ^{13}C NMR spectroscopy technique using a specifically ^{13}C-labeled α,α'-trehalose will definitely be an unequivocal technique for determination of the anomeric configuration of the enzymically released D-glucose units. This technique makes it possible to study in vivo in metabolism of 1,1'-^{13}C-labeled trehalose.

We have previously reported that a phytopathogenic fungus, *Rhizoctonia solani* stores much trehalose in the mycelium, and that the trehalase inhibitor, validamycin A, significantly inhibits its hydrolysis in vivo. *R. solani* is one of the most typical examples of a fungus that grows rapidly by transporting nutrients from the basal part to the tip through long streches of hyphae. It is thought that trehalose plays a significant role in the supply of nutrient to the tip of the hyphae. Herein, we describe the time-course of the biosynthesis of trehalose in the presence or absence of a trehalase inhibitor and the isolation of α,α'-[1,1'-^{13}C]trehalose.

EXPERIMENTAL

Materials.—D-[1-^{13}C]Glucose (99 atom% ^{13}C) was purchased from Isotec, Inc. (Miamisburg, OH, USA). Validamycin A was kindly provided by Takeda Chemical Industries (Osaka, Japan).

Chromatography.—HPLC was performed under the following conditions: column, YMC PA-5, 4.6 × 150 mm; solvent system, 75:25 (v/v) acetonitrile–water; flow rate, 2.0 mL min^{-1}; and detection by refractive index detector.

NMR analysis.—^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Jeol JNM-GX 400 spectrometer in D_2O containing sodium 3-(trimethylsilyl)propionate (TSP) as an internal standard with acquisition times of 3.277 s for ^1H NMR and 0.819 s for ^{13}C NMR.

Mass spectrometry.—FABMS was performed in the positive-ion mode using a Jeol JMX-DX300 JMA-DA5000 spectrometer. A sample was dissolved in water and loaded into glycerol on a stainless steel target.

Organism and culture conditions.—A strain of *Rhizoctonia solani* TKF 44, maintained on potato–glucose agar, was inoculated into 100 mL of GY medium composed of 1% D-glucose or D-[1-^{13}C]glucose, 0.3% $(NH_4)_2SO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.05% yeast extract (pH 5.7) in the presence or absence of 0.005% validamycin A (50 μg/mL) and incubated at 27°C with shaking.

Extraction and measurement of trehalose in mycelia.—Mycelia grown on 100 mL of GY medium were filtered through a glass filter, washed with water, and dried with acetone and ether. The dried mycelia were heated in 50% MeOH (15 mL) under reflux for 1 h. The resulting extracts were filtered through Celite, the filtrate was evaporated, and the residue was dissolved in 2 mL of water. After centrifugation at 3000 rpm for 10 min, the amount of trehalose in the supernatant (Sup-A) was determined by HPLC.

Isolation of α,α'-[1,1'-^{13}C]trehalose.—*R. solani* was grown in a GY medium (100 mL) containing D-[1-^{13}C]glucose (1 g) and validamycin A (5 mg) for 4 days at 27°C. Sup-A prepared as described above was deionized with Amberlite MB-3

resin, the effluent and washings were evaporated, and the residue was dissolved in a small amount of water. This solution was applied to a Bio-Gel P-2 column (1 × 140 cm) and eluted with water. The fraction size was 1.7 mL. Fractions 43–46 were collected and lyophilized to give α,α'-[1,1'-^{13}C]trehalose (25 mg).

RESULTS

The time-course of the yield of biomass in the presence or absence of validamycin A is shown in Fig. 1. These growth experiments were conducted with replicate sets. The trehalase inhibitor, validamycin A, had no significant effect on the mass of the fungal growth.

Fig. 2 shows the time-course of trehalose content in mycelia grown in the presence or absence of the inhibitor.

In both cases, the trehalose content in mycelia reached a maximum at four days of culture. Throughout the culture period, the trehalose content in the culture with validamycin A increased to about 1.4-fold that of the control culture. Thus validamycin A depressed the decay of intracellular trehalose in the growing culture, as well as in the resting cell system[6].

After α,α'-[1,1'-^{13}C]trehalose was extracted from the mycelia as described in the Experimental section, it was treated with Amberlite MB-3 resin and then readily isolated by gel filtration using Bio-Gel P-2 as shown in Fig. 3.

^{13}C NMR spectra of 1,1'-^{13}C-labeled trehalose and the nonlabeled trehalose, which was isolated from mycelia grown in a GY medium containing nonlabeled D-glucose, are shown in Fig. 4.

As shown in Fig. 4 (panel a), the enlargement of the C-2 signal revealed the splitting ($J_{C,C}$ 45.5 Hz) between C-1 and C-2. Enrichment ratios (Table I) were calculated from the relative intensity of C-5,5' taken as 1.0. From the labeling experiment with D-[1-^{13}C]glucose, C-1 and/or C-1' of trehalose were labeled with

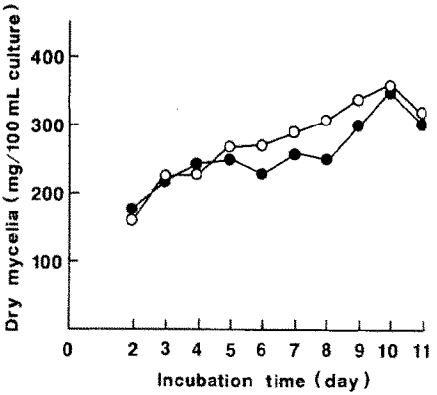

Fig. 1. Time-course of the yield of biomass of organism in the presence or absence of validamycin A: ○, no addition of validamycin A: ●, addition of validamycin A (50 μg/mL).

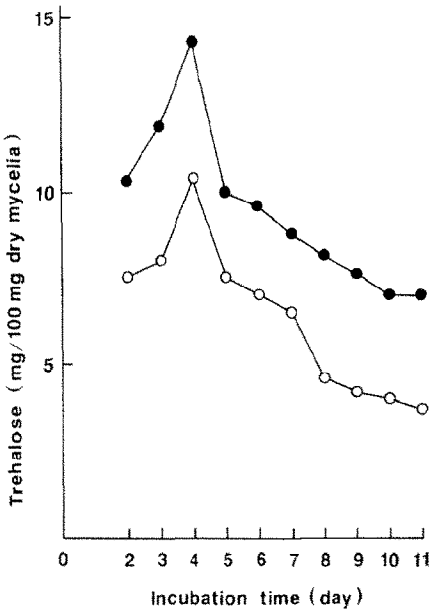

Fig. 2. Time-course of trehalose content in organism mycelia in the presence or absence of validamycin A: ○, no addition of validamycin A; ●, addition of validamycin A (50 μg/mL).

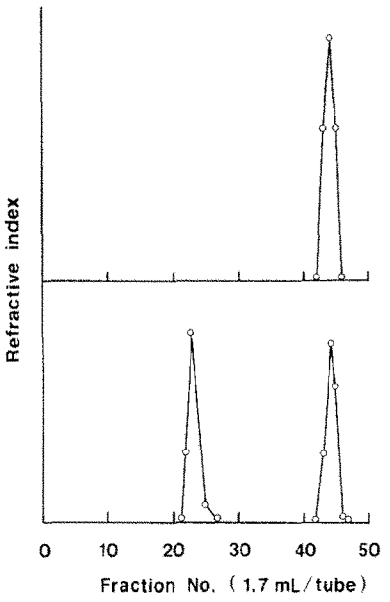

Fig. 3. Bio-Gel P-2 chromatography of α,α'-[1,1'-^{13}C]trehalose extracted from the mycelia. Elution of the column (1 × 140 cm) with water was monitored by a refractive index detector: (top) authentic sample of trehalose; (bottom) Sup-A deionized with Amberlite MB-3 resin as described in the Experimental section.

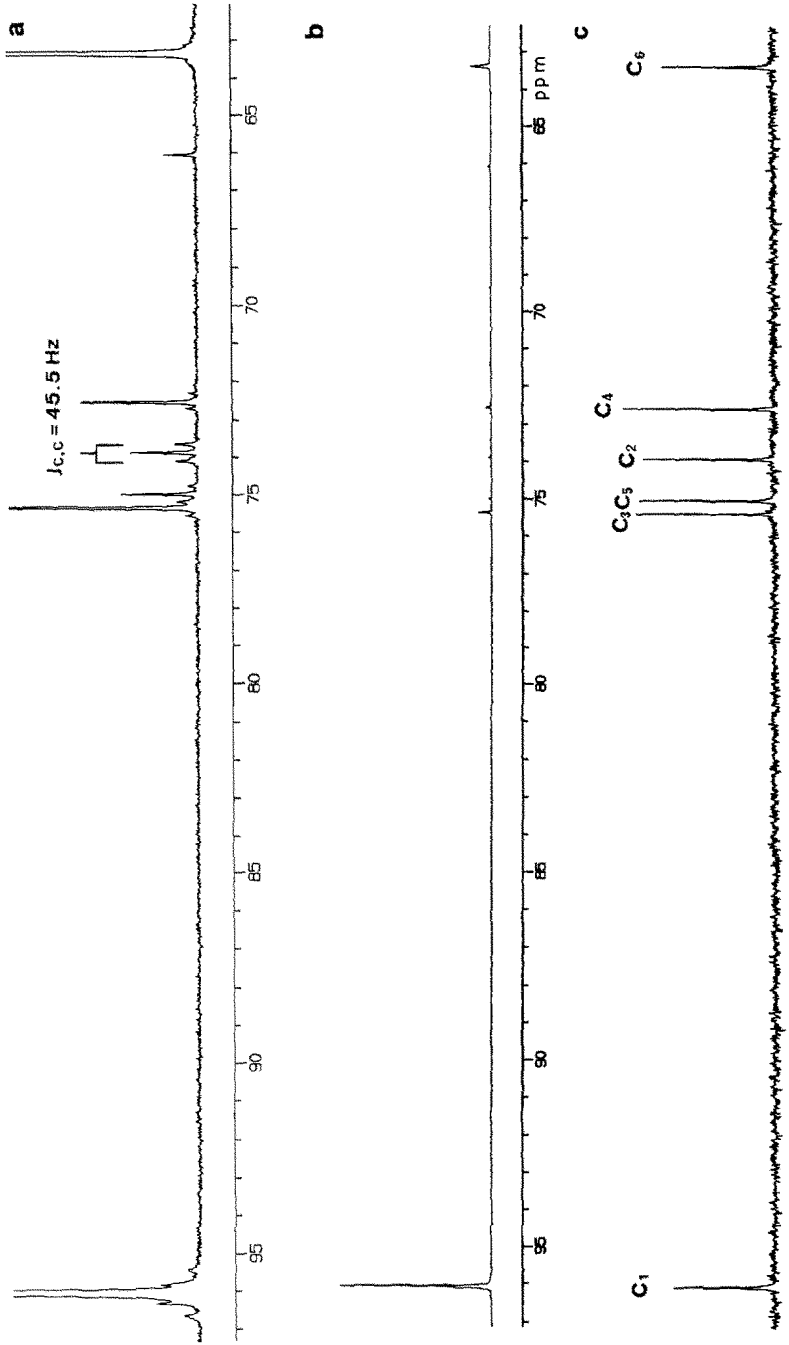

Fig. 4. ^{13}C NMR spectra of α,α'-[1,1'-^{13}C]trehalose: (a and b), ^{13}C-labeled trehalose, recorded with 2400 and 128 scans, respectively; (c) nonlabeled trehalose isolated from the mycelia, recorded with 128 scans.

TABLE I

Relative ^{13}C-enrichment in trehalose biosynthesized from D-[1-^{13}C]glucose

Carbon No.	Chemical shift (ppm)	Enrichment ratio [a]
1,1′	96.08	44.7
2,2′	73.89	1.2
3,3′	75.37	3.9
4,4′	72.56	1.4
5,5′	75.01	1.0
6,6′	63.38	7.1

[a] Enrichment ratios were calculated from the relative intensity of C-5,5′ taken as 1.0.

an enrichment ratio of 44.7 by C-1 of D-glucose in the manner expected for the biosynthetic route of α,α'-trehalose[7]. However, enhancement was observed at C-3,3′ and C-6,6′ with enrichment ratios of 3.9 and 7.1, respectively. This suggests that a proportion of the D-glucose unit in trehalose is reconstituted from the C-3 fragment, and, furthermore, that trehalose unlabeled at either C-1 or C-1′, or both of them, is contained in a given preparation. The ^{13}C-labeling rate at C-1 of trehalose was determined by integration of the anomeric protons in the ^{1}H NMR spectrum (Fig. 5, panel a). This experiment proved that 76% of C-1 and/or C-1′ of trehalose had been labeled. In addition, the $J_{C\text{-}1,H}$ value (172 Hz) observed in ^{13}C-labeled trehalose was consistent with the expected value (175 Hz) for an equatorial CH bond as reported by Defaye et al[5].

We also characterized the ^{13}C-labeled trehalose preparation by FABMS. The relative proportions of zero, singly and doubly labeled trehaloses were calculated from the relative abundance of the molecular ions (M + H). As shown in Fig. 5, our ^{13}C-labeled trehalose preparation was estimated to comprise of 12% of zero-labeled trehalose (m/z 343), 27% of the singly labeled trehalose (m/z 344), and 61% of the doubly labeled trehalose (m/z 345).

DISCUSSION

We have previously reported that a phytopathogenic fungus, *Rhizoctonia solani* stores trehalose in mycelia in remarkably high concentrations, and a trehalase inhibitor, validamycin A, depresses the decay of intracellular trehalose in the resting cell system[6].

In the present study, we found that validamycin A depressed the decay of intracellular trehalose without affecting the fungal specific growth rate. The ^{13}C NMR spectrum of trehalose isolated from mycelia proved that C-3,3′ and C-6,6′ (enrichment ratios of 3.9 and 7.1, respectively) in addition to C-1,1′ (enrichment ratio of 44.7) were labeled by C-1 of D-glucose. The enrichment of C-3,3′ or C-6,6′ suggests that a proportion of the D-glucose unit is reconstituted from the C-3 fragment derived from D-glyceraldehyde 3-phosphate through the glycolytic path-

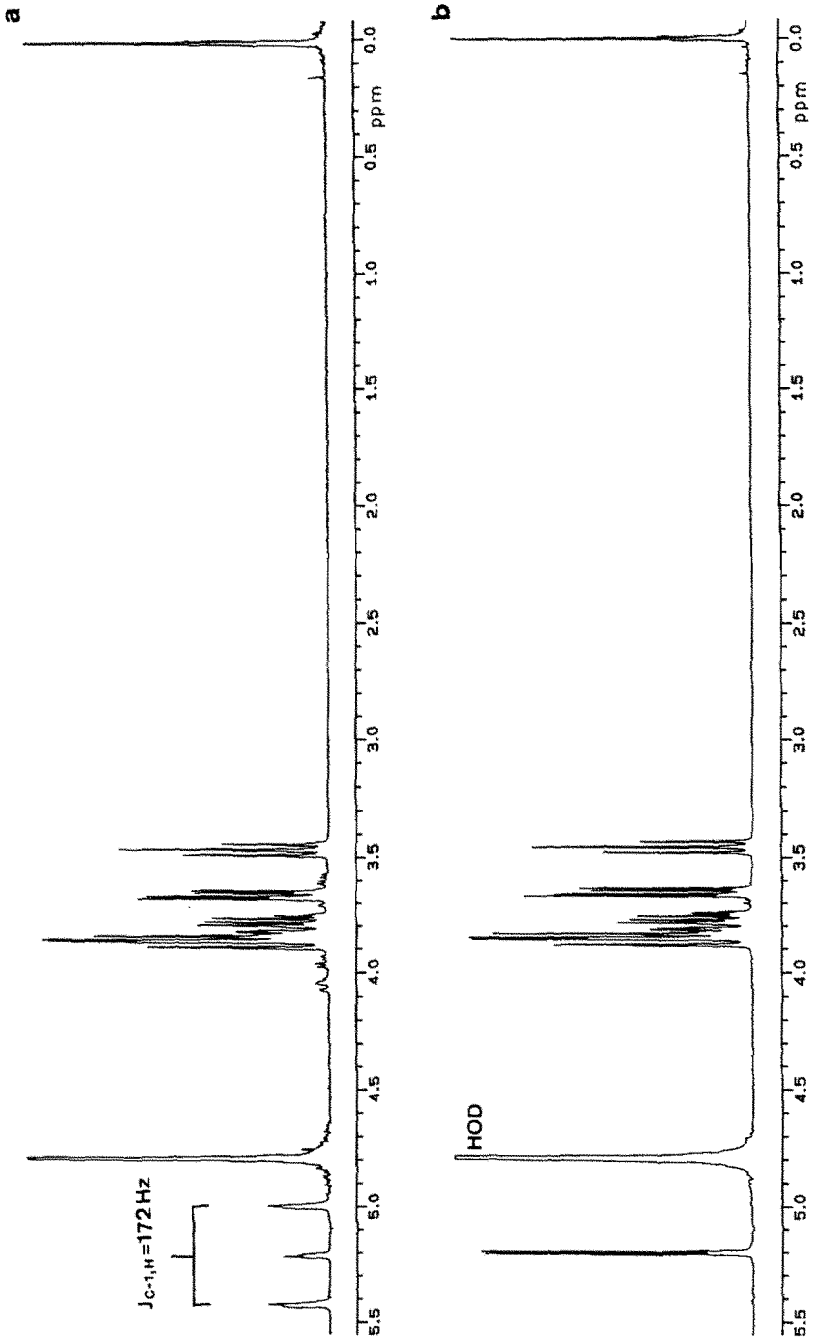

Fig. 5. ^{1}H NMR spectra of α,α'-[1,1'-^{13}C]trehalose: (a) ^{13}C-labeled trehalose; (b) nonlabeled trehalose isolated from the mycelia.

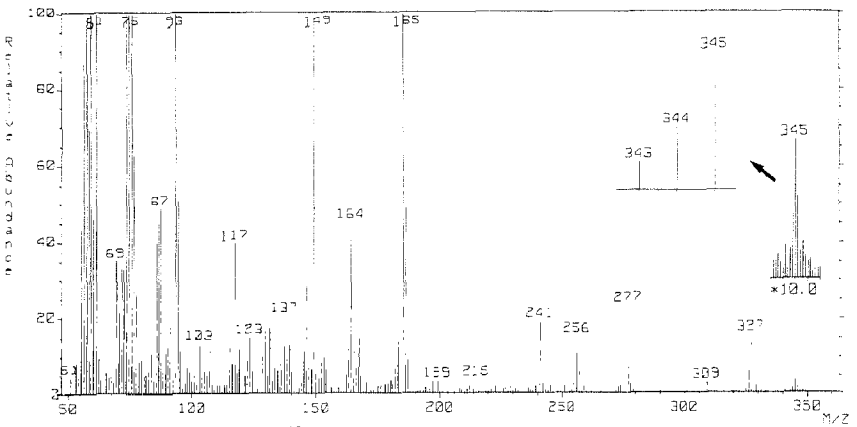

Fig. 6. FAB-mass spectrum of a ^{13}C-labeled trehalose preparation.

way. Furthermore, we estimated the content of α,α'-[1,1′-^{13}C]trehalose at ~60% from mass spectrometry.

Iida and Kajiwara[8] reported that D-[1-^{13}C]glucose injected intra-abdominally into live *Gryllodes sigillatus*, the Japanese Kamado criket, was converted into α,α'-[1,1′-^{13}C]trehalose, which was isolated by HPLC. Their trehalose preparation is thought to be a mixture of native trehalose in the insect and one synthesized in vivo from D-[1-^{13}C]glucose, and the percent ^{13}C-labeling in the preparation has not been characterized.

The use of specifically ^{13}C-labeled α,α'-trehalose should permit the in vivo study of the metabolic fate of trehalose in various organisms by using ^{13}C NMR spectroscopy.

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REFERENCES

1 A.D. Elbein, *Adv. Carbohydr. Chem. Biochem.*, 30 (1974) 227–256.
2 S. Friedman, *Annu. Rev. Entomol.*, 23 (1978) 389–407.
3 K.H. Clifford, *Eur. J. Biochem.*, 106 (1980) 337–340.
4 E.D. Hehre, T. Sawai, C.F. Brewer, M. Nakano, and T. Kanda, *Biochemistry*, 21 (1982) 3090–3097.
5 J. Defaye, H. Driguez, B. Henrissat, and E. Bar-Guilloux, *Carbohydr. Res.*, 124 (1983) 265–273.
6 N. Asano, T. Yamaguchi, Y. Kameda, and K. Matsui, *J. Antibiot.*, 40 (1987) 526–532.
7 E. Cabib and L.F. Leloir, *J. Biol. Chem.*, 231 (1958) 259–275.
8 K. Iida and M. Kajiwara, *J. Chromatogr.*, 515 (1990) 573–575.