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Note

Enzymic formation of di-D-fructofuranose 1,2′:2,1′-dianhydride by *Aspergillus fumigatus*

KUNIJI TANAKA, KIYOMI SONOBE, TAKAO UCHIYAMA, AND TOSHIO MATSUYAMA
Department of Biology, Osaka Kyoiku University, Tennoji-ku, Osaka 543 (Japan)
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It has been shown that three kinds of difructose dianhydride are produced from inulin by acid hydrolysis[1–4]. Recently, one of these, di-D-fructofuranose 1,2′:2,3′-dianhydride has been proved to be formed from inulin with an extracellular enzyme of *Arthrobacter ureafaciens* by an intramolecular transfructosylation reaction[5–7]. This finding led us to examine the enzymic formation of difructose dianhydrides by a similar reaction using an inulin-containing culture liquid of *Aspergillus fumigatus*[8]. The formation, in very low yield, of yet another isomer, termed *A. fumigatus* difructose dianhydride, was observed, but its structure could not be ascertained, nor could the responsible enzyme be isolated. However, it was observed during this study that an enzyme preparation from an autolyzate of the mycelia produced a difructose dianhydride different from both di-D-fructofuranose 1,2′:2,3′-dianhydride and the *A. fumigatus* difructose dianhydride. This Note describes its identification.

In the paper-chromatographic examination of the product of degradation of inulin by the mold enzyme, spraying with the anthrone–phosphoric acid reagent[9] showed a spot having a mobility intermediate between that of di-D-fructofuranose 1,2′:2,3′-dianhydride and D-fructose, and slightly lower than that of the *A. fumigatus* difructose dianhydride (see Fig. 1A). The corresponding spot was not detected by the alkaline silver nitrate spray reagent[10] (see Fig. 1B), suggesting a nonreducing sugar.

To isolate this nonreducing compound, the enzymic digest was degraded with yeast, and chromatographed successively on active carbon, ion-exchange resins, and Bio-Gel P-2. The resulting product was crystallized from ethanol and purified by recrystallization. It was nonreducing toward the Somogyi–Nelson reagent[11] and nonfermentable. The melting point, mixed melting point, and optical rotation suggested that it is di-D-fructofuranose 1,2′:2,1′-dianhydride reported by Jackson and Goergen[1]. This identification was supported by the preparation of the hexa-*O*-acetyl and -methyl derivatives, which had properties identical with those of the corresponding derivatives of di-D-fructofuranose 1,2′:2,1′-dianhydride.

As the enzyme used for the present study was not pure, its mode of action is not established. However, it may be expected that the enzymic reaction is also an

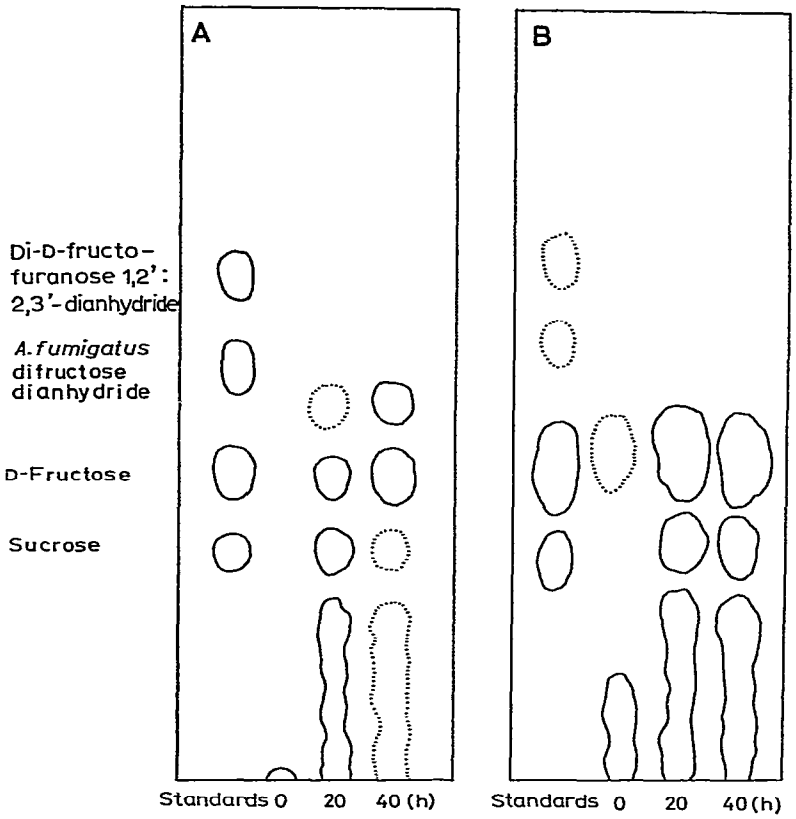

Fig. 1. Paper chromatograms of enzymic formation of a nonreducing sugar from inulin with *A. fumigatus*: (A) sprayed with anthrone–phosphoric acid reagent; (B) sprayed with alkaline silver nitrate reagent. The spots outlined with a dotted line indicate a weak reaction.

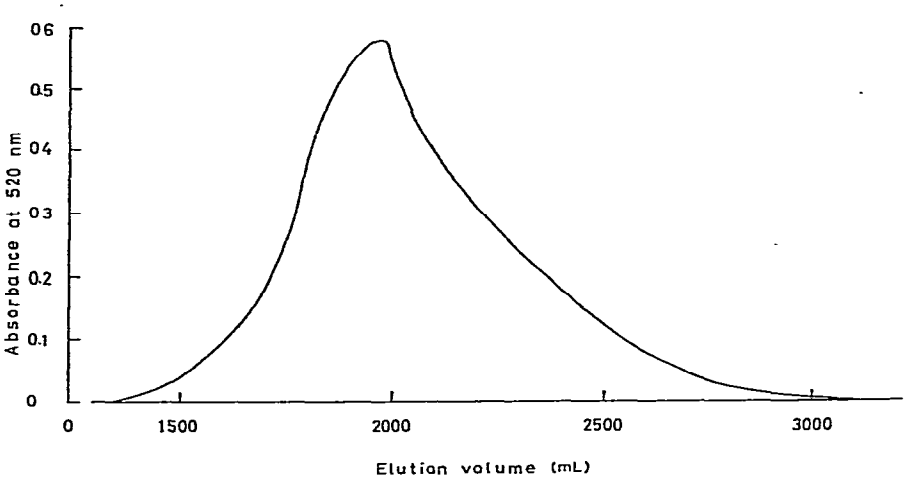

Fig. 2. Elution of the nonreducing sugar product with 5% ethanol from a column of Celite–carbon.

intramolecular transfructosylation reaction as described[5] for inulinase II. It is interesting to note the formation of a (2→1)-α-D-fructosyl linkage[12] from a β-D-(2→1)-linked inulin molecule.

EXPERIMENTAL

General methods. — Melting points are uncorrected. The optical rotations were determined with a Jasco DIP 180 type polarimeter with a 1.0-dm light path.

Cultivation of the mold and enzyme preparation. — The culture medium was a Czapeck-inulin medium composed of inulin (10 g) from dahlia tubers (Sigma Chemical Co., Lot 95C-8075), sodium nitrate (2 g), dipotassium hydrogenphosphate (1 g), magnesium sulfate heptahydrate (0.5 g), and ferric chloride (1 mg) per L of distilled water, adjusted to pH 5.0 with 0.5M hydrochloric acid. Agar (15 g) was added for the stock culture medium. The mold, isolated in our laboratory from soil and identified as *Aspergillus fumigatus* Fresenius by Dr. Junta Sugiyama of Mitsubishi-kasei Co., was cultivated for 6 days at 30° in 500-mL Roux flasks containing 150 mL of the medium. The mycelia were treated three times with ice-cold acetone, twice with diethyl ether, dried in a desiccator (calcium chloride) under diminished pressure, and stored (silica gel) at −24° until use. A 5.4-g portion of the acetone–ether dried material was ground in a mortar with quartz sand and 0.2M sodium phosphate buffer (108 mL,pH 5.65), and the homogenate was mixed with toluene (22 mL), and incubated for 21.5 h at 30°. After centrifugation for 30 min at 10 000 *g* and 0°, the supernatant was twice dialyzed at 5° against 0.02M sodium phosphate buffer (1 L, pH 5.65) for 2 days. A small amount of sediment was removed by centrifugation, and the supernatant (98 mL) was used as enzyme solution.

Conditions of the enzyme reaction and isolation of the reaction product. — The reaction mixture, containing dahlia inulin (1.6 g) and enzyme solution (80 mL) in 0.05M Tris–malate buffer (160 mL, pH 7.0), was incubated under toluene at 37°. Controls (vol. 4.0 mL), from which inulin or enzyme was omitted, were incubated similarly. The 40.5-h enzymic digest was heated for 20 min at 80°, and then cooled in cold water. After removal of the toluene by evaporation at 40° under reduced pressure, a suspension of bakers' yeast (7.8 g, prewashed with water[5]) in distilled water (30 mL) was added, and the mixture was incubated for 20 h at 37°. The yeast was removed by centrifugation. The product was found, by paper chromatography, to be the major sugar component of the solution, and its amount (~240 mg) was estimated by the resorcinol–hydrochloric acid method[13] on the basis of D-fructose. The solution was chromatographed on a column (4.5 × 33.8 cm) containing 60 g of Tokusei Shirasagi carbon (Takeda Pharma. Indust. Co.) and 120 g of Celite No. 535 (Johns-Manville Co.). The procedure was carried out as reported[5], except that the elution of the column with 5% ethanol was at a flow rate of 4 mL/min, and 20-mL fractions were collected. The fractions were monitored with the resorcinol–hydrochloric acid reagent[13]. The fractions containing the product were pooled, treated with IR-120 (H^+) and IR-45 (OH^-) ion-exchange resins, concentrated to 3 mL at

40° under diminished pressure, and fractionated by gel-filtration on a column (2.57 × 100 cm) of Bio-Gel P-2 (Bio-Rad Laboratories, 200–400 mesh), 5-mL fractions being collected at the flow rate of 12 mL/h. The fractions containing the product, which was estimated as just described, were combined and concentrated at 40° under diminished pressure to a thick syrup. A small volume of absolute ethanol was added, and an insoluble material was removed by filtration on a G-4 glass filter. The clear solution was slowly evaporated in a desiccator over calcium chloride at 5° to give crystals (yield, 85 mg). Three recrystallizations from ethanol yielded 24.3 mg of purified compound, m.p. 162–162.5°, $[\alpha]_D^{20}$ +27.9° (*c* 0.56, water).

Anal. Calc. for $C_{12}H_{20}O_{10}$: C, 44.45; H, 6.20. Found: C, 44.19; H, 6.38.

Methylation. — The compound (29.8 mg) was methylated by the method of Hakomori[14] with a slight modification[5] to give 31.7 mg of dried syrup, $[\alpha]_D^{20}$ +22.3° (*c* 0.736, chloroform); lit.[3] $[\alpha]_D^{20}$ +23.7° (chloroform) for 3,4,6,3′,4′,6′-hexa-*O*-methyl di-D-fructofuranose 1,2′:2,1′-dianhydride.

Anal. Calc. for $C_{18}H_{32}O_{10}$: C, 52.93; H, 7.90. Found: C, 52.90; H, 8.32.

Acetylation. — The compound (49.2 mg) was acetylated under the same conditions as described previously[5], with the exception that the reaction mixture was neutralized with sodium hydrogencarbonate after addition of crushed ice with shaking, to yield 64.1 mg, m.p. 138°, $[\alpha]_D^{20}$ +0.51° (*c* 2.73, chloroform).

Anal. Calc. for $C_{24}H_{32}O_{16}$: C, 49.99; H, 5.59. Found: C, 49.78; H, 5.60.

Comparison with authentic compounds. — Di-D-fructofuranose 1,2′:2,1′-dianhydride and its 3,4,6,3′,4′,6′-hexa-acetate were prepared by the method of Jackson and Goergen[1]. The free sugar showed m.p. 160–161°, $[\alpha]_D^{20}$ +27.8° (water), and the acetate m.p. 136°, $[\alpha]_D^{20}$ +0.51° (*c* 2.50, chloroform): lit.[1] m.p. 164°, $[\alpha]_D^{20}$ +27.0° (water) for the free sugar, and m.p. 137°, $[\alpha]_D^{20}$ +0.54° (chloroform) for the acetate. Di-D-fructofuranose 1,2′:2,3′-dianhydride, prepared by the enzymic method[15], showed m.p. 162°, $[\alpha]_D^{20}$ +136° (water). *A. fumigatus* difructose dianhydride, prepared by cultivation of the mold in an inulin medium, showed m.p. 201–203°, $[\alpha]_D^{20}$ +95.8° (*c* 1.13, water); details for its preparation will be published later.

Paper chromatography. — Portions (0.5 mL) of the reaction mixtures were withdrawn at 20-h intervals, heated in a boiling water-bath for 3 min, and cooled in cold water. Aliquots (10 μL) of the sample solutions were evaporated on a sheet of Whatman No. 1 filter paper and developed twice with ascending 6:4:3 (v/v) 1-butanol–pyridine–water[16].

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