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Note

Chromatographic procedures for isolating α,β-trehalose formed during the preparation of β,β-trehalose

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α,β-Trehalose (α-D-glucopyranosyl β-D-glucopyranoside) was needed in order to confirm the identity of one of the reversion products of D-glucose catalyzed by glucoamylase, to determine the kinetics of its conversion into D-glucose with glucoamylase, and to examine its separation from α,α- and β,β-trehalose, as well as from other disaccharides, by high-performance liquid chromatography[1,2]. Mother liquors from a Koenigs–Knorr preparation of β,β-trehalose octaacetate[3,4] were available, and as these were known to contain α,β-trehalose octaacetate[3], it was decided to attempt the isolation of α,β-trehalose from this source. The use of chromatographic procedures for the separation and analysis of the various trehalose forms facilitated the isolation of unequivocally pure α,β-trehalose and the measurement of an accurate optical rotation and melting point for it.

EXPERIMENTAL

Optical rotations were measured for 2% solutions at 589 nm in a 0.1-dm tube with a Perkin–Elmer Model 241 automatic polarimeter. Melting points were determined with a DuPont 1090 thermal analyzer. Thin-layer chromatography (t.l.c.) of acetylated compounds was performed on Brinkmann* (Westbury, NY) silica gel with 2:1 diethyl ether–petroleum ether; detection of separated compounds was accomplished by spraying the t.l.c. plates with 50% (v/v) aqueous sulfuric acid followed by heating at 110°. Capillary gas–liquid chromatography of trimethylsilyl derivatives of sugars was performed as described by previous authors[5]. Effluent

*Use of brand names over others not mentioned does not imply recommendation by the U. S. Department of Agriculture.

from chromatographic separations was monitored for D-glucose with 3,5-dinitrosalicyclic acid reagent[6], and for total carbohydrate with phenol–sulfuric acid[7]. Nuclear magnetic resonance (n.m.r.) spectra (^{13}C and ^{1}H) were recorded for α,β-trehalose dissolved in methanol-d_4, with a 300-MHz Nicolet NT300 n.m.r. spectrometer. Chemical shifts were based on tetramethylsilane. Samples were dried under vacuum and redissolved in methanol-d_4 three times.

2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**1**) (244 g, 0.60 mol) was prepared in crystalline form from anhydrous D-glucose (125 g, 0.69 mol) as described by Lemieux[8] except that the recrystallization step was omitted. 2,3,4,6-Tetra-*O*-acetyl-β-D-glucose (**2**) (87 g, 0.25 mol) was prepared from **1** (135 g, 0.33 mol)[4] using active silver carbonate[9]. Crude **1** (20.5 g, 0.05 mol) and **2** (17.4 g, 0.05 mol) were treated with mercuric cyanide (12.5 g, 0.05 mol) to give crystalline β,β-trehalose octaacetate (**3**) (10.1 g, 15 mmol)[4]. The ether was evaporated from the mother liquor of the crystallization of **3** to give a syrup (27.5 g). The syrup was deacetylated with sodium methoxide (75 mg, 14 mmol) in methanol (500 mL) for 30 min at reflux. The solution was evaporated *in vacuo* to a syrup which was dissolved in water (100 mL). The solution was applied to a column (500 × 50 mm) of Fisher coconut charcoal[10] (400 g; 50–200 mesh), and elution was performed with 2% (v/v) aqueous ethanol (2 L) followed by 10% ethanol (2 L). The effluent was concentrated at 40° *in vacuo* to ~100 mL, and the concentrate was applied to a column (200 × 20 mm) of Dowex-1 (OH^-) resin (100–200 mesh) which was eluted with water (150 mL). The nonreducing, aqueous solution that resulted was

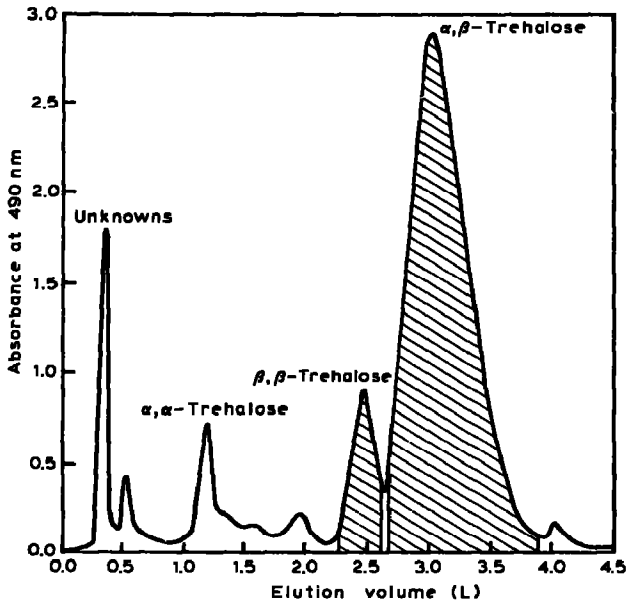

Fig. 1. Elution pattern of a trehalose mixture (250 mg) from a column (26-mm i.d. × 280-mm) of AG1-X4 (OH^-) anion-exchange resin eluted with water at 3 mL/min. Hatching indicates pools of α,β- and β,β-trehalose.

evaporated to a syrup at 40° *in vacuo*, and the syrup was dissolved in methanol (15 mL). After being kept for 16 h at −20°, crystals (2.9 g, 33 mmol) of α,β-trehalose were obtained. Recrystallization yielded purer product. Alternatively, dried syrup (250 mg) from the Dowex-1 eluate was further purified by passage through a column (280 × 26 mm) of Aminex AG1-X4 (OH^-) resin (400 mesh) (Bio-Rad, Richmond, CA), which was eluted with water at 3 mL/min. The α,β-trehalose peak was pooled and concentrated at 50° *in vacuo* to 100 mL using a 3-L single-effect evaporator, and was then freeze-dried to give 150 mg. Collection of the β,β-trehalose peak yielded 14 mg.

RESULTS AND DISCUSSION

Compound **3** was obtained in a yield of 30%, which is comparable to the 31.5% yield reported previously[3,4]. Examination by t.l.c. of the mother liquors from the preparation of **3** showed the presence of two compounds; the faster-moving compound (R_F 0.67) had the same mobility as compound **2** and the slower-moving component (R_F 0.40) had a mobility similar to that of compound **3** (R_F 0.47). The deacetylated mixture was analyzed by chromatographic[5] and spectrophotometric[6,7] techniques, and found to contain 65% of glucose, 2% of α,α-trehalose, 31% of α,β-trehalose, and 2% of β,β-trehalose. After removal of the glucose by means of Dowex-1 resin, the mixture contained 3.8% of α,α-trehalose, 81.8% of α,β-trehalose, 12.0% of β,β-trehalose, and 2.4% of an unknown compound (see Fig. 1). Thus, the composition of the reaction mixture prior to the isolation of crystalline **3** was roughly 45% of **1** plus **2**, 1% of α,α-trehalose octaacetate, 22% of α,β-trehalose octaacetate, and 32% of β,β-trehalose octaacetate. Good agreement was found in the analytical data from four individual experiments.

The efficient separation of **3** from the corresponding α,β isomer by crystallization from ethereal solution is comparable to that of methyl tetra-*O*-acetyl-β-D-mannopyranoside from its α anomer[11].

Helferich and Weis[3] recognized that the mother liquor from the crystallization of **3** contained a considerable amount of compound **2**, which they chose to remove as the benzylamine adduct prior to isolation of α,β-trehalose octaacetate. We chose instead to remove D-glucose from the deacetylated mixture and to recover the trehaloses by chromatographic separation on coconut charcoal; this procedure was rapid and efficient. Small amounts of D-glucose that contaminated the α,β-trehalose mixture were removed by passage through Dowex-1 (OH^-) resin. In this manner, overall recovery of α,β-trehalose after crystallization was 17%, higher than the 9% yield of Helferich and Weis[3], but lower than the 40% yield of Sharp and Stacey[12] using tetra-*O*-acetyl-β-D-glucopyranosyl fluoride, and the 26% yield obtained by Marquez and Sotillo[13] using **1**, penta-*O*-acetyl-β-D-glucopyranose instead of **2**, and mercuric bromide instead of mercuric cyanide. The latter two articles[12,13] did not report the amounts of α,α and β,β isomers which resulted under their conditions. Micheel and Hagel[14] also obtained **3** and its α,β isomer in yields

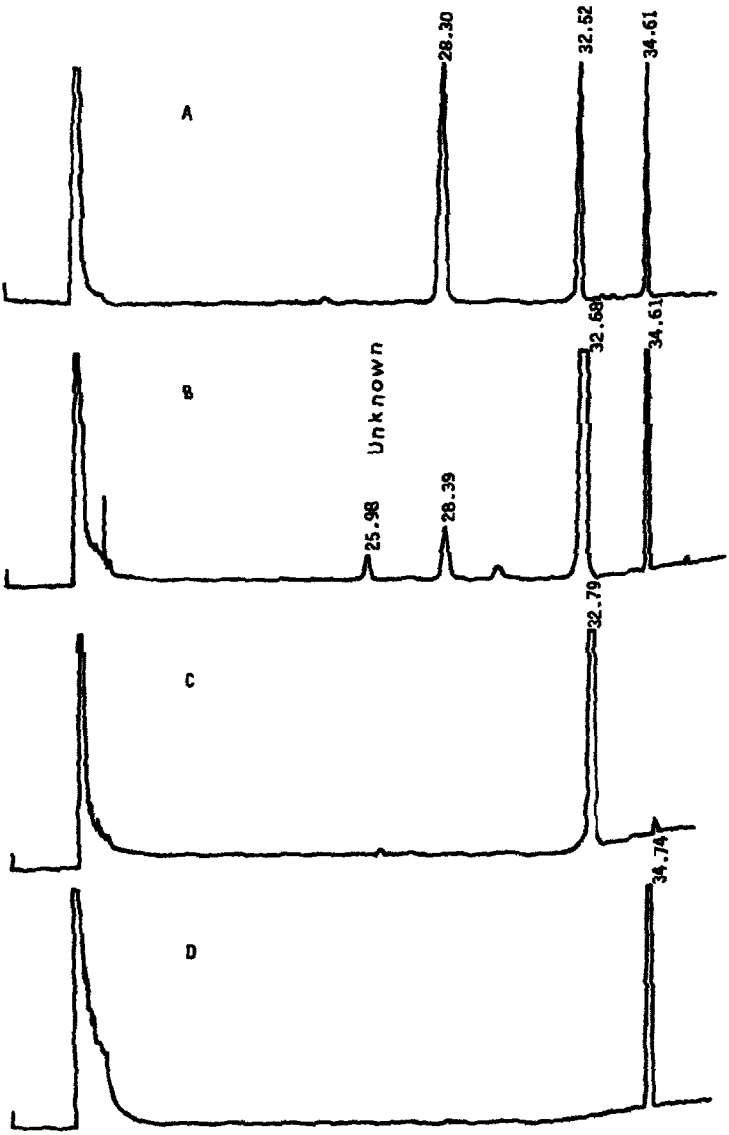

Fig. 2. Capillary gas–liquid chromatograms of trimethylsilated samples of (A) trehalose standard, (B) trehalose mixture before chromatography on a column of anion-exchange resin, (C) α,β-trehalose pool from chromatography on a column of anion-exchange resin, (D) β,β-trehalose pool from chromatography on a column of anion-exchange resin. Chromatography was conducted on a capillary column (256 μm i.d. × 30 m) of fused silica coated with DB-5 liquid phase using He as carrier gas at 220 mm/s. Oven temperature was 217° until 31 min, when it was increased at 10°/min to 250°. Injector temperature was 270°, detector temperature was 280°, and sample size was 1.5 μL. Numerals denote min after injection.

of 9.7 and 18%, respectively, from **1** with mercuric cyanide in moist acetone.

α,β-Trehalose could also be separated from α,α- and β,β-trehalose and other impurities in the effluent from the Dowex-1 column by using a column filled with Aminex AG1-X4 (OH^-) resin (see Fig. 2). Chittenden[15] had separated α,α- from α,β-trehalose with Dowex-1-X2 (OH^-) resin (200–400 mesh), and Onishi and Karasawa[16] separated all three trehalose with Dowex-1-X4 (OH^-) resin (200–400 mesh), both using water as the column eluant. The order of elution reported by the latter[16] was α,α-trehalose followed by α,β- and then β,β-trehalose, whereas Chittenden[15] reported an elution order of α,α- and then α,β-trehalose. The elution order observed in this work differs from that of Onishi and Karasawa[16], with β,β-trehalose being eluted before α,β-trehalose (see Fig. 2). Liquid chromatography purified α,β- and β,β-trehalose to 99.5% or greater (see Fig. 1).

The yield of α,β-trehalose using only chromatographic technique was 16%, compared to 17% by crystallization. Crystallization is the better choice for isolating gram quantities, but column chromatography is very effective for smaller amounts.

The physical constants of α,β-trehalose prepared by a variety of methods are considerably different. The product obtained by crystallization had m.p. 147° and $[\alpha]_D^{22}$ +78° (*c* 2, H_2O), while that prepared by liquid chromatography had m.p. 149° and $[\alpha]_D^{22}$ +83.5° (*c* 2, H_2O). These values differ from m.p. 80° and $[\alpha]_D^{18}$ +70° (*c* 0.04, H_2O) of Sharp and Stacey[12], and from the values of m.p. 178–179° and $[\alpha]_D^{22}$ +136° (*c* 5, H_2O) reported by Marquez and Sotillo[13]. The optical rotation value of Marquez and Sotillo[13] is approximately twice the +70° predicted theoretically by Hudson[17], while that of Sharp and Stacey[12] is the same. When the supernatant liquor from the crystallization of **3**, which contained all three trehalose forms (see Fig. 1B), was deacetylated, the D-glucose removed by charcoal and Dowex-1 chromatography, and a single (instead of a double) crystallization performed, the product had m.p. 80° (dec.) and $[\alpha]_D^{20}$ +69.4°, similar to the values[12] of Sharp and Stacey, suggesting that their α,β-trehalose preparation may have been contaminated with the other trehalose forms. The melting points and rotations of **3** {m.p. 181°, $[\alpha]_D^{22}$ −17.5° (*c* 2, $CHCl_3$)}, and of β,β-trehalose {m.p. 138–140°, $[\alpha]_D^{22}$ −40° (*c* 2, H_2O)} were in good agreement with values published earlier[18].

The ^{13}C-n.m.r. spectrum of the chromatographically purified α,β-trehalose dissolved in methanol-d_4 had 12 peaks, besides those for methanol (see Fig. 3). The two peaks of interest have chemical shifts of 105.18 and 101.99 p.p.m. and

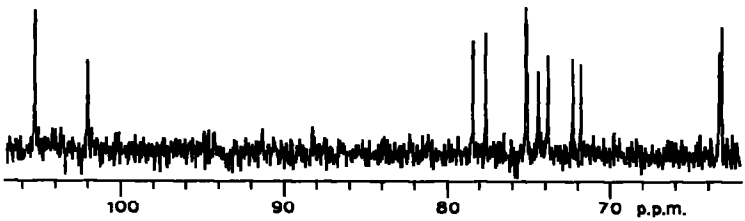

Fig. 3. ^{13}C-N.m.r. spectrum of chromatographically purified α,β-trehalose dissolved in methanol-d_4.

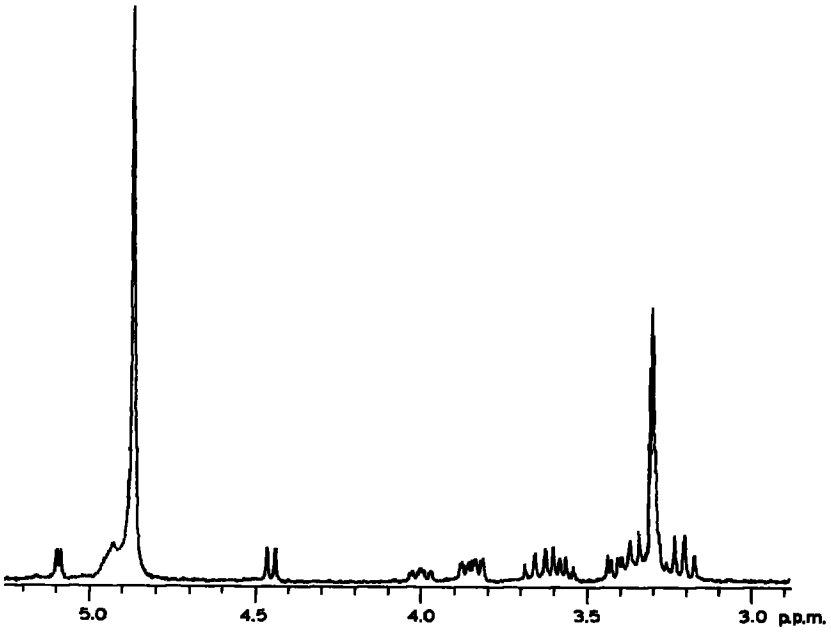

Fig. 4. ^{1}H-N.m.r. spectrum of chromatographically purified α,β-trehalose dissolved in methanol-d_4.

represent C-1 atoms linked equatorially and axially to glycosidic oxygen, respectively. The eight peaks from 78.4 through 71.7 p.p.m. represent the C-2 through C-5 atoms of the two glycosyl residues, and the two peaks at 63.23 and 63.08 p.p.m. correspond to the two C-6 atoms.

The ^{1}H-n.m.r. spectrum had two doublets of nearly equal height and area, at 5.09 and 4.45 p.p.m., corresponding to axial and equatorial protons attached to the two C-1 atoms (see Fig. 4). The peaks at 4.90 and 4.92 p.p.m. are attributed to D-glucosyl hydroxyl protons that had been only partially exchanged with deuterium. The large signal at 3.3 p.p.m. is assigned to the incompletely deuterated methyl group of the methanol-d_4. Integration of all other peaks indicated that there is a total of 12 protons attached to the C-2 through C-6 atoms of the two D-glucosyl residues.

The n.m.r. spectra of the purified preparation, as well as the knowledge that it is nonreducing and is composed exclusively of D-glucosyl unequivocally establish it as α,β-trehalose.

Because analysis showed the presence of a considerable amount of **2**, either unreacted material or that derived from **1**, in the mother liquor from crystallization of **3**, further study of the reaction using chromatographic analysis[1-5] of the products could lead to improved yields of both **3** and its α,β isomer. However, from results reported by Garegg *et al.*[19] on Koenigs–Knorr glycosylations, the use of mercuric bromide in combination with mercuric cyanide, instead of mercuric cyanide alone, would not be expected to alter significantly the relative amounts of the isomeric forms of the products.

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