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A Crystalline Mannobiose from the Enzymatic Hydrolysis of Guaran[1]

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By enzymatic hydrolysis of guaran there is obtained in 7% yield a crystalline mannobiose which is shown to be 4-(β-D-mannopyranosyl)-β-D-mannopyranose. This is the first isolation of the mannobiose. Its occurrence as a hydrolytic fragment of guaran suggests that β-1,4'-glycosidic linkages are present between D-mannose units in the polysaccharide.

Guaran,[2] the principal component of guar seed endosperm, consists of approximately 36% D-galactose anhydride and 64% D-mannose anhydride. Information concerning the structure of this polysaccharide has been obtained from physical measurements of triacetate films,[3] from periodate oxidation,[4,6] from methylation investigations[5,6] and from X-ray examination of highly oriented films.[7]

These combined data indicate that guaran consists of a chain of D-mannose units with D-galactose residues attached as single unit side-chains. However, it is evident that this derived structure, at best, is only a rough conception of the molecular configuration. To provide a more detailed picture of the guaran structure, the exact linkage between the sugar residues must be established. The manner in which the various units are linked can be determined to a large degree by isolation and characterization of the oligosaccharide fragments which are obtained from guaran on partial depolymerization. This paper reports the first of several crystalline oligosaccharides obtained through the enzymatic hydrolysis of guaran.

An enzyme preparation capable of bringing about a rapid hydrolysis of guaran has been extracted from germinated guar seeds and the optimum conditions for the hydrolytic action of the enzyme have been determined.[8] When an aqueous solution of guaran is treated with the enzyme preparation, hydrolysis proceeds to about 65% of completion. The hydrolysate can be conveniently separated into a monosaccharide fraction and several oligosaccharide fractions by the charcoal chromatographic procedure of Whistler and Durso.[9] Monosaccharides constitute about 65% of the hydrolysate and most of the D-galactose is found as free sugar. After removal of monosaccharides from the charcoal column, there can be eluted with 2.5% ethanol a disaccharide in 7% yield which crystallizes from methanol–butanol as tufts of needles. This substance is shown to be the mannobiose, 4-(β-D-mannopyranosyl)-β-D-mannopyranose. On methylation and hydrolysis of the fully methylated product there is obtained 2,3,4,6-tetramethyl-D-mannose and 2,3,6-trimethyl-D-mannose. On oxidation of the mannobiose to the corresponding mannobionic acid and methylation there is produced on hydrolysis equal molar quantities of 2,3,4,6-tetramethyl-D-mannose and 2,3,5,6 - tetramethyl - γ - D - mannonolactone. The presence of a β-glycosidic linkage in the mannobiose is suggested by the fact that the sugar is not hydrolyzed by emulsin which is a known source of α-mannosidase.[10] Mutarotation in a positive direction when the sugar is dissolved in water indicates that it crystallized in the β-configuration. Since the mannobiose is not produced when the enzyme preparation from germinated guar seeds acts on a solution of D-mannose, the isolated disaccharide is a true fragment of the guaran and, therefore, the glycosidic linkage present in this disaccharide must also be present in the guaran molecule.

Experimental

Enzyme Preparation.—Two kg. of guar seed was germinated, extracted with water and the centrifugate fractionated with ammonium sulfate.[8] The enzyme fraction which salted out between ammonium sulfate concentrations of 10 and 40% was air-dried and ground in a mortar.

Hydrolysis of Guar Flour.—Twenty-five grams of guaran was suspended in 1250 ml. of water in a Waring blendor. The mixture was stirred 8 hr. in an oil-bath at 95°, cooled and the *p*H was adjusted to 5.0 with 1 *N* hydrochloric acid. The resulting solution was placed in a 35° constant temperature bath and 1 g. of enzyme preparation was added. The hydrolysis mixture was stirred constantly and at the end of 24 hr. an additional 1 g. of enzyme preparation was added. After 70 hr. the hydrolysate was neutralized with sodium bicarbonate and filtered. The reducing power was determined at intervals during the hydrolysis by iodimetric titration of 1-ml. aliquots. In 70 hours, when hydrolysis had ceased, the reducing value indicated that the guaran was 65% hydrolyzed. Similar results were obtained when crude guar flour was used.

Chromatography of Hydrolysates.—The neutralized hydrolysate was chromatographed on Darco–Celite columns[9] with 600 ml. of hydrolysate being placed on each of two 44 × 260 mm. columns. Monosaccharides were desorbed with 1.5–2 l. of water. This is about 500 ml. more than is required to bring the optical rotation of the effluent to zero. Next the disaccharide was desorbed with 2 l. of 2.5% ethanol.

Partial Characterization of Products.—The weight of dried sirup from the water eluate represented 65% of the original guaran. On analysis of the sirup for D-galactose[2] by the mucic acid procedure and for D-mannose[2] by the phenylhydrazone procedure, values indicating 60.5% D-galactose and 39.3% D-mannose were obtained. On partial crystallization of the water fraction from 80% methanol, D-galactose was obtained in a yield equivalent to 80% of the amount originally present in guaran. The sugar had the accepted melting point and specific optical rotation.

The carbohydrate content of the 2.5% ethanol effluent represented 7% of the original guaran. Analysis for galactose showed no more than a trace to be present. Iodimetric titration values were those accepted for disaccharides. The dry amorphous solid was dissolved in a minimum amount of water and 100 ml. of absolute methanol was added. Butanol

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was then added until the solution became cloudy, after which it was evaporated on the steam-bath until flocculation started. On standing at room temperature, tufts of needles formed. After several recrystallizations they gave a melting point of 193.5–194.0°; $[\alpha]^{25}D$ −7.7° (extrapolated to zero time) → −2.2 (final), (*c*, 0.9 in water).

Anal. Calcd. for $C_{12}H_{22}O_{11}$: C, 42.1; H, 6.4. Found: C, 41.9; H, 6.5.

Methylation of 2.5% Ethanol Effluent.—Four grams of dried sirup of the 2.5% effluent was dissolved in a minimum amount of water and acetone was added until the solution became cloudy. The solution was methylated using 16 ml. of dimethyl sulfate and 40 ml. of 30% sodium hydroxide. It was kept under an atmosphere of nitrogen and was stirred vigorously at room temperature for 2 hours; then the temperature was slowly raised to 35–40° and maintained there with constant stirring for 6 hours. The temperature was raised to 55° and 2 ml. of dimethyl sulfate and 5 ml. of 30% sodium hydroxide were added each half-hour for 4 hours. The contents of the reaction flask were neutralized with 20% sulfuric acid and extracted with chloroform. The total yield of sirup was 2.1 g.

This was dissolved in 30 ml. of methyl iodide, 2 g. of freshly prepared silver oxide was added and the mixture refluxed. Two grams of silver oxide was added each half-hour for 4 hours. The mixture was concentrated to dryness and was extracted with chloroform. After evaporation of the chloroform, the sirup was remethylated again in this same fashion. The final yield was 2.1 g.

Hydrolysis and Identification of Products.—The methylated sirup was dissolved in 50 ml. of 5% aqueous hydrochloric acid and heated under reflux at 95–100° until the optical rotation reached a constant value. Then the solution was neutralized with sodium bicarbonate and filtered. The filtrate was extracted with chloroform, and the extract (A) was evaporated to dryness under vacuum. The water portion from the extraction was evaporated to dryness, extracted with boiling chloroform, and the chloroform extract (B) evaporated to dryness under vacuum.

To sirup A were added 0.37 g. of aniline and 2.24 ml. of absolute ethanol. This solution was refluxed at 80° for 3 hours and after nucleation with an authentic specimen of 2,3,4,6-tetramethyl-D-mannopyranose anilide, it crystallized. After three recrystallizations from absolute ethanol, the melting point was 145–146.5° and was not depressed on admixture with an authentic specimen.

Anal. Calcd. for $C_{16}H_{25}O_5N$: C, 61.7; H, 8.0; N, 4.5. Found: C, 61.7; H, 8.1; N, 4.6.

To sirup B were added 1.5 ml. of dry benzene and 0.19 g. of aniline. This solution was refluxed for 2.5 hr. at 80°, dried and triturated with dry ether. Crystals were obtained and were recrystallized from low-boiling (65–67°) petroleum ether. The melting point was 126–127°; and was unchanged when the crystals were mixed with an authentic specimen of 2,3,6-trimethyl-D-mannose anilide.

Oxidation of the Mannobiose.—To a solution of 10.15 g. of crystalline mannobiose in 500 ml. of water were added 15 g. of calcium benzoate and 2 ml. of bromine. The mixture was shaken vigorously and stored for 48 hr. in the dark at room temperature with occasional shaking. Then the solution was aerated until colorless and filtered. It was heated to 50° and 6.5 g. of oxalic acid was added. After cooling, the solution was filtered, and chromatographed one-half on each of two charcoal–Celite (1:1) columns 44 × 260 mm. The columns were washed with water until free of benzoic acid and then with 20% ethanol. This effluent contained positive rotating material and was concentrated to dryness under vacuum; yield 8.24 g.

Methylation and Hydrolysis of Oxidized Mannobiose.—The oxidized sirup was methylated with dimethyl sulfate and sodium hydroxide by the same procedure as described for the methyl glycoside above. The yield of sirup extractable by chloroform was 1.92 g. The water portion from the extraction was dried under vacuum, filtering off salts as necessary, and was methylated a second time as above. The yield of sirup extractable by chloroform was 5.13 g. The two sirups were combined, dissolved in methyl iodide and subjected to three methylations by the Purdie procedure as described above. The final yield of dry sirup was 5.0 g.

The fully-methylated oxidized product was dissolved in 100 ml. of 5% aqueous hydrochloric acid and refluxed in an oil-bath for 4 hours during which the optical rotation became constant. Then the mixture was neutralized with barium carbonate, filtered, concentrated to dryness and extracted with chloroform (D). The residue was dissolved in water, acidified with 1 *N* hydrochloric acid, evaporated to a sirup, dried completely by use of ethanol and benzene, and extracted with boiling ether (C). The chloroform extract (D) was evaporated to dryness, washed 4 times with 3 *N* sodium carbonate, and dried again; yield 1.35 g. (D). The sodium carbonate washings were dried and extracted with boiling ether; these extracts were combined with (C) and the whole evaporated to dryness under vacuum; yield 1.6 g. (C).

Identification of Fragments.—Fraction (C) was heated at 100° for one-half hour to complete lactonization. The lactone was crystallized from a mixture of ethyl ether and petroleum ether (b.p. 90–100°) and was recrystallized from petroleum ether. The physical properties agreed with those reported for 2,3,5,6-tetramethyl-D-mannono-γ-lactone[11]; m.p. 107.5–108.0°; $[\alpha]^{25}D$ + 65.1° (initial) → + 61.0° (9 days) (*c*, 0.7 in water).

Anal. Calcd. for $C_{10}H_{18}O_6$: OCH_3, 53.0. Found: OCH_3, 53.1.

The sirupy fraction (D) was treated with alcoholic aniline and the characteristic crystalline anilide of 2,3,4,6-tetramethyl-D-mannose was formed. It showed no lowering of melting point (145–146°) on admixture with an authentic sample.

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