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THE PREPARATION OF 6-*O*-α-D-GLUCOPYRANOSYLCYCLOHEXAAMYLOSE*

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ABSTRACT

A branched oligosaccharide was prepared by limited action of *Bacillus macerans* cycloamylose glucanotransferase (BME) on waxy corn starch. This branched oligosaccharide was treated with BME in the presence of sodium dodecyl sulfate. Various branched cycloamyloses were formed as major products, with cyclohexa- and cyclohepta-amylose (α- and β-cyclodextrins) as minor products. The cycloamyloses and branched cycloamyloses were converted into a mixture of 6-*O*-α-D-glucopyranosylcycloamyloses and D-glucose by the joint action of glucoamylase and *Aspergillus oryzae* alpha amylase (Taka-amylase A). 6-*O*-α-D-Glucopyranosylcycloamyloses were separated from the mixture by charcoal-column chromatography and purified by paper chromatography. 6-*O*-α-D-Glucopyranosylcyclohexaamylose crystallized from water, and its iodine complex formed dichroic spherocrystals.

INTRODUCTION

The Schardinger dextrins (cycloamyloses) are cyclic (1→4)-α-D-glucans produced by the action of *Bacillus macerans* cycloamylose (cyclomaltooligosaccharide) glucanotransferase (BME) (EC 2.4.1.19) on starch[1,2]. The main cyclic products are cyclohexa-, cyclohepta-, and cycloocta-amylose. In addition to these purely (1→4)-linked, cyclic molecules, various branched cycloamyloses (G_n-cycloamyloses, where *n* indicates the number of α-D-glucopyranosyl groups in the side chain) have been reported[3,4,5], but the yields have been so low that it has not been practical to prepare them pure in quantity.

In a previous report[6] it was shown that addition of sodium dodecyl sulfate (SDS) during the enzymolysis greatly enhanced the formation of cyclohexaamylose and repressed formation of cyclohepta- and cycloocta-amylose. With branched or

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chemically modified substrates, SDS enhanced formation of the corresponding branched or modified cyclohexaamylose. Action of glucoamylase (EC 3.2.1.3) on the mixed, branched, cycloamyloses converted the various branched cycloamyloses into a mixture of G_1-cycloamyloses. Purification of G_1-cyclohexaamylose from such a mixture was complicated, however, because this preparation contained significant amounts of cyclohexa- and cyclohepta-amylose, which interfered with the chromatographic separation.

The present paper reports an improved method of preparation of G_1-cyclohexaamylose in which a highly branched malto-oligosaccharide was used as the substrate for BME, the various G_n-cyclohexaamyloses were converted into G_1-cyclohexaamylose by action of glucoamylase, and cyclohexa, and cycloheptaamylose were degraded by joint action of *Aspergillus oryzae* alpha amylase and glucoamylase. Chromatographic isolation afforded the pure, crystalline G_1-cyclohexaamylose, and it was shown to be 6-*O*-*α*-D-glucopyranosylcyclohexaamylose.

EXPERIMENTAL

Materials. — BME was prepared and partially purified through a starch-adsorption step as previously described[7]. The lyophilized enzyme had an activity of 16 Tilden and Hudson units (THU)[1] per mg. "Glucozyme", a crude preparation of glucoamylase (powder, 3 IU mg^{-1}), was kindly donated by Nagase Sangyo Co., Ltd., Osaka, Japan. "Kokulase", a crude Taka-amylase preparation (powder, 30 IU mg^{-1}), was purchased from Sankyo Co., Ltd., Tokyo, Japan. Activated charcoal for chromatography was purchased from Wako Jyunyaku Co., Ltd., Osaka, Japan. Before use it was suspended in M hydrochloric acid and kept for 2 h in a boiling-water bath and overnight at room temperature. The charcoal was then filtered off, washed with distilled water until it was acid-free, and air-dried. SDS was obtained from Sigma, St. Lowis, Missouri, U.S.A. Other chemicals used were of analytical grade.

Charcoal-column chromatography. — Acid-treated charcoal was packed in a glass column (5.6 cm diameter, 43 cm high, fitted with a fritted disk) to a depth of 30 cm. The column was pretreated with 3 L of 6% 1-butanol and 4 L of water, and a solution of 25 g of the sample was placed on the column. The column was eluted with 10 L of water to remove glucose. To collect cycloamyloses, it was eluted with 6% 1-butanol at a flow rate of 100 mL per 5 min. SDS was adsorbed onto the column (1 g of charcoal was able to remove 280 mg of SDS). The SDS was not eluted by 6% 1-butanol. SDS was detected by Epton's method[8] and the presence of sugars was determined by the Molisch test.

Paper chromatography. — Sample solutions were spotted onto Whatman No. 3 filter paper, 20 cm high and 20 or 25 cm wide, and the spotted paper was irrigated three times with 5:3:4 1-propanol–1-butanol–water at 70°, with 4 h for each ascent. Cycloamyloses were revealed by dipping the chromatogram in a freshly prepared solution of 0.5% iodine in 90% acetone, and air drying. Appropriate in-

tensities for visualization of cycloamylose spots were obtained with 25 μg of cyclohexaamylose, 100 μg of cycloheptaamylose, 200 μg of cyclooctaamylose, and 100–400 μg of the branched cycloamyloses. Preparative paper-chromatography was performed by streaking the sample on the paper, irrigating and staining as described, excising the individual colored bands and eluting them with water. By this means, it was possible to purify 37 mg of G_1-cyclohexaamylose on a single sheet of paper.

High-pressure liquid chromatography (l.c.). — The chromatograph used was an ALC-GPC 201 instrument (Waters Associates Inc., Milford, Mass., U.S.A.), and the conditions were as follows: solvent, 65% acetonitrile; flow rate, 2 mL/min; detection, refractive index (r.i.); attenuation, 8×; and columns: Whatman Co, Pell PAC [precolumn, 2.1 mm (i.d.) × 7 cm long] and Waters Associates μBondapak carbohydrate-analysis column [3.9 mm (i.d.) × 30 cm]. Injected samples (50 μL) contained 0.5–1.5 mg of carbohydrate in 65% acetonitrile.

Preparation of branched malto-oligosaccharides. — The branched malto-oligosaccharides were prepared by suspending waxy maize starch (200 g) in 1 L of water containing 2400 THU of BME. The mixture was heated in a boiling-water bath for 20 min, and then autoclaved for 30 min at 120°. After cooling, the pH was adjusted to 6.7 with M sodium hydroxide, and 4 mL of 0.5M calcium chloride was added. The solution was treated with 3200 THU of BME and kept for 24 h at 40° with constant stirring. The mixture was evaporated to one half by boiling, cooled, and treated with 10 mL of 1,1,2,2-tetrachloroethane and 10 mL of bromobenzene. The mixture was agitated overnight at 10° and centrifuged at 8000*g* for 10 min. The supernatant solution, adjusted to pH 6.7 with M sodium hydroxide, was treated with 1600 THU of BME for 24 h at 40° with stirring. The mixture was again treated with 5 mL of 1,1,2,2-tetrachloroethane and 5 mL of bromobenzene, and a small amount of precipitate was removed by centrifugation. The supernatant solution was retreated by the same method and evaporated to dryness at 90° (yield 50 g).

Preparation of G_1-cyclohexaamylose. — Branched maltooligosaccharides (50 g) and 5 g of SDS were dissolved in 1 L of hot water. The mixture was cooled to 40°, 2 mL of 0.5M calcium chloride and 9000 THU of BME were added, and the mixture was kept for 24 h at 40°, pH 5–6. Paper chromatography of the mixture (Fig. 1) showed that it contained various kinds of branched cyclohexaamyloses as major components. Cyclohexaamylose, cycloheptaamylose, linear oligosaccharides, and branched, acyclic glucans were present as minor components. In comparison with controls containing no SDS during the enzymolysis, the amount of cycloamylose in the mixture with SDS was extremely high.

The total mixture was treated with 1 g (powder) of Glucozyme (crude glucoamylase) for 24 h at 40° to degrade acyclic glucans and the oligosaccharide side-chains of branched cycloamyloses. This treatment gave, in addition to the expected D-glucose and G_1-cycloamyloses, various kinds of oligosaccharides and branched cycloamyloses.

The mixture (500 mL) was applied to a charcoal column as described in the

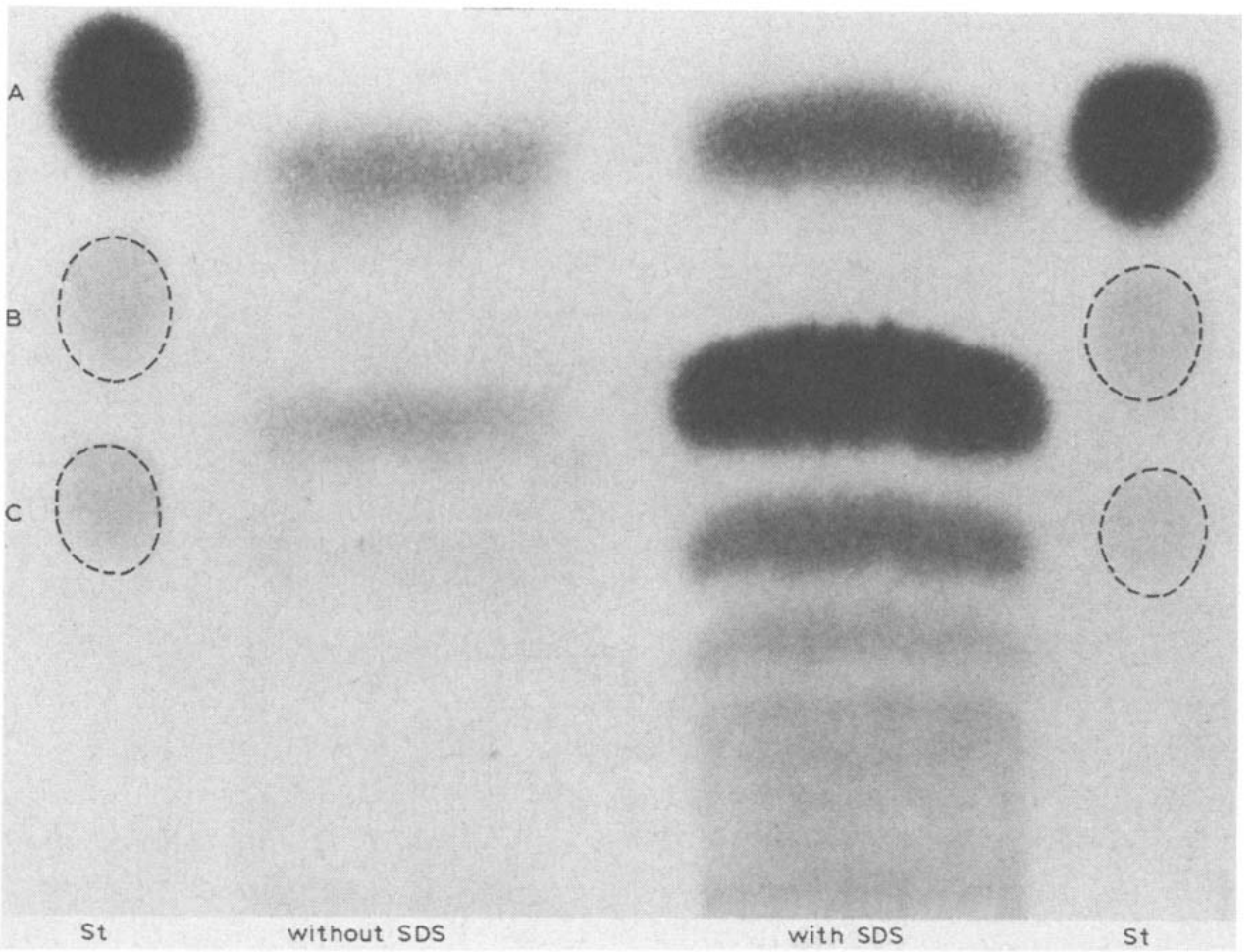

Fig. 1. Paper chromatogram of BME digests of branched maltooligosaccharides, with and without added SDS. St: standards of cyclohexaamylose (A), cycloheptaamylose (B), and cyclooctaamylose (C). Each digest (200 μL) with (right) and without (left) SDS, containing 10 mg of carbohydrate, was streaked in a 5-cm wide band, irrigated, and stained by the iodine–acetone dip-method. By this method cyclohexaamylose gives a blue stain, cycloheptaamylose is yellow, cyclooctaamylose is orange, G_1-cyclohexaamylose is brown-purple, and other branched cycloamyloses are brown. Bands in the digest without SDS are: top, blue; middle, weak brown-purple; lower, faint brown. Bands in the digest with SDS are, from the top downwards: blue, very strong brown-purple, and brown.

section on charcoal-column chromatography, and glucose and SDS were removed to obtain a crude, branched cycloamylose preparation, whose l.c. profile is shown in Fig. 2 (OR). The preparation contained G_1-cyclohexaamylose as a major component (I in Fig. 2); the yield was 84.2% (42.1 g from 50 g of branched maltooligosaccharides).

The OR component (10 g) was treated with glucoamylase (powder, 326 mg) for 2 days at 40° at pH 5–6 (without pH adjustment) at a carbohydrate concentration of 10% (digest G). Another part of OR (10 g) was treated with Taka-amylase A (powder, 326 mg) and glucoamylase (powder, 320 mg) at the same time, and under the same conditions, as the glucoamylase treatment (digest GT).

RESULTS AND DISCUSSION

Digests G and GT were compared after removing D-glucose by use of a charcoal column (Fig. 3). As shown in Fig. 2, peak IV of OR disappeared by the action

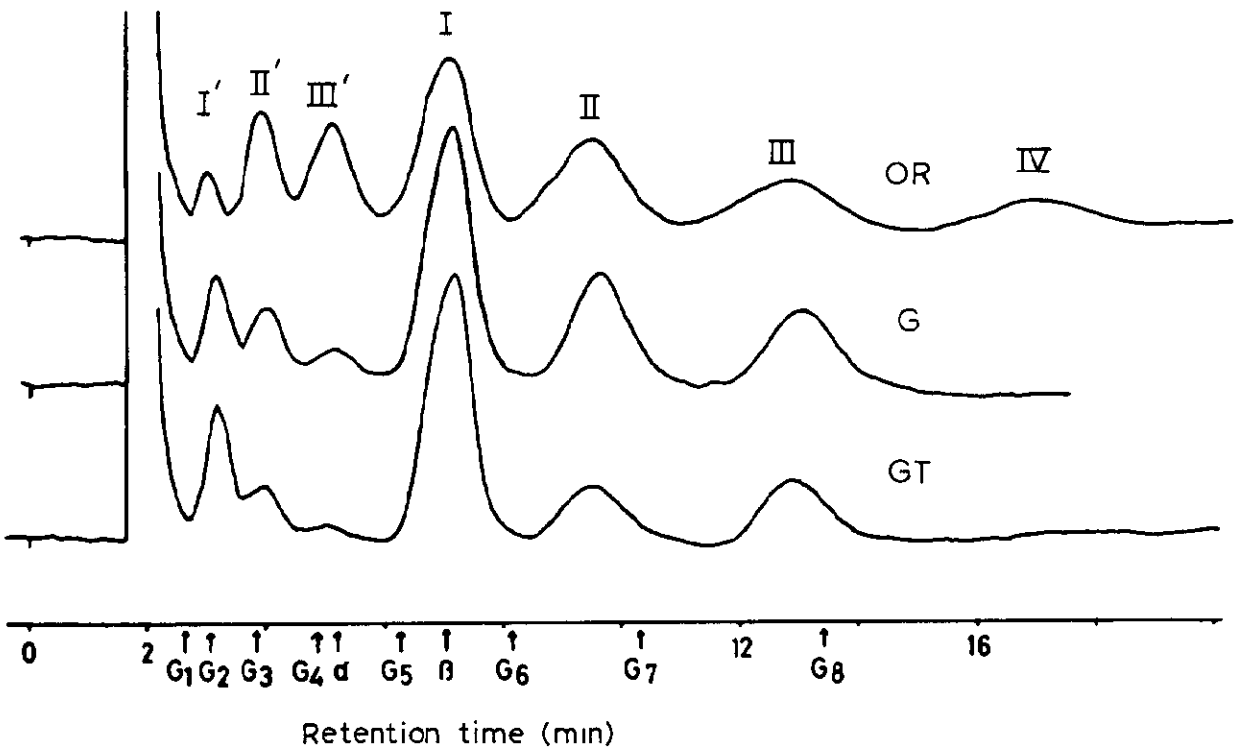

Fig. 2. L.c. profiles of stages of preparation of G_1-cyclohexaamylose. Refractive-index detector response *vs.* retention time. OR: 6% 1-butanol eluate from charcoal column of the digest from Fig. 1 with SDS. G: OR retreated with glucoamylase and eluted with 6% 1-butanol from a charcoal column. GT: OR treated with glucoamylase and Taka-amylase A, and then eluted with 6% 1-butanol from a charcoal column. Solvent, for l.c., 65% acetonitrile. Retention times of various reference materials are indicated along the abscissa. G_1, G_2, *etc.* are D-glucose, maltose, etc.; α is cyclohexaamylose; β is cycloheptaamylose. Branched oligosaccharides not degraded by enzyme action are possibly present in peaks I′, II′, and III′. In peak I, G_1-cyclohexaamylose; in II, G_2-cyclohexaamylose and G_1-cycloheptaamylose; in III, G_3-cyclohexaamylose, G_2-cycloheptaamylose and G_1-cyclooctaamylose; and in IV, G_4-cyclohexaamylose, G_3-cycloheptaamylose, and G_2-cyclooctaamylose.

of glucoamylase (G), and peaks II and III were smaller in GT than those in G. This is because peak IV contains cycloamyloses with having or longer branches, which are degraded by glucoamylase, and peaks II and III contain G_1-cycloheptaamylose and G_1-cyclooctaamylose, respectively, which are resistant to the action of glucoamylase, and which might be slightly susceptible to the action of Taka-amylase A. Peak II also contains G_2-cyclohexaamylose and peak III contains G_3-cyclohexaamylose and G_2-cycloheptaamylose, which are degraded by glucoamylase to G_1-cyclohexaamylose and G_1-cycloheptaamylose. Peak II showed the same iodine stain and R_F value as G_1-cycloheptaamylose on a paper chromatogram. Peaks I′, and II′, and III′ contained mainly low-molecular-weight oligosaccharides.

Pure G_1-cyclohexaamylose was prepared by streaking 400 μL (total sugar, 80 mg) of GT on an ascending paper chromatogram. After irrigation, the spots were located by the iodine dip-method. The G_1-cyclohexaamylose and G_1-cycloheptaamylose spots were outlined with a pencil, cut out, and eluted with water. The eluate was applied to a small charcoal column (1 cm diameter × 20 cm, maximum amount of charge 500 mg of carbohydrate) to remove iodine and other soluble inorganic material. After the column had been washed with water, the carbohydrate eluted from the column by 6% 1-butanol was concentrated by vacuum evaporation and dried in a vacuum oven overnight at 95° (350 mg from 10 sheets). The purified

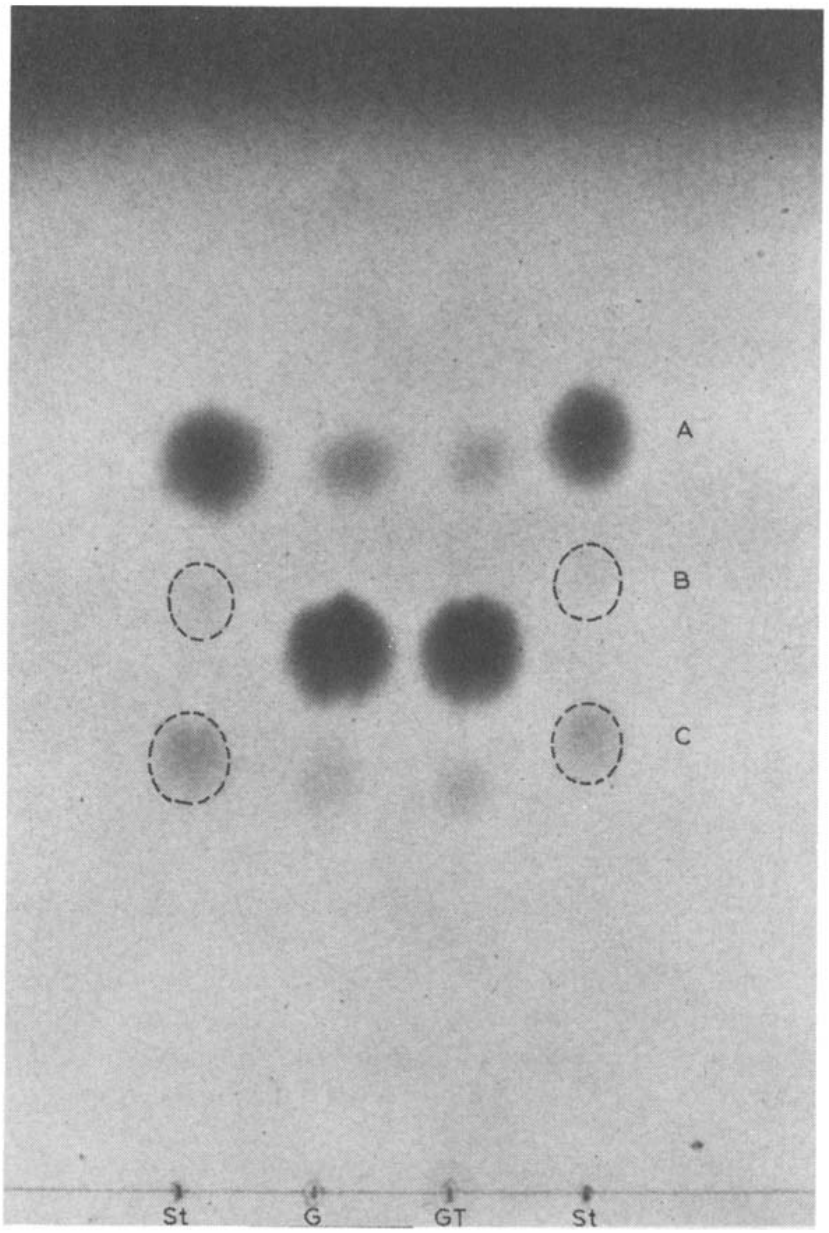

Fig. 3. Paper chromatogram of preparations G and GT from Fig. 2. Staining by the iodine–acetone dip-method. Standards (St) are: A, cyclohexaamylose; B, cycloheptaamylose, C, cyclooctaamylose. Spot colors in G and GT, from the top downwards: blue, brown-purple, and light brown.

G_1-cyclohexaamylose preparation gave a single peak by l.c. (Fig. 4). The preparation was degraded by the action of rabbit muscle amylo-(1→6)-glucosidase, which specifically degrades a 6-*O*-*α*-D-glucopyranosyl linkage attached to cyclohexaamylose, to form equimolar amounts of D-glucose and cyclohexaamylose. Attempted purification of G_1-cycloheptaamylose by paper chromatography gave a product showing two peaks in l.c. One of these corresponded to G_1-cycloheptaamylose and the other was unknown, possibly G_1-cyclooctaamylose. A 40% solution of G_1-cyclohexaamylose in water, stored in a desiccator over silica gel for a week, gave needle-shaped crystals (Fig. 5). Treatment of a solution of G_1-cyclohexaamylose on a microscope slide with a solution of iodine–potassium iodide gave dichroic spherocrystals (Fig. 6).

Confirmation of the structure of the G_1-cyclohexaamylose preparation as 6-O-*α*-D-*glucopyranosylcyclohexaamylose.* — Methylation analysis was performed by the method of Hakomori[9]. Crystalline G_1-cyclohexaamylose (5 mg) was dissolved in dimethyl sulfoxide (2 mL) under nitrogen and then methylated with methylsul-

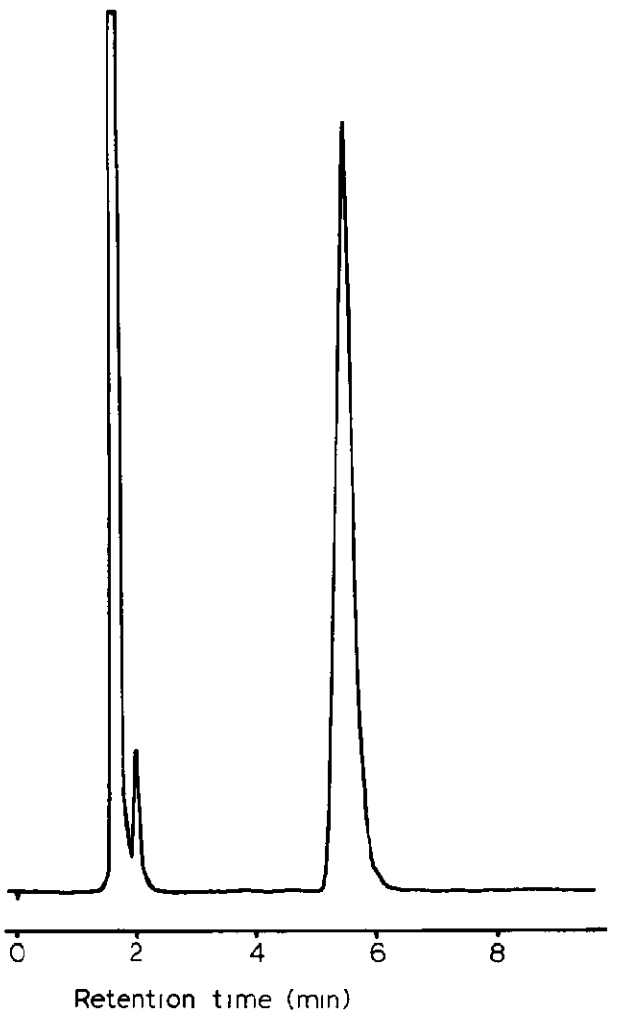

Fig. 4. L.c. of G_1-cyclohexaamylose. Refractive-index detector-response *vs.* retention time. The peaks at a retention time of 1.5–2 min are due to the solvent; that at 5.4 min is G_1-cyclohexaamylose. Sample, 10 μL of 8% G_1-cycloheptaamylose, solvent, 65% acetonitrile; attenuation, ×8.

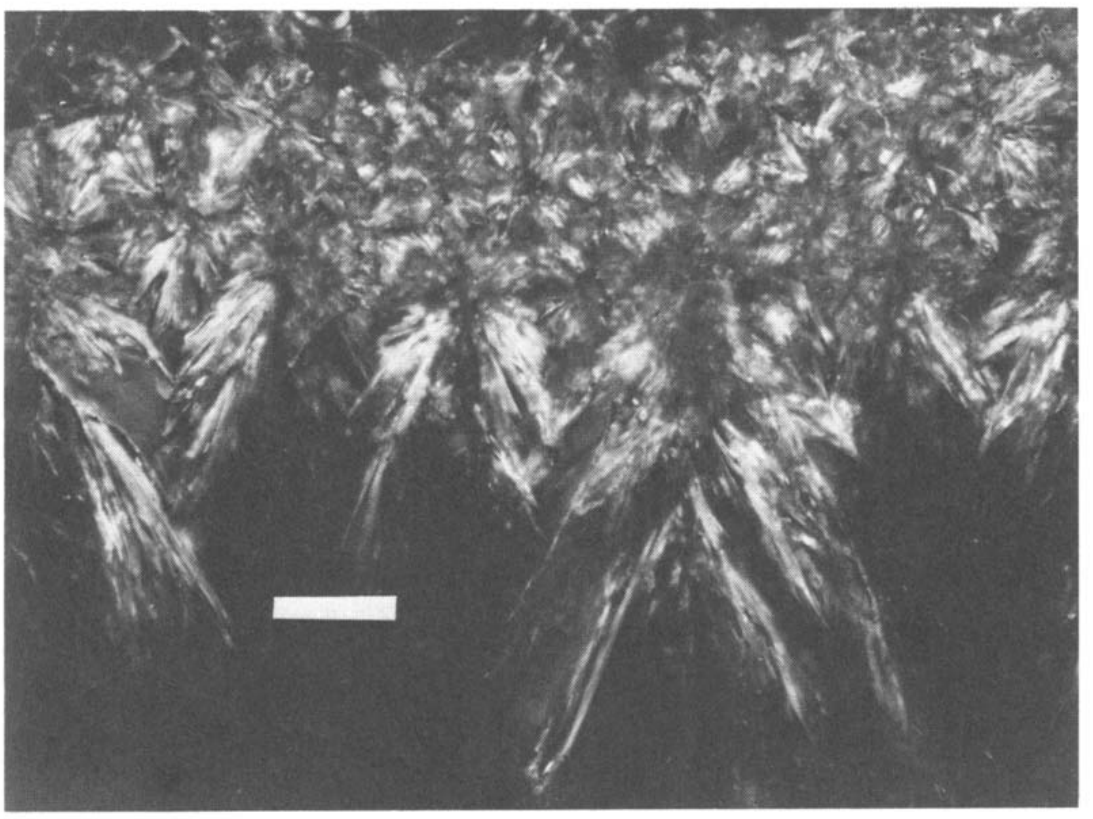

Fig. 5. Crystals of G_1-cyclohexaamylose from water, crossed polarizers. The bar is 50 μm long.

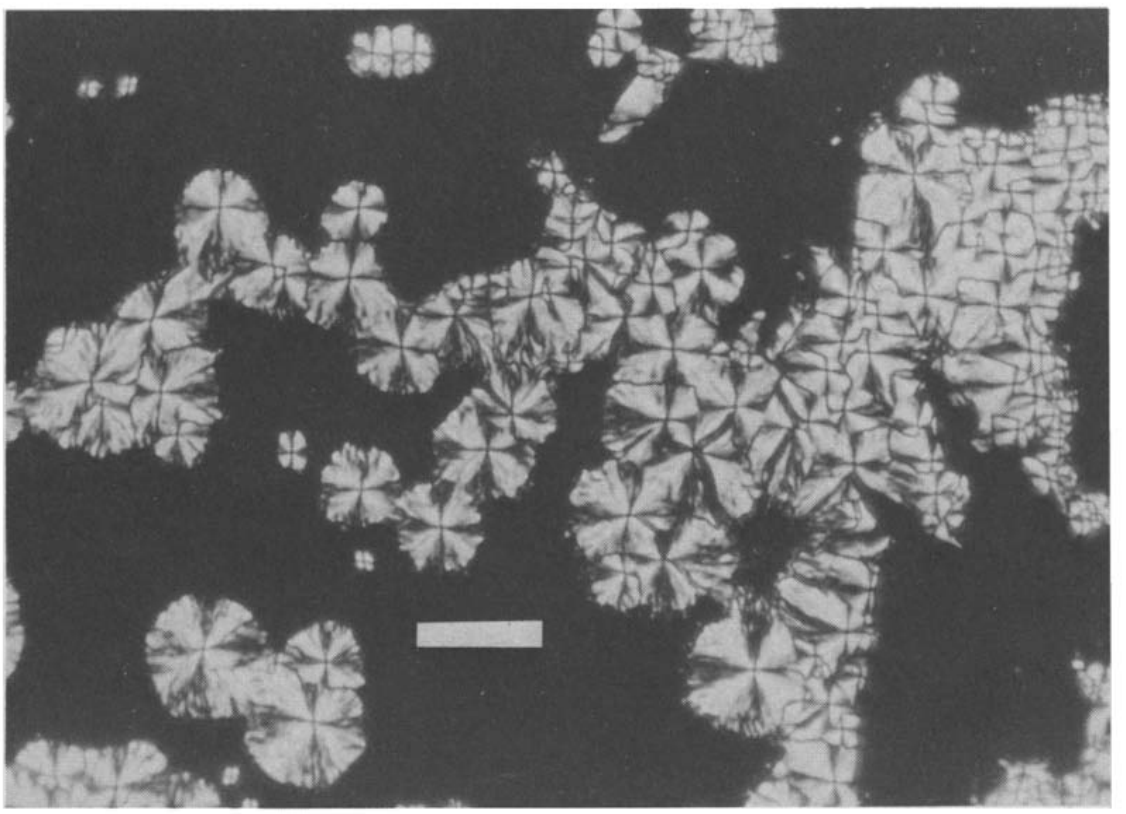

Fig. 6. Spherocrystals of the iodine–potassium iodide complex of G_1-cyclohexaamylose, crossed polarizers. The bar is 50 μm long. After being kept longer, the complex forms needle crystals. When observed in polarized light, the spherocrystals show moderate dichroism, colorless-brown; the needles are strongly dichroic, colorless-black; maximum absorption occurs when the plane of polarization is parallel to the radius of the spherocrystal or to the crystal-needle axis (sign of dichroism, positive).

finyl carbanion (0.5 mL) and methyl iodide (0.5 mL). After the methylation had been completed. the mixture was applied to a column of Sephadex LH-20, and the fractions of derivatized G_1-cyclohexaamylose were collected and evaporated. I.r. analysis indicated methylation to be incomplete, and therefore the procedure was repeated.

The completely methylated G_1-cyclohexaamylose was hydrolyzed by heating

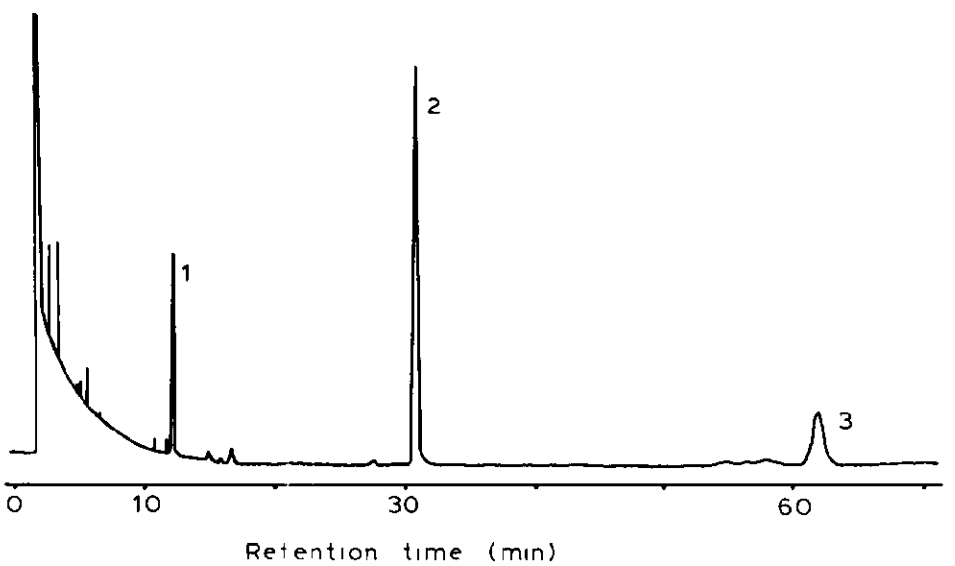

Fig. 7. Gas-chromatographic analysis of G_1-cyclohexaamylose preparation by methylation. Peak 1: 2,3,4,6-tetra-*O*-methylglucitol diacetate; peak 2: 2,3,6-tri-*O*-methylglucitol triacetate; peak 3: 2,3-di-*O*-methylglucitol tetraacetate.

it with 90% formic acid for 2 h at 100°, followed by heating with M trifluoroacetic acid for 1 h at 121°. The methylated sugars so obtained were converted into the corresponding alditol acetates and the latter analyzed by g.l.c. G.l.c. was performed with a Shimadzu GC-6AM gas chromatograph equipped with a flame-ionization detector and a surface-coated, open-tubular glass capillary column coated with Silar-10C (G-SCOT, 30 m × 0.28 mm, Gasukuro-kogyo Co., Tokyo, Japan). The column was connected to the gas chromatograph via a Shimadzu CLH-6 all-glass splitter (splitting ratio = 1:59). Fig. 7 shows a g.l.c. profile of a mixture of the alditol acetates derived from the methylated G_1-cyclohexaamylose. As shown in Fig. 7, peaks 1, 2, and 3 were determined to the 2,3,4,6-tetra-*O*-methylglucitol diacetate, 2,3,6-tri-*O*-methylglucitol triacetate, and 2,3-di-*O*-methylglucitol tetraacetate from their relative retention-times (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol = 1.00). Their molar ratios calculated by e.c.r. theory[10] were 1.07:5.25:1.00 (2,3-:2,3,6-:2,3,4,6-) and this ratio is in approximate agreement with the theoretical ratio for the alditol acetates formed from 6-*O*-α-D-glucopyranosyl cyclohexaamylose.

For Smith degradation, the G_1-cyclohexaamylose (2 mg) was oxidized with 0.02M sodium periodate (2 mL) for 5 days at 4° in the dark. The mixture was made neutral with barium carbonate and the oxidized polysaccharide was reduced with sodium borohydride overnight at room temperature. The resulting polyalcohol was completely hydrolyzed with M trifluoroacetic acid (1 mL) for 1 h at 100° and the product reduced with sodium borohydride. The alditol acetates formed after acetylation were analyzed by g.l.c.

Fig. 8 shows a g.l.c. profile of a mixture of the polyalcohols derived from G_1-cyclohexaamylose by Smith degradation. Peaks 1 and 2 were determined to be glycerol and erythritol, derived from branching glucose residue and the cyclic

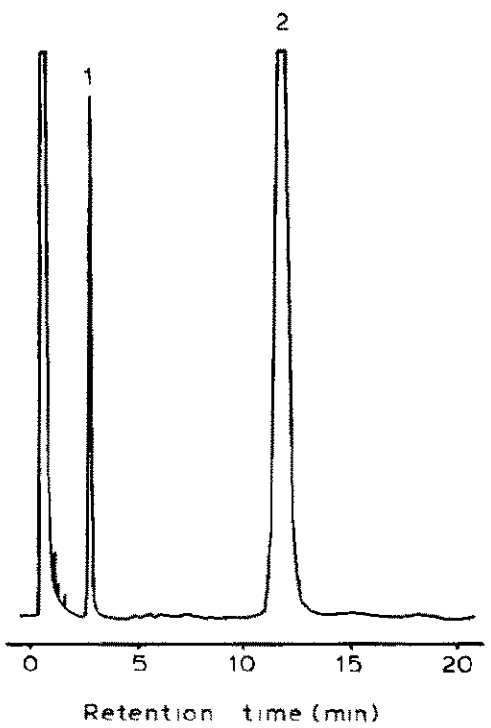

Fig. 8. Gas-chromatographic analysis of G_1-cyclohexaamylose preparation by Smith degradation. Peak 1: glycerol; peak 2: erythritol.

glucose residues of G_1-cyclohexaamylose, repectively. The molar ratio of erythritol to glycerol was calculated to be 5.8 from the response factors.

This ratio is in approximate agreement with the theoretical ratio (6.0) for the polyalcohol formed from 6-*O*-α-D-glucopyranosylcyclohexaamylose.

The results of methylation analysis, Smith degradation, and enzymic degradation all indicated the structure of the cycloamylose derivated to be 6-*O*-α-D-glucopyranosylcyclohexaamylose.

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