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Reduction of the Products of Periodate Oxidation of Carbohydrates. II. A New Method for the End-group Assay of Amylopectin[1]

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Amylopectin (waxy corn starch) has been subjected to prolonged periodate oxidation with sodium periodate and the polyaldehyde so formed reduced with sodium borohydride to the corresponding polyalcohol. Hydrolysis of the latter gives glycerol, erythritol, glycolic aldehyde and a small amount of D-glucose. The amounts of glycerol and erythritol are determined, after paper chromatographic separation, by periodate oxidation and determination of the formaldehyde so formed by means of the chromotropic acid method. The ratio of non-terminal to terminal non-reducing glucose units calculated from these results agrees reasonably well with those obtained by methylation and by periodate oxidation. The method has also been applied to the β-amylase limit dextrin prepared from waxy corn starch.

Amylopectin, the branched component of starch, has been the subject of many investigations in an effort to elucidate its fine structure. One of the most notable has been the application of the method of end-group assay,[2,3] which is based upon the complete methylation of all available hydroxyl groups followed by hydrolysis and determination of the ratio of 2,3,4,6-tetra-*O*-methyl-D-glucose to 2,3,6-tri-*O*-methyl-D-glucose. This method has been applied very successfully to native starch which is a mixture of amylose and amylopectin, and also to waxy corn starch which contains 98–99% amylopectin. Now that methods for the separation of amylose and amylopectin are fairly successfully worked out[4,5] there have been some recent studies on pure amylopectin fractions using the Haworth method. The ratio of non-terminal to terminal non-reducing units reported for the amylopectin fraction of most starches is 18–24[6] as determined by methylation. A more recent and perhaps more widely used method, due to its simplicity, makes use of periodate oxidation.[7–9] In this method, the terminal glucose residues having adjacent α, β, γ-triol groups, when treated with an alkali periodate salt, give rise to formic acid which can be titrated readily.[10] The results agree very well with those obtained by the methylation procedure. A third method[11] employing a system of enzymes applied separately and successively to polysaccharides such as amylopectin or glycogen has been devised in an effort to establish their molecular architecture.

This paper is concerned with the development of a new method[12] for the end-group assay of amylopectin. The procedure described herein is based upon the oxidation of the amylopectin by periodate to the corresponding polyaldehyde which is then reduced with sodium borohydride[13] to the polyalcohol. Hydrolysis of the latter gave a mixture consisting of glycerol, erythritol, glycolic aldehyde and a small amount of D-glucose. The glycerol, erythritol and D-glucose were separated chromatographically and a crystalline derivative of each component was prepared.

The glycerol is derived from the terminal non-reducing glucose units while the erythritol arises from the non-terminal units (see Fig. 1). The origin of the glucose is not yet clear but the possibility has to be considered[12] that it arises from glucose units linked in a different manner from those units already recognized, namely, those joined through positions 1 and 4 and through positions 1, 4 and 6.

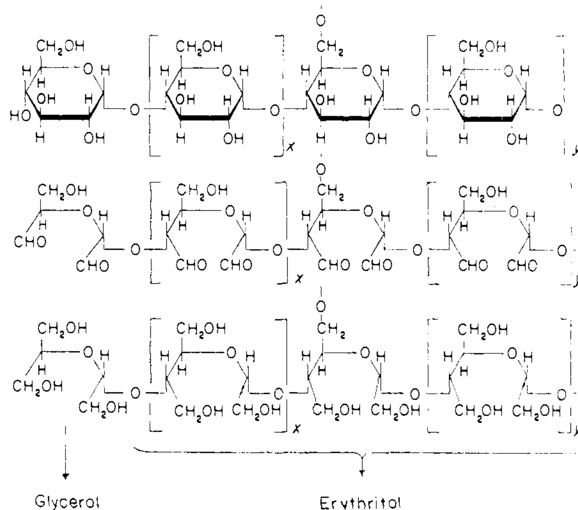

Fig. 1.

The amounts of glycerol and erythritol were determined quantitatively, after separation by paper chromatography, by oxidation with periodate and determination of the formaldehyde so formed by means of chromotropic acid.[14] The

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molar ratio of these two compounds was calculated and made the basis for determining the ratio of non-terminal to terminal non-reducing units of the molecule.

Waxy corn starch was found by this method to have a ratio of non-terminal to terminal non-reducing units of 16 as compared to 19 or 20 by methylation or periodate studies, respectively. The β-limit dextrin of waxy corn starch was found to have an average chain length of 5 by this method as compared to 6 deduced from the formic acid produced by periodate oxidation. The ratios of non-terminal to terminal non-reducing units of the β-limit dextrins obtained from other amylopectins have been in the range of 10–12.[15]

It is apparent that this method will be applicable to other types of polysaccharides in which the terminal non-reducing sugar residues react in a different manner from the other anhydro sugar units comprising the main body of the polymer.

Experimental

Determinations of the Average Chain Length of Amylopectin.—Defatted[4] and dried waxy corn starch (20 g.) was gelatinized at 68–75° and allowed to cool slowly to room temperature and mixed with a solution of sodium periodate (85.6 g.), and the volume adjusted to 1000 ml. giving a final concentration of 0.4 *M* with respect to periodate ion. The reaction mixture was shaken thoroughly and quickly cooled to 2–4° and kept at this temperature in the dark.[16] At suitable intervals an aliquot was removed, treated with excess ethylene glycol and the formic acid titrated with 0.0105 *N* sodium hydroxide using phenolphthalein as the indicator.[10,16] The periodate consumption was determined simultaneously on another sample, after appropriate dilutions, by the usual arsenite method[8] using 0.01 *N* iodine solution. Blank determinations were carried out in each case. The average chain length as determined by the amount of formic acid produced was found to be 19–20. The consumption of periodate proceeded much more slowly than formic acid production and was allowed to oxidize for 48 days at which time the consumption was 1.10 moles per anhydroglucose unit.

Isolation of the Waxy Corn Starch Polyaldehyde.—The insoluble polyaldehyde was isolated by centrifugation, washed with distilled water and dried by solvent exchange using absolute ethanol, petroleum ether and finally in a vacuum desiccator to constant weight (yield 12 g.).

Preparation and Isolation of the Waxy Corn Starch Polyalcohol.—The polyaldehyde (5 g.) was suspended in water (300 ml.) and sodium borohydride (1 g. in 25 ml. of water) was added in portions with vigorous stirring and the alkaline reaction mixture left overnight. An additional quantity of sodium borohydride (0.5 g.) was added and the reaction mixture was allowed to stand another 6 hours. An aliquot of the reaction mixture after acidification to destroy sodium borohydride was non-reducing to Fehling solution. The solution was neutralized with glacial acetic acid and concentrated under diminished pressure to a small volume.

Hydrolysis of the Waxy Corn Starch Polyalcohol.—To the above solution concentrated hydrochloric acid was added until the *p*H was approximately 1. The acidic solution was refluxed gently for 10 hours, cooled, neutralized, and deionized by passage through cation exchange (Amberlite IR 120)[17] and anion exchange (Duolite A4)[18] columns. The neutral solution was concentrated under diminished pressure to a sirup which was dissolved in a small volume of ethanol. Paper partition chromatographic analysis using phenol saturated with water at room temperature as the irrigating solvent showed the presence of glycerol, erythritol and a small amount of glucose. Similar results were obtained using 1-butanol:ethanol:water (4:1:5).

Quantitative Determination of Erythritol and Glycerol.—A portion of the above sirupy hydrolysate (0.914 g.) of the polyalcohol was dissolved in ethanol and made up to 10 ml. An aliquot of this solution (0.1 ml.) was placed on a sheet of Whatman No. 1 filter paper (8″ × 22″) and the constituents separated by partition chromatography using 1-butanol:ethanol:water (4:1:5) for 36 hours. The components, located by spraying marginal marking strips with Tollens reagent, were each extracted from the unsprayed central portion of the chromatogram with water (20 ml.).

The determination of the amount of glycerol and erythritol was carried out using 15 ml. of the glass wool filtered eluate containing glycerol and 4 ml. of the similarly filtered erythritol extract, by oxidation with periodate and determination of the amount of formaldehyde so formed colorimetrically.[14] An appropriate blank for the glycerol and erythritol was carried out simultaneously on a pieee of paper cut from the irrigated portion of a chromatogram containing no glycerol or erythritol. The intensity of color formed was determined by a Coleman Junior Spectrophotometer and the concentration of glycerol and erythritol ascertained by reference to standard curves. The results showed that the glycerol content of the hydrolysate of the polyalcohol was 28 mg. or 3.06% while the erythritol content of the hydrolysate was 562.5 mg. or 61.5% of the sirup. The molar ratio of the glycerol to erythritol was therefore 1:15.

Identification of Erythritol, Glycerol and D-Glucose.—A portion of the sirup from the hydrolyzed polyalcohol was separated using several large sheets (22.5″ × 18.25″) of Whatman No. 3 filter paper and an irrigating solvent of 1-butanol:ethanol:water (4:1:5) for 48 hours. The relative positions of the glycerol, erythritol and glucose were determined as follows: one-half inch vertical strips were cut from each edge of the chromatogram and also 1/4″ vertical strips from portions one-third and two-thirds across the sheet. The strips were sprayed with Tollens reagent and heated at 130° for one to two minutes. Using the markers, the areas of the various sheets containing the glycerol and erythritol were excised and the individual components eluted with 70% ethanol. The eluted material in each case was filtered and concentrated *in vacuo* to a sirup.

Isolation of Erythritol.—The sirup obtained from the erythritol fraction of the large scale sheet chromatographic separation was dissolved in a small volume of ethanol and upon cooling the erythritol crystallized, m.p. and mixed m.p. 120–122° (after recrystallization from ethanol).

Preparation of Tetra-*O*-tosyl-erythritol.—To an ice-cold solution of the above erythritol (0.114 g.) in anhydrous pyridine (2 ml.), *p*-toluenesulfonyl chloride (0.780 g.) was added. The reaction mixture was allowed to stand 24 hours at room temperature and poured into water (10 ml.). Upon cooling, the derivative crystallized and the crystals were washed with water followed by ethanol and dried in air. Recrystallization from acetone–ethanol gave tetra-*O*-tosylerythritol, m.p. and mixed m.p. 165–166° in agreement with the recorded value.[19]

Anal. Calcd. for $C_{32}H_{34}O_{12}S_4$: C, 52.03; H, 4.6. Found: C, 52.21; H, 4.72.

Preparation of Tri-*O*-tosylglycerol.—The sirup obtained from the glycerol fraction of the large scale sheet chromatographic separation was dissolved in a small volume of ethanol, filtered and concentrated to a sirup (0.115 g.).

To an ice-cold solution of this sirup (0.115 g.) in anhydrous pyridine (2 ml.), *p*-toluenesulfonyl chloride (0.781 g.) was added. The reaction mixture was kept for 24 hours at room temperature and poured into ice-water (10 ml.). The aqueous layer was decanted and the gum-like product was washed several times with cold water. It was dissolved in hot ethanol (2–3 ml.) and rapidly cooled whereupon the derivative separated as an oil. The latter was dissolved in hot acetone (2 ml.), a drop or two of ethanol added and the solution left overnight. The crude crystals were recrystallized three times from acetone–alcohol to give tri-*O*-tosylglycerol, m.p. and mixed m.p. 103–105°.

Preparation of the *p*-Nitroanilide of D-Glucose.—The sirup from the glucose fraction of the large scale sheet chromatographic separation was found to be slightly contaminated with a small amount of erythritol. It was redissolved in ethanol (1 ml.) and rechromatogrammed on a single sheet of Whatman No. 3 filter paper in the same manner as before. The eluate was filtered and concentrated to a sirup.

When this sirup was treated with acidified methanolic *p*-

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nitroaniline[20] it afforded D-glucose *p*-nitroanilide, m.p. and mixed m.p. 180–182° (after recrystallization from methanol), $[\alpha]^{23}D$ −191° in pyridine (*c* 0.5).

Preparation of the β-Limit Dextrin of Waxy Corn Starch.—Waxy corn starch (50 g.) was degraded[21] using β-amylase prepared[22] from soybean flour.

Determination of the Extent of β-Amylolysis.—The method devised for the rapid estimation of the rate and extent of the reaction of β-amylase on the amylopectin involved determination of the maltose liberated spectrophotometrically in the following manner. To an aliquot of the amylopectin-enzyme solution (5 ml.) ethanol (5 ml.) was added and the mixture containing the precipitated dextrin was shaken thoroughly. The precipitate was removed by centrifugation and the supernatant liquid adjusted to 10 ml. A portion of this solution (1 ml.) was diluted to 250 ml. and an aliquot (2 ml.) in duplicate was used in the spectrophotometric determination by the phenol-sulfuric method of Smith, *et al.*,[23,24] as follows: 0.1 ml. of 80% aqueous phenol was added to 2 ml. of the maltose solution (containing 20–60 micrograms) followed by the rapid addition of concentrated sulfuric acid (5 ml.). After thorough mixing, the solution was allowed to cool for at least 10 minutes, and the absorbance measured at 490 mμ in a Coleman Junior Spectrophotometer against an appropriate reagent blank. The blank was prepared by mixing water (5 ml.), ethanol (5 ml.), and carrying out the same dilutions as in the sample. The amount of maltose was determined by reference to a standard curve.

The amount of maltose liberated after 24 hours was 37% of the theoretical and was constant for 144 hours. The partially degraded amylopectin was precipitated by the addition of an equal volume of absolute alcohol, isolated by centrifugation and dissolved in distilled water (500 ml.). The solution was treated in the same manner as mentioned previously with a freshly prepared enzyme solution. The action of the β-amylase was followed as before. The total amount of maltose liberated from both treatments was 62.6% (31.3 g.). The constant value of 62.6% maltose liberated is in good agreement with values quoted in the literature.[21,25,26]

A control experiment was carried out on a sample of waxy corn starch without the addition of β-amylase in order to determine whether there were any free sugars present in the substrate that would give rise to color when the spectrophotometric determination was performed. The result was negative.

Isolation and Purification of the β-Limit Dextrin.—After boiling for 10 minutes in order to inactivate the enzyme and precipitate the protein, the solution was cooled and centrifuged. The supernatant liquid was poured into an equal volume of absolute ethanol which precipitated the β-limit dextrin. After keeping in the cold room (2°) overnight in order to complete the precipitation, the β-limit dextrin was removed by centrifugation, redissolved in hot water, cooled and reprecipitated by the addition of ethanol as before. This reprecipitation procedure was repeated twice after which the β-limit dextrin was centrifuged, resuspended in absolute ethanol and dried by successive washings with absolute ethanol, acetone and petroleum ether. The white amorphous powder was dried *in vacuo* to constant weight to give a yield of 8.0 g. or 16%.

Periodate Oxidation of β-Limit Dextrin.—A solution of the dextrin (5.0 g.) in water (50 ml.) was mixed with a solution of sodium periodate (21.4 g.) in water (150 ml.) and the volume adjusted to 250 ml. giving a final periodate concentration of 0.4 *M*. The reaction mixture was kept at 2–6° in the dark. The formic acid production and periodate consumption were followed in the manner already described. The average chain length of the dextrin as determined by formic acid production was 6, a value that was essentially constant from 24 to 144 hours.[27,28] The periodate consumed per anhydroglucose unit after 24 hours was 0.95 mole increasing slowly to 1.09 moles after 144 hours.

Reduction of β-Dextrin Polyaldehyde with Sodium Borohydride and Hydrolysis of the Polyalcohol.—The polyaldehyde was reduced and hydrolyzed, neutralized, deionized and concentrated under diminished pressure to a sirup as described above for amylopectin. Glycerol, erythritol and D-glucose were qualitatively identified as before.

Quantitative Determination of Glycerol and Erythritol.—The method was identical with that employed for the waxy corn starch. The results of the analyses showed that the glycerol content of the hydrolysate of the β-limit dextrin polyalcohol was 77 mg. or 5.25% based on the weight of the sirupy hydrolysate, and the erythritol content was 381 mg. or 26% of the sirupy hydrolysate. The molecular ratio of glycerol to erythritol was therefore 1:4.

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ST. PAUL, MINNESOTA

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