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MOLECULAR STRUCTURE OF UNMODIFIED AND CHEMICALLY MODIFIED MANIOC STARCHES*

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ABSTRACT

Unmodified (**1**) and a modified (**2**, *O*-2-hydroxypropyl distarch phosphate) manioc starch were hydrolyzed with pullulanase, beta-amylase, and/or amyloglucosidase and the hydrolyzates fractionated on Sephadex G-50. The amylopectin of **1** had one chain of $\overline{\text{d.p.}}$ 45 to 7.5 chains of $\overline{\text{d.p.}}$ 15. Debranching of **2** with pullulanase yielded only one half of the $\overline{\text{d.p.}}$ 15 chains obtained from **1**. The further beta-amylolysis of the debranched **2** was incomplete, although all of the debranched $\overline{\text{d.p.}}$ 15 chains were converted into maltose and maltotriose. Beta-amylolysis of **1** and **2** was 61 and 34%, respectively, and hydrolysis by amyloglucosidase was 93 and 49%, respectively. The relative amounts of the chromatographed fractions suggest that about one-half of the $\overline{\text{d.p.}}$ 15 chains of **2** contained no modifying groups. A model depicting the possible sites of 2-hydroxypropyl and/or phosphate groups on the modified manioc amylopectin molecule is presented.

INTRODUCTION

Techniques employing hydrolytic enzymes and gel-filtration chromatography have been useful in elucidating the molecular structure of many starches[1–12]. In this work, these methods have been utilized in structural studies of manioc (tapioca) starch and a chemically modified manioc starch, hydroxypropyl distarch phosphate. This derivative is crosslinked with phosphate groups and monosubstituted with 2-hydroxypropyl groups. This modified starch is an important component of many processed foods. Its susceptibility toward alpha amylase[13–17] and granular structure[18–22] have been described. However, the location of the modifying groups on the macromolecule has not been reported. By comparing the results obtained for the unmodified and modified manioc starches, the location of the modifying groups on the macromolecule may be specified.

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

EXPERIMENTAL

Materials. — *O*-(2-Hydroxypropyl)distarch phosphate manioc (**2**, Stein, Hall and Co., Inc., New York, N.Y.) having a molar substitution (m.s.) of 0.045 hydroxypropyl groups, and unmodified manioc starch (**1**) were used in this study. The moisture contents of **2** and **1** were 11.1 and 14.9%, respectively. Amylose contents were determined[23]. The iodine-absorption spectra were determined on mixtures containing 4 ml of starch solution (60 μg of starch per ml, boiled for 3 min to solubilize the starch) and 0.1 ml of iodine solution (0.2% iodine + 2.0% potassium iodide in water)[24].

Starch suspensions (1% in deionized water) were boiled in 100-ml flasks for 3–5 min with constant agitation. To ensure that **2** was solubilized, the effect of autoclaving the boiled starch-suspension for 1 h at 2.5 bars was evaluated. After autoclaving, both the boiled and the boiled and autoclaved samples were treated with pullulanase or amyloglucosidase, as described later. The sample that had been autoclaved and boiled was not hydrolyzed to any greater extent than that which had only been boiled. Thus boiling for 5 min was adopted for all preparations of **2**. The samples of **1** were boiled for 3 min. After boiling, the starch solutions were cooled to 37° and the enzyme digests prepared immediately. In all instances, boiled, deionized water was used. The scheme followed is shown in Fig. 1.

Enzymic degradation. — Debranching of the starches with pullulanase (pullulan 6-glucanohydrolase, EC. 3.2.1.41) was performed essentially as by Mercier and Whelan[4]. The compositions of the digests (P_1) were (per ml): 0.5 ml of 1% starch solution, 0.2 ml of 0.2M citrate–phosphate buffer, pH 5.0 (ref. 25), 0.3 ml of water, and 2 μl (0.5 IU) of a pullulanase solution that had been purified by the method of Mercier *et al.*[26]. Blanks were prepared without the enzyme. The surfaces of each

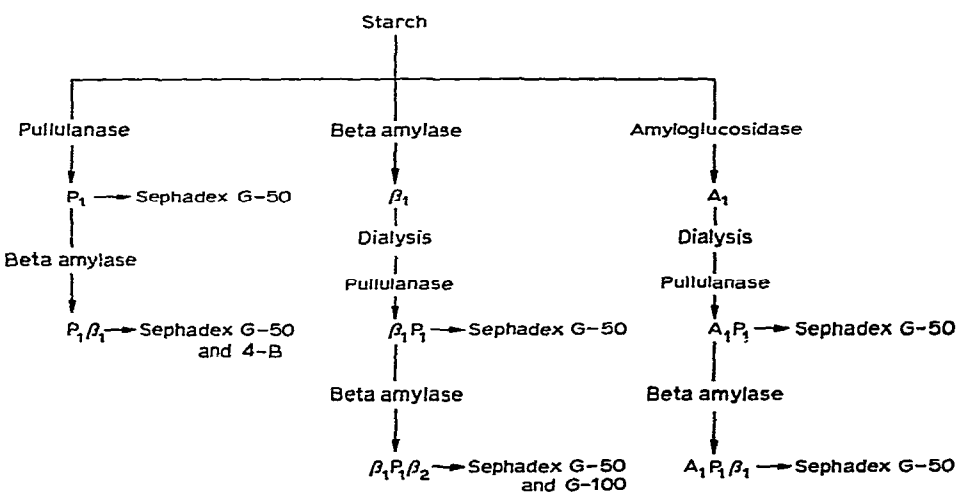

Fig. 1. Sequence of enzymic and chromatographic methods used to study the structure of manioc starch.

preparation were covered with toluene to prevent bacterial growth and the flasks were incubated at 37°. Hydrolysis was monitored by the Nelson method[27]. When the reducing capacity became constant (23–29 h), the enzyme was inactivated by placing the digestion flask for 20 min in boiling water. The degree of hydrolysis was expressed as the average unit chain-length ($\overline{c.l.}$), and was calculated as:

$$\frac{\text{polysaccharide in digest (glucose equivalents)}}{\text{reducing capacity (glucose equivalents)}}$$

Beta-amylolysis of the debranched starches ($P_1\beta_1$) was effected by adding 0.1 vol of 0.1% barley beta-amylase [(1 → 4)-α-D-glucan maltohydrolase, EC. 3.2.1.2, Fluka A.D., Buchs S.G., Switzerland] in water (6 IU/ml) to 1.0 vol of the P_1 digest. Digests were incubated at 37° and hydrolysis monitored as already described. When the reducing capacity became constant (18–24 h), the flasks were placed for 10 min in boiling water to inactivate the enzyme. The percent of beta-amylolysis was calculated as:

$$\frac{\text{reducing capacity (maltose equivalents)}}{\text{polysaccharide in digest (glucose equivalents)}} \times 100.$$

Beta-limit dextrins (β_1) were prepared by adding (per ml): 0.5 ml of 1% starch solution, 0.1 ml of 0.2M acetate buffer, pH 4.8 (ref. 25), 0.3 ml of water, and 0.1 ml (0.6 IU) of 0.1% barley beta-amylase solution. Blanks were prepared in which an equal volume of water replaced the beta-amylase solution. The surfaces of digests and blanks were covered with toluene and incubated at 37°. When the reducing capacity became constant (22–24 h), the beta amylase was inactivated as already described. The digest was dialyzed against several volumes of water at 2° until maltose was no longer detectable in the dialyzate. The maltose-free, beta-limit dextrin was lyophilized. A 1% solution of beta-limit dextrin was debranched with pullulanase (β_1P_1) and subsequently hydrolyzed with beta amylase ($\beta_1P_1\beta_2$) under the conditions already described for P_1 and β_1P_1.

The beta-limit dextrins (β_1P_1 and $\beta_1P_1\beta_2$) from **2** differed structurally from those from **1** (see Results). Therefore another exoenzyme, amyloglucosidase [(1 → 4)-α-D-glucan glucohydrolase, EC 3.2.1.3], was utilized in lieu of beta amylase to prepare the limit dextrins. The amyloglucosidase-limit dextrins (A_1) were prepared by combining (per ml): 0.5 ml of 1% starch solution, 0.25 ml of 0.2M acetate buffer (pH 4.8), 0.25 ml of water, and 6.3 μg (0.11 IU) of amyloglucosidase from *R. niveus* (crystalline, Seikagaku Kogyo Co Ltd., Tokyo, Japan). The enzyme contained no alpha-amylase activity. Digests and blanks were layered with toluene and incubated at 37°. When the reducing capacity became constant (40–48 h), the enzyme was inactivated by placing the flask for 15 min in boiling water. The percent hydrolysis was calculated as:

$$\frac{\text{reducing capacity (glucose equivalents)}}{\text{polysaccharide in digest (glucose equivalents)}} \times 100.$$

Compound **1** was almost completely hydrolyzed, but **2** was not. Therefore, the amyloglucosidase-limit dextrin from **2** was dialyzed, debranched with pullulanase (A_1P_1), and subsequently treated with beta amylase ($A_1P_1\beta_1$) as already described above for the beta-limit dextrins.

Analysis of the digests. — Total polysaccharide in the digests was determined by hydrolysis with amyloglucosidase–alpha amylase from *A. niger*[28] and quantification of the D-glucose formed by D-glucose oxidase–peroxidase[29]. As the modifying groups had been shown to inhibit amyloglucosidase (see Results), the polysaccharide concentration in the digests of **2** was determined by the anthrone method described by Loewus[30] and modified by Tollier[31] as follows. Anthrone reagent was prepared by dissolving in a dark bottle 2.0 g anthrone in 100 ml of redistilled ethyl acetate. Digest (2 ml, containing 30–120 μg of carbohydrate) was pipetted into 25 × 200 mm test tubes. The tubes were placed in a rack that maintained them at ~30° from the vertical, and 0.5 ml of anthrone reagent was added to each tube. The rack of tubes was placed in an ice-bath and 5.0 ml of concentrated sulfuric acid (density 1.83) was carefully added so that the acid ran down the side of the tube and formed a layer under the sample–anthrone reagent-mixture. Each tube was agitated individually and immediately placed for exactly 12 min in boiling water. The tube was returned to the ice bath, cooled to 20°, and the absorbance read at 625 nm. The amount of carbohydrate in each tube was calculated from a standard curve prepared with 0–60 μg/ml solutions of D-glucose.

All of the digests were fractionated on a calibrated column[4] (2.54 × 100 cm) of Sephadex G-50 at 20–25°. Elution was in the ascending direction at 20 ml per h with 10mM phosphate buffer (pH 7.0) containing 0.001% sodium azide. In several instances, fractionation was also conducted on Sephadex G-100 and Sepharose 4-B under the same conditions. Fractions of 8–10 ml were collected. The α-D-glucans in each fraction were hydrolyzed by amyloglucosidase[28] and the resulting D-glucose determined with D-glucose oxidase[29]. As **2** was not completely hydrolyzed by amyloglucosidase, the carbohydrate concentration in each fraction was also determined by the orcinol–sulfuric acid method at 420 nm, with a Technicon Autoanalyzer.

The elution profiles denote the weight percent (mg/100 mg) recovered in each fraction plotted against the elution volume. Each experiment was repeated twice. In all instances, recoveries from the columns were > 88%.

RESULTS

The iodine-absorption spectra of **1** and **2** revealed an absorption maximum for both starches at 590–595 nm, but the height of the peak for **2** was lower than for **1** (Fig. 2). The amylose content of **1** was 17%. No amylose was detectable in **2** by the iodine-binding method.

Conversion of starch into glucose by amyloglucosidase–alpha amylase was 98% for **1** but only 70% for **2**. Similar, relative hydrolysis-values have been reported for **2** and **1** by using pancreatic alpha amylase and fungal alpha amylase–amyloglucosidase

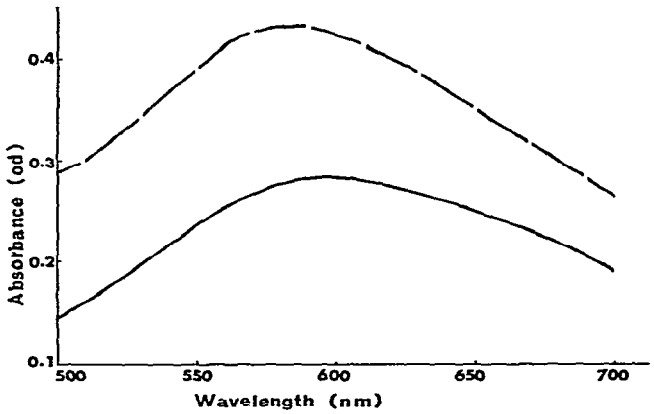

Fig. 2. Iodine-absorption spectrum of *O*-(2-hydroxypropyl) distarch phosphate (—) and unmodified manioc (---) starches.

preparations[15,16]. Obviously, the modifying groups interfere with the amylolytic activity of several enzymes.

The elution profile of pullulanase-debranched **1** (P_1) is shown in Fig. 3A. Three peaks are evident. The peak at the void volume (V_0) comprised about 20% of the total polysaccharide (Table I) and was principally amylose. Peaks at $\overline{d.p.}$ 45 and 15 were present in the relative concentration of 1 to 2.5. When calculated on a molecular-weight basis, there were approximately 7.5 of the $\overline{d.p.}$ 15 for each of the $\overline{d.p.}$ 45. Except for a small amount (2%) of the material at the V_0, all of the debranched polysaccharide was converted into maltose and maltotriose by beta amylase ($P_1\beta_1$). Thus the fractions of $\overline{d.p.}$ 45 and 15, and most of the material at the V_0 were linear.

Beta-amylolysis of **1** was 61%. The elution profile of the debranched, beta-

TABLE I

CHARACTERISTICS OF PULLULANASE-DEBRANCHED, UNMODIFIED STARCH (1) AND *O*-(2-HYDROXYPROPYL) DISTARCH PHOSPHATE (2)[a]

Component	1	2
P_1		
$\overline{c.l.}$[b]	25	53
$\overline{d.p.} > 60$	20	67
$\overline{d.p.}$ 45	21	7
$\overline{d.p.}$ 15	53	24
$P_1\beta_1$		
Beta amylolysis of P_1	104	50
$\overline{d.p.} > 60$	2	47

[a]Unless otherwise noted, all values expressed as%. [b]$\frac{\text{polysaccharide in digest (glucose equivalents)}}{\text{reducing capacity (glucose equivalents)}}$.

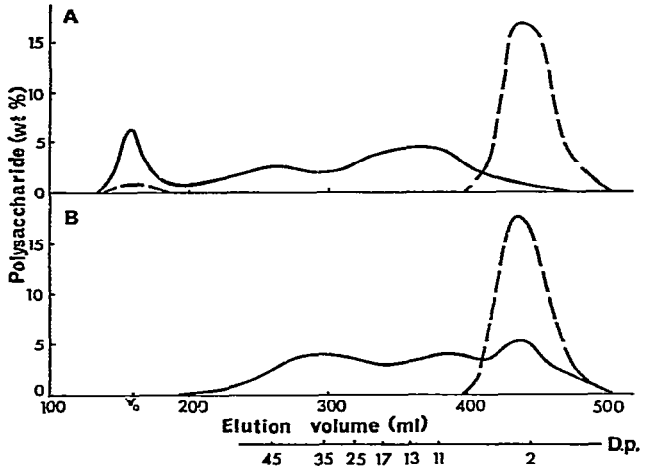

Fig. 3. Elution profiles from Sephadex G-50 of unmodified manioc starch. Polysaccharide concentration in each fraction was determined by the amyloglucosidase–D-glucose oxidase method. A: Debranched (P_1 —), P_1 hydrolyzed with beta amylase ($P_1\beta_1$ ---); B: debranched, beta-limit dextrin (β_1P_1 —), β_1P_1 hydrolyzed with beta amylase ($\beta_1P_1\beta_2$ ---).

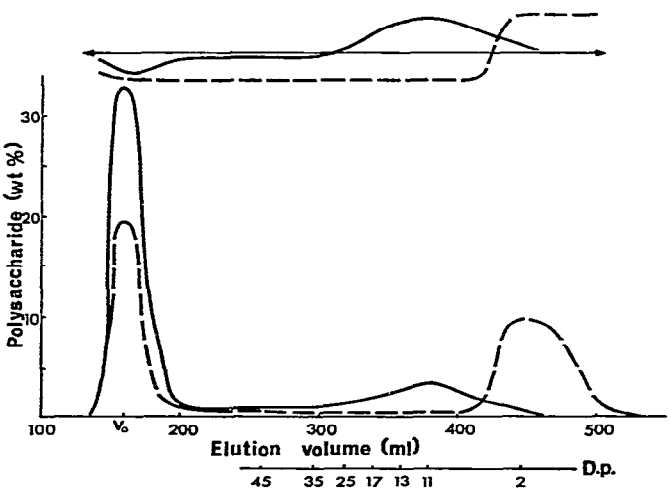

Fig. 4. Elution profiles from Sephadex G-50 of *O*-(2-hydroxypropyl) distarch phosphate manioc. Polysaccharide concentration in each fraction was determined by the amyloglucosidase–D-glucose oxidase and the orcinol methods. Bottom curves are from orcinol values. Top curves denote the ratio of amyloglucosidase–D-glucose oxidase to orcinol values in each fraction and in the unfractionated digest (↔). Debranched (P_1 —), P_1 hydrolyzed with beta amylase ($P_1\beta_1$ ---).

limit dextrin (β_1P_1) (Fig. 3B) reveals a similar pattern to that reported for maize and waxy-maize starches[8,10], except for the small amount of undebranched material remaining at the V_0 for maize. The fact that no such polysaccharide was observed at the V_0 for β_1P_1 manioc suggests that branched amylose does not exist in manioc starch.

The peak at $\overline{\text{d.p.}}$ 30–35 represents the $\overline{\text{d.p.}}$ 45 chains that had been shortened by beta amylase during preparation of the beta-limit dextrin. The broad, bimodal peak at $\overline{\text{d.p.}}$ 2–10 constitutes the maltosyl and maltotriosyl stubs remaining after the outer chains had been shortened by beta amylase, as well as the inner chains of the amylopectin. Beta-amylolysis of the debranched, beta-limit dextrin was 104%, verifying the linearity of the inner and outer chains of the amylopectin of **1**.

The debranching of **2** by pullulanase was not complete. The elution profile of the P_1 from Sephadex G-50 (Fig. 4) reveals peaks only at the V_0 and at $\overline{\text{d.p.}}$ 15. There was no distinct peak at $\overline{\text{d.p.}}$ 45. When compared to **1**, only about half of the amount of $\overline{\text{d.p.}}$ 15 was generated (Table I). Of the pullulanase-treated starch, 67% was eluted at the V_0. As **1** contained 17% of amylose, this result indicates that the remaining 50% not debranched by pullulanase was amylopectin. Manioc starch has about 83% amylopectin, and thus some 60% of the amylopectin had been modified sufficiently to inhibit hydrolysis by pullulanase.

The amyloglucosidase–D-glucose oxidase method and the orcinol or anthrone methods were used to determine the amount of carbohydrate in each digest of **2**. The amount obtained by the amyloglucosidase method was always less than that determined by the orcinol or anthrone procedures. Amyloglucosidase–D-glucose oxidase and orcinol were used to determine the polysaccharides in each fraction from Sephadex G-50. The ratio of values obtained by the two methods for each fraction was compared to the same ratio for the total digest. These results are shown at the top of Fig. 4. If the ratio of a particular fraction was lower than that for the total digest (↔ at the top of Fig. 4), the number of modifying groups per mg of polysaccharide presumably would be higher in that fraction than in the total digest. Conversely, if the ratio for the fraction were higher than for the total, the degree of modification would be lower in the fraction than in the total digest. This procedure does not give an absolute measure of the hydroxypropyl and/or phosphate content of each fraction, but it does facilitate a comparison of fractions (and peaks) on the basis of relative concentration of modifying groups. Thus the curve at the top of Fig. 4 suggests that the polysaccharide eluted at the V_0 had a higher than average number of modifying groups. Conversely, the $\overline{\text{d.p.}}$ 15 polysaccharide contained a much lower than average degree of modification.

Beta-amylolysis of the pullulanase-debranched **2** ($P_1\beta_1$) was only 50% as compared with 104% for the $P_1\beta_1$ from **1** (Table I). All of the $\overline{\text{d.p.}}$ 15 material was hydrolyzed to maltose or maltotriose, confirming that this fraction did not contain modifying groups (Fig. 4). The proportion of the high-molecular-weight fraction eluted at the V_0 decreased from 67% in P_1 to 47% in $P_1\beta_1$. The 20% hydrolyzed by beta amylase may represent unmodified amylose and/or unmodified parts of the amylopectin that were resistant to pullulanase but were hydrolyzed by beta amylase. The amyloglucosidase–D-glucose oxidase:orcinol ratios were below the value for the $P_1\beta_1$ digest for the entire elution profile, except for the maltose–maltotriose peak. At that point, the values for total carbohydrate obtained by the two methods for each fraction were equal, implying that maltose and maltotriose did not contain modifying

TABLE II

CHARACTERISTICS OF EXOENZYME LIMIT-DEXTRINS FROM UNMODIFIED STARCH (1) AND *O*-(2-HYDROXYPROPYL) DISTARCH PHOSPHATE (2)[a]

	1	2
Beta amylase		
Beta amylolysis of starch (β_1)[b]	61	34
β_1P_1		
c.l.[c]	10	26
d.p. > 60	0	34 (52)
d.p. 20–60	14 (36)[d]	14 (21)
d.p. < 20	25 (64)	18 (27)
$\beta_1P_1\beta_2$		
d.p. > 60	0	19 (29)
d.p. 20–60	0	18 (27)
d.p. 4–19	0	10 (15)
d.p. 2–3	39 (100)	19 (29)
Amyloglucosidase		
Hydrolysis of starch (A_1)[e]	93	49
A_1P_1		
c.l.		43
d.p. > 60		26 (51)
d.p. 20–60		13 (25)
d.p. < 20		12 (24)
$A_1P_1\beta_1$		
d.p. > 60		7 (14)
d.p. 20–60		20 (39)
d.p. 4–19		8 (16)
d.p. 2–3		16 (31)

[a]Unless otherwise noted, all values expressed as % of original starch;

[b] $\frac{\text{reducing capacity (maltose equivalents)}}{\text{polysaccharide in digest (glucose equivalents)}} \times 100$;

[c] $\frac{\text{polysaccharide in digest (glucose equivalents)}}{\text{reducing capacity (glucose equivalents)}}$; [d]Values in parentheses are % of total polysaccharide in the digest; [e] $\frac{\text{reducing capacity (glucose equivalents)}}{\text{polysaccharide in digest (glucose equivalents)}} \times 100$.

groups. In an attempt to further characterize the enzyme-resistant fraction at the void volume (V_0) on Sephadex G-50, the $P_1\beta_1$ was fractionated on a Sepharose 4-B column that had been calibrated with dextran standards (Pharmacia Fine Chemicals, Uppsala, Sweden). Two peaks were apparent. The peak corresponding to the V_0 peak from Sephadex G-50 was eluted from Sepharose 4-B after the dextran of 70,000 molecular weight. Thus the V_0 component on Sephadex G-50 had a molecular weight of ~10,000–70,000. Obviously, it represented partially degraded amylopectin and/or amylose.

The beta-amylolysis of **2** was 34% (Table II). This constituted only 55% of the

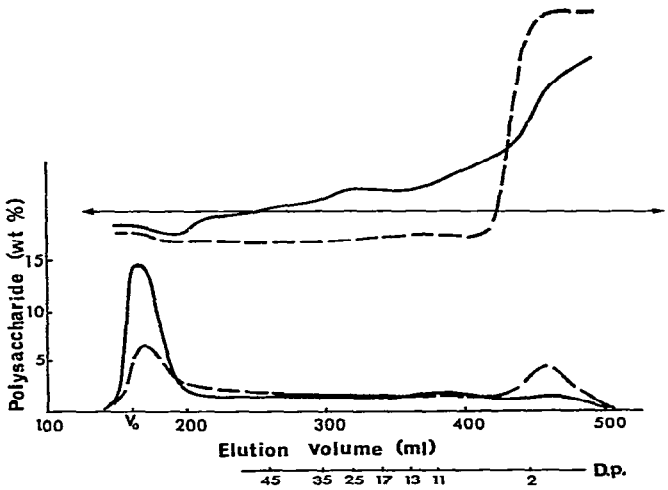

Fig. 5. Elution profiles from Sephadex G-50 of *O*-(2-hydroxypropyl)distarch phosphate manioc. Polysaccharide concentration in each fraction was determined with the amyloglucosidase–D-glucose oxidase and the orcinol methods. Bottom curves are from orcinol values. Top curves represent the ratio of amyloglucosidase-D-glucose oxidase values to orcinol values in each fraction and in the unfractionated digest (↔). Debranched beta limit dextrin (β_1P_1 —), β_1P_1 hydrolyzed with beta amylase ($\beta_1P_1\beta_2$ ---)

amount of hydrolysis obtained with **1**. This means that either the amylose and/or some of the outer chains of the amylopectin contained modifying groups.

The elution pattern from Sephadex G-50 for the debranched beta-limit dextrin (β_1P_1) is shown in Fig. 5. About one-half of the beta-limit dextrin (34% of the original starch) was not debranched with pullulanase and was eluted at the V_0. As compared with **1** (Fig. 3B), there was no distinctive peak at $\overline{\text{d.p.}}$ 35. There was a trail from the V_0 peak to the end of the profile, implying the presence of a heterogeneous molecular-weight population in the digest. Subsequent beta-amylolysis of the β_1P_1 decreased the amount at the V_0 to 19% (Table II). When the $\beta_1P_1\beta_2$ was fractionated on a column of Sephadex G-100, no peak was evident at the V_0. Apparently the V_0 fraction from Sephadex G-50 had an approximate molecular weight of $< 100{,}000$, and was thus composed of partially hydrolyzed amylopectin and/or amylose.

The amyloglucosidase hydrolysis of **2** was only 49%, as compared with 93% for **1**. Hydrolysis products were confirmed by paper chromatography to be only "dextrins" of high molecular weight and D-glucose. As with the other exoenzyme utilized (beta-amylase), the modifying groups interfered with the amyloglucosidase activity.

Debranching of the amyloglucosidase-limit dextrin (A_1) was not complete. An elution profile similar to that for β_1P_1 (Fig. 5) was obtained when the A_1P_1 was fractionated on Sephadex G-50. About 26% of **2** was eluted at the V_0, as compared with 34% in the β_1P_1 fractionation (Table II). After the peak at V_0, there was a trail

over the entire elution-volume, with a slight peak evident at $\overline{\text{d.p.}}$ 15. When the debranched amyloglucosidase-limit dextrin (A_1P_1) was subjected to beta amylase, the elution profile from Sephadex G-50 was essentially the same as that for $\beta_1P_1\beta_2$. The V_0 peak was decreased, but the small $\overline{\text{d.p.}}$ 15 peak was still evident.

DISCUSSION

The molecular structure of unmodified manioc amylopectin has many similarities to that of the amylopectin of potato, maize, waxy maize, wheat, and rice starches[8-10, 12]. The linear chains generated by debranching fall into two discrete populations: those of $\overline{\text{d.p.}}$ 15 and of $\overline{\text{d.p.}}$ 45. The ratio of 7.5 $\overline{\text{d.p.}}$ 15 chains to each chain of $\overline{\text{d.p.}}$ 45 is higher than potato[9], but lower than cereal starches[10]. The average chain length ($\overline{\text{c.l.}}$) of 25 in the P_1 is similar to that of other amylose-containing starches but, as expected is lower than that of the waxy starches[32,34]. The percent of beta-amylolysis was similar to that reported for maize[8], but slightly higher than that for waxy maize and potato[8,10,33]. Debranching of the beta-limit dextrin produced populations of $\overline{\text{d.p.}}$ 2–10 and 30–35. This result indicates that the outer chains beyond the (1 → 6) branch points were $\overline{\text{d.p.}}$ 15. The $\overline{\text{d.p.}}$ value of 2–10 of the β_1P_1 suggests that the inner chains vary from $\overline{\text{d.p.}}$ 4–10. The $\overline{\text{d.p.}}$ 2–3 originate from the maltose and maltotriose stubs remaining at the (1 → 6) branch points after beta-amylolysis (β_1). The $\overline{\text{c.l.}}$ in the β_1P_1 of 10 (Table II) agrees with reported values for debranched beta-limit dextrin from potato[32], maize, and waxy maize[8]. Beta-amylolysis of the debranched beta-limit dextrin, and the lack of any hydrolysis products other than maltose and maltotriose, confirms that the debranched polymers were entirely linear.

The identity of the high-molecular-weight fraction of **1** remaining after debranching and beta-amylolysis (Fig. 3A) is unknown. To our knowledge, it has not been reported for any other starches. Mercier[8] reported that a small proportion of polysaccharide (4.6%) remained at the V_0 when a debranched, beta-limit dextrin from maize was fractionated on Sephadex G-50; she tentatively identified it as branched amylose. Others[35,36] have demonstrated the presence of slightly branched amylose in potato, wheat, and oat starches. Greenwood and Thomson[33] reported 95% beta-amylosis for tapioca (manioc) amylose, considerably higher than the 72–84% determined for other starches. In our work, we have not obtained any evidence that the amylose of manioc starch is branched. The most plausible explanation for the material at the V_0 after $P_1\beta_1$ is that it represents undebranched amylopectin. Abdullah *et al.*[37] observed a similar characteristic for waxy-maize amylopectin. They suggested that the surface density of the amylopectin molecule was too great for the pullulanase to penetrate into the interior of the molecule, resulting in incomplete debranching. Once the surface density was decreased by beta amylase, the pullulanase could penetrate more readily into the interior. This theory would explain why the debranching of the beta-limit dextrin of manioc was complete (Fig. 3B), whereas it was incomplete when the starch was treated with pullulanase only (Fig. 3A).

The difference between the iodine-absorption profiles of **1** and **2** undoubtedly

reflects the influence of the modifying groups on the ability of the amylose to form a helix and to complex with iodine. Furthermore, amperometric titration yielded 17% amylose for **1** but nothing for **2**. These results support the conclusions of Colburn and Schoch[38] that the standard iodine-affinity methods do not yielded accurate results for starches whose molecular structures have been modified by esterification or etherification.

About 50% of the $\overline{d.p.}$-15 chains of the **2** amylopectin are not modified. French[7] described a cluster model for amylopectin that contained alternating amorphous and crystalline regions. He suggested that the crystalline areas were comprised of the outer chains of amylopectin, with a $\overline{d.p.}$ of 12. From studies on acid-treated (lintnerized) potato starch, Robin *et al.*[9] also have suggested that the closely packed, outer chains of amylopectin, ~15 D-glucose residues long, can form starch crystallites. These crystalline regions would be impervious to the reagents utilized in the modification reactions, and consequently fewer chemical modifications would occur inside the crystalline regions.

The derivative **2** evaluated contained both 2-hydroxypropyl groups and phosphate crosslinks. The level of crosslinking was very low (less than one phosphate per 1000 glucose residues) and thus it is unlikely that the phosphate groups exert a significant quantitative effect on enzyme activity. On the other hand, there were ~45 hydroxypropyl groups per 1000 D-glucose residues in the starch. Beta-amylase activity is terminated by modified D-glucose residues[39]. Assuming that beta amylase is inhibited by hydroxypropylation of the residue, the 47% of the starch at the V_0 after $P_1\beta_1$ must contain all of the hydroxypropyl groups. Thus the effective concentration on the modified segments could be as high as one hydroxypropyl group for every 10 D-glucose residues. If the modified residues were multiply substituted (namely, two or more 2-hydroxypropyl groups per residue), the number of substituted residues would be less. However, Banks *et al.*[40] have shown that the amount of multiple substitution by 2-hydroxyethyl groups increased for both amylose and amylopectin when the molar substitution (m.s.) was greater than 0.45. When the m.s. was 0.45, the D-glucose residues were monosubstituted. The starch used in our study had a m.s. of 0.045. Thus it is highly probable that **2** was not multiply substituted.

Amyloglucosidase and beta amylase are both exoenzymes. Beta amylase would be stopped by α-D-(1 → 6) linkages, leaving either a maltosyl or maltotriosyl stub. Amyloglucosidase could hydrolyze the (1 → 6) linkages, assuming that the adjacent linkage toward the reducing end of the molecule was (1 → 4). After hydrolyzing the (1 → 6) linkage, it would continue to hydrolyze α-D-(1 → 4) bonds in an exoenzyme pattern until it reached a modified residue. Pazur and Kleppe[41] have shown that amyloglucosidase from *A. niger* is essentially inactive toward methyl α-glucoside. In addition, amyloglucosidase activity is inhibited by phosphate esterified on C-6 (ref. 42). Thus, as more hydrolysis was attained with amyloglucosidase than with beta amylase, the difference must be due to hydrolysis of the (1 → 6) linkages and, subsequently, some of the inner chains. This would imply that some segments of the inner chains were not modified. Since we noted earlier that a maximum of one per ten D-glucose residues

may contain 2-hydroxypropyl groups, the hydrolysis of part of the inner chains by amyloglucosidase as far as the first *O*-(2-hydroxypropyl)-D-glucose residue is not surprising.

The manner in which modifying groups inhibit pullulanase is unknown. In theory, if some of the chains of $\overline{d.p.}$ 15 were modified near the (1 → 6) branch points and others were not, a population of $\overline{d.p.}$ 15 polymers would be expected in the P_1. This was observed, but no distinctive $\overline{d.p.}$ 2–10 population was evident in the β_1P_1 of 2. Together with the amyloglucosidase results, this would suggest that the attachment of pullulanase to the substrate is inhibited by modifying groups on the inner or outer chains near the branch points. According to the amylopectin model of Robin *et al.*[9], amorphous regions are rich in α-D-(1 → 6)-linkages. It is likely that, during the chemical modification of the starch, the modification reagents penetrate more readily into the amorphous areas. Thus a higher than average concentration of modifying groups would be expected on the macromolecule near the branch points.

A model depicting the probable locations of the modifying groups on the manioc amylopectin molecule is shown in Fig. 6. In constructing this model, we have disregarded the amylose fraction. Assuming that the amylose is modified to the same degree as amylopectin (namely, 1–2 modified residues per 20 D-glucose residues), and that the modification is uniform over the entire amylose molecule, the maximum amount of hydrolysis by an exoenzyme of a d.p. 1500 amylose would be 1.3%

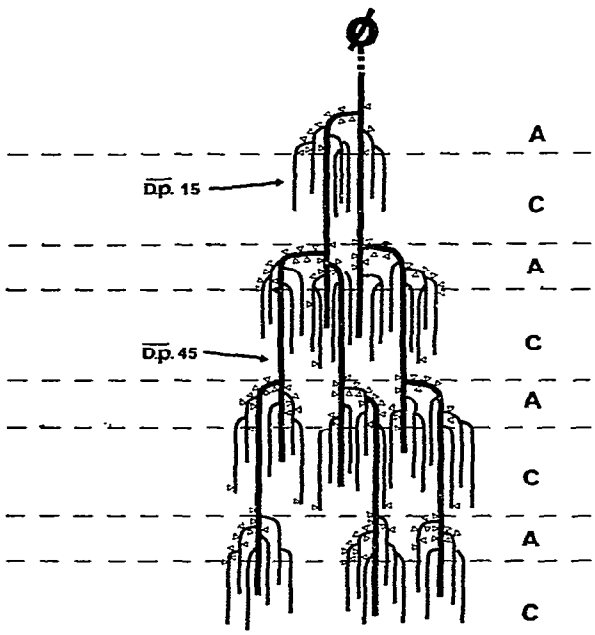

Fig. 6. Proposed structure of modified manioc amylopectin. △ indicates location of modifying groups. (A) Amorphous region, (C) crystalline region.

(20/1500). In addition, pullulanase acts only on amylopectin. This model is based on the cluster model of French[7], as further developed by Robin *et al.*[9]. We have shown here that manioc amylopectin has 7.5 of the $\overline{\text{d.p.}}$ 15 for each of the d.p. 45. Thus each $\overline{\text{d.p.}}$ 15–$\overline{\text{d.p.}}$ 45 cluster would have approximately 160 D-glucose residues. If, as suggested earlier, one-tenth of the residues were modified, there would be 16 modifying groups per cluster. Most of these would be located in the amorphous region rich in α-D-(1 → 6) linkages. The debranched, exoenzyme limit-dextrins contained a small amount of chains ($\overline{\text{d.p.}}$ 15) that were not hydrolyzed by further treatment with beta amylase (Fig. 5). Consequently a few modifying groups must be located at the nonreducing ends of the $\overline{\text{d.p.}}$ 15 chains. The precise location of the modifying groups in the α-D-(1 → 6) regions must await the definition of the receptor-site requirements for pullulanase. Nevertheless, it is clear from these results that the 2-hydroxypropyl groups are concentrated in the amorphous regions of the manioc amylopectin molecule and that about 50% of the $\overline{\text{d.p.}}$ 15 chains contain no modifying groups. These unmodified chains may be located in the inner regions of the molecule, where crystallinity would be the greatest.

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