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# STRUCTURAL FEATURES OF NAEGELI AMYLODEXTRIN AS INDICATED BY ENZYMIC DEGRADATION*

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ABSTRACT

Naegeli amylodextrin is the insoluble residue remaining after prolonged treatment of native starch granules with strong aqueous acid. The Naegeli amylodextrin from waxy maize starch was separated by gel chromatography on Sephadex G-50 into three fractions. Although the fractions were heterogeneous, their average structures were examined by enzymic degradation with porcine-pancreactic alpha amylase, beta-amylase, and pullulanase. The results show that Fraction I (highest molecular weight) has complex branching, Fraction II (major component, d.p. ~25) contains about one branch per molecule, and Fraction III (d.p. ~12) is mostly linear. Formation of these acid-resistant fractions may be explained as arising from a cluster model of amylopectin in which the outer chains are in a double-helical, crystalline arrangement.

## INTRODUCTION

Naegeli amylodextrins[2] are produced by protracted acid hydrolysis of native starch granules under such conditions that the starch granules maintain much of their gross form and crystallinity[3]. Although a great decrease occurs in the molecular size of the starch molecules, to d.p. 25–30, those facets of starch structure that are important in granule crystallinity are largely resistant to acid treatment, whereas the amorphous, gel phase is more rapidly eroded.

Previously, we regarded such amylodextrins as being essentially linear. They are readily soluble in hot water, giving clear, non-viscous solutions. On cooling or by treatment with methanol or other de-watering agents, such solutions give crystalline or spherocrystalline deposits with super-sharp X-ray diffraction patterns (S. Kiku-

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moto and D. French, unpublished results). Moreover, during the acid treatment, they become increasingly susceptible to degradation by beta amylase, another criterion of linearity. However, we now find that the beta limit does not approach 100%, even after very extensive acid treatment. Also, hydrolysis by porcine-pancreatic alpha amylase gives both singly and multiply branched oligosaccharides, and the debranching enzyme pullulanase gives a significant degree of hydrolysis. These facts taken together indicate that some branch points in starch granules occur in structural regions that are very resistant to acid hydrolysis.

In the exploratory part of this study, we used mainly potato-starch treated under the conditions described by Naegeli[2]. However, it became quite obvious that the amylose component of potato starch was confusing the picture, and in any case this linear component could tell us little or nothing about branching. Therefore, to eliminate the amylose complication, we have subsequently used mainly waxy-maize starch, which consists only of amylopectin and yet has a fairly high degree of granule crystallinity.

In this study, we have examined the structure of waxy-maize and potato amylodextrin by fractionation on Sephadex G-50 and by analysis of the fractions by using porcine-pancreatic alpha amylase, beta amylase, and pullulanase. Our results indicate that there are three classes of polymer in amylodextrin. Fraction I, high molecular weight, is multiply branched; Fraction II, d.p. 25, is singly branched; and Fraction III, d.p. 12, is linear.

After the completion of this work, Robin *et al.*[4a] reported on a study of "Lintnerized" potato starch; namely, native potato-starch granules treated with 2.2M hydrochloric acid at 35°. Extensively Lintnerized starch is synonymous with Naegeli amylodextrin. They[4] found that, after 40 days, the insoluble residue consisted of peak II, d.p. 25, branched, and peak III, d.p. 15, linear. In another study, Robin *et al.*[4b] showed that Lintnerized waxy maize starch gave results basically similar to those with potato starch. Their results corroborated our previous proposals[1] for the structures of peaks II and III as well as for the "cluster" formula for amylopectin.

## MATERIALS AND METHODS

*Preparation of amylodextrin.* — Each starch type (waxy-maize and potato starch) was treated by suspending 50 g (dry basis) of starch in 2 L of 15% sulfuric acid. The mixtures were kept at room temperature and resuspended daily. The used sulfuric acid was siphoned off every 30 days and replaced by fresh 15% sulfuric acid. After 3 months, the acid-resistant residue (amylodextrin) was collected by filtration and washed with water until free of acid.

*Elution diagram of amylodextrin on Sephadex G-50.* — Waxy-maize amylodextrin (5 mg, air dry) was dissolved by boiling in 0.4 mL of water. The cooled solution was applied to the top of a column (0.8 × 120 cm) of Sephadex G-50. The column was eluted with water and the eluate separated by a drop-counting fraction collector into tubes containing 15 drops each. The carbohydrate content of each

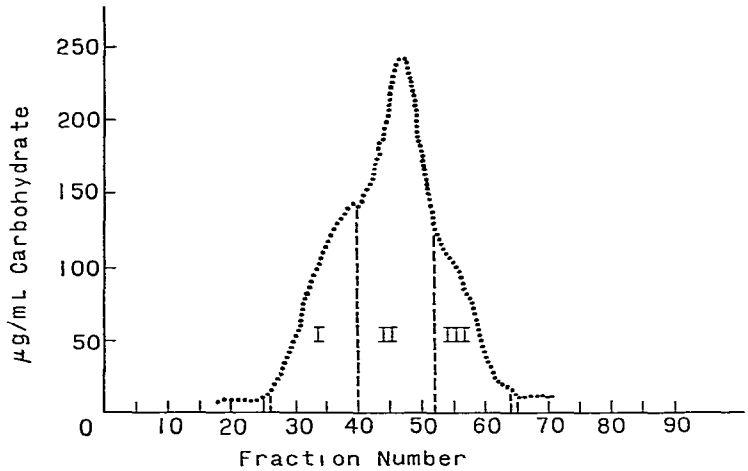



Fig. 1. Elution diagram of waxy maize-starch amylodextrin on Sephadex G-50. The Roman numerals indicate Fractions I, II, and III.

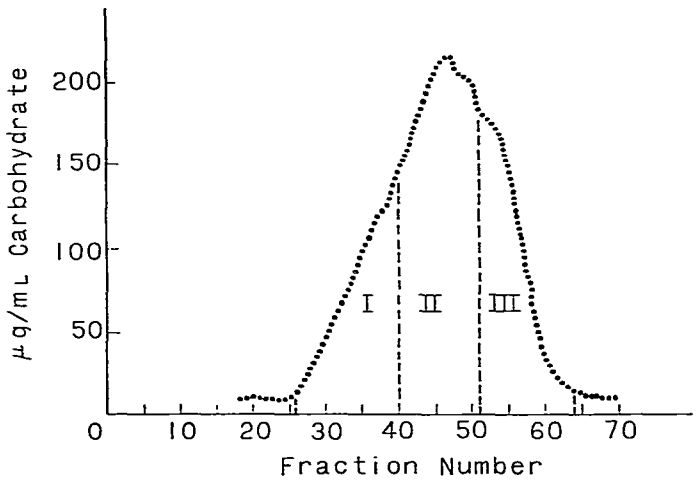



Fig. 2. Elution diagram of potato-starch amylodextrin on Sephadex G-50. The Roman numerals indicate arbitrary Fractions I, II, and III.

tube was determined by an automated phenol–sulfuric acid method[5]. The elution diagram is shown in Fig. 1. Several additional 5-mg separations were made in exactly the same way. The eluate from each fractionation was divided into three poorly-resolved fractions: Fraction I, tubes 26–40; Fraction II, tubes 41–52; and Fraction III, tubes 53–64. After the tubes for each fraction from a given run had been pooled, the solution was evaporated to dryness under vacuum at 35°, and the dry residue was dissolved in 1 mL of water for enzymic analysis. Carbohydrate analysis[5] indicated that Fraction I contained 1.3 mg, Fraction II 2.3 mg, and Fraction III 0.8 mg.

Potato amylodextrin was fractionated in the same way. The elution diagram is given in Fig. 2.

*Enzymes.* — Twice-crystallized porcine-pancreatic alpha amylase and sweet-potato beta amylase were obtained from Worthington Biochemical Corp. Crude glucoamylase from *Aspergillus niger* (Diazyme 160) was a gift from Miles Laboratories, Inc. Pullulanase was prepared from *Aerobacter aerogenes* (*Klebsiella aerogenes*) NRRL B-3388 by the method of Wallenfels *et al.*[6]. Distilled water, redistilled from glass, was used throughout for all enzyme digests and column elutions. The unit (U) of enzyme activity is that amount which under controlled conditions catalyzes one μmol of bond cleavage per min.

*Action of porcine-pancreatic alpha amylase, beta amylase, and pullulanase on the fractions.* — (A). *Porcine pancreatic alpha amylase.* Each of Fractions I, II, and III from an individual run was treated with 120 μL (220 U) of porcine-pancreatic alpha amylase solution. After 48 h of incubation at 40°, the enzyme action was stopped by heating in a boiling-water bath. Approximately half of the digest of each fraction was applied as a single spot to a paper chromatogram.

B. *Beta amylase.* The procedure as in A was repeated except that each fraction was treated with 120 μL (45 U) of beta amylase solution. After 24 h of incubation at 40°, the enzyme reaction was stopped by heating in a boiling-water bath, and ~0.5 mL of the digest of each fraction was applied as a single spot to a paper chromatogram.

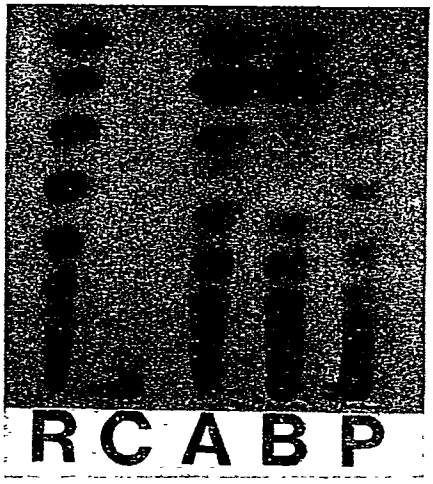
C. *Pullulanase.* Each fraction from a single run was treated with 200 μL (0.8 U) of pullulanase solution. After 72 h of incubation at 40°, the enzyme reaction was stopped by heating in a boiling-water bath, and ~0.5 mL of each digest was applied as a single spot to a paper chromatogram.

D. *Untreated control.* Each fraction from one additional run was evaporated to dryness, and the concentrated eluate dissolved in 1 mL of water. This solution (~0.5 mL) was applied to a chromatogram for a control.

*Paper chromatography.* — The oligosaccharide mixture was spotted on paper (Whatman 3MM) and subjected to multiple ascents (three times) with 6:4:4 butanol–pyridine–water at 65°. The dried chromatogram was dipped in an enzyme suspension made by dissolving 1 g of Diazyme 160 in 200 mL of 200mM pyridine–acetic acid buffer (pH 4.8), and then adding 800 mL of acetone. The wet chromatogram was hung in air for 1–2 min to evaporate the acetone and allowed to incubate[3] in a stainless-steel moist chamber for 30 min in an oven at 65°. The chromatograms were dried, and the carbohydrates revealed by the silver-dip method[9].

## RESULTS AND DISCUSSION

*Fractionation of amylodextrin.* — In our original fractionation with potato starch amylodextrin (Fig. 2), it was not obvious that there were any "breaks" in the elution diagram from Sephadex G-50. With waxy-maize starch amylodextrin, however, there were hints of breaks at about tube numbers 40 and 52. Hence, we chose



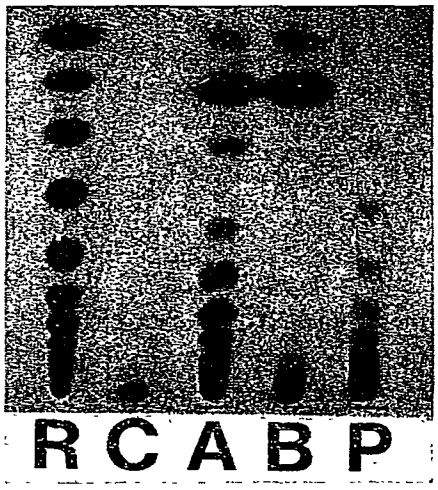




Fig. 3. Action of porcine-pancreatic alpha amylase, beta amylase, and pullulanase on Fraction I of waxy-maize amylodextrin. R: reference series, from top, down: D-glucose, maltose, maltotriose, etc.; C: untreated control; A: product of action of porcine-pancreatic alpha amylase; B: product of action of sweet-potato beta amylase: P: treatment by pullulanase.

Fig. 4. Action of porcine-pancreatic alpha amylase, beta amylase, and pullulanase on Fraction II of waxy-maize amylodextrin. Channel identification as in Fig. 3.

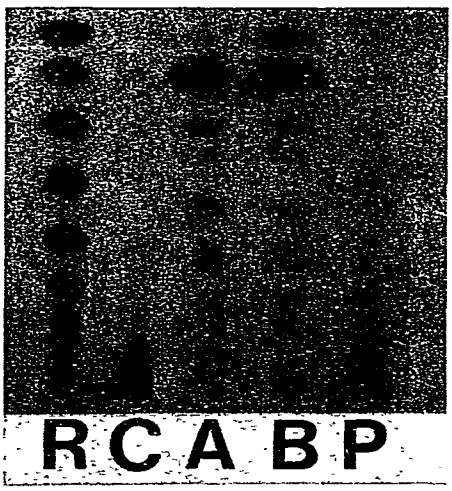



Fig. 5. Action of porcine-pancreatic alpha amylase, beta amylase, and pullulanase on Fraction III of waxy-maize amylodextrin.

to divide the total material into three fractions (I, II, and III) as indicated in Fig. 1. Determination of the reducing value of these fractions indicated that Fraction I had d.p. ~35, Fraction II ~25, and Fraction III ~12.

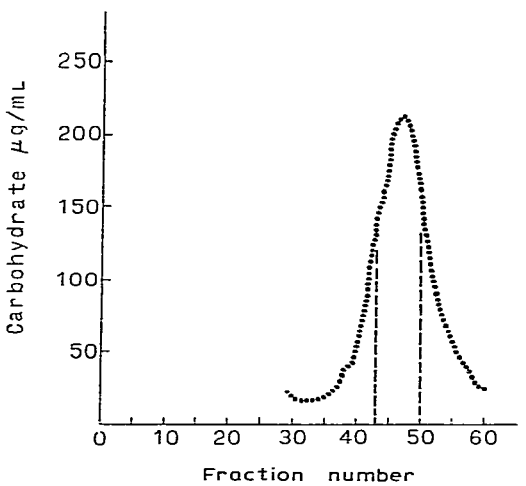
*Action of enzymes on the fractions.* — The experimental results are shown in Figs. 3–5. Fraction I of waxy-maize amylodextrin was converted by porcine-pancreatic

alpha amylase into D-glucose, $G_2$*, $G_3$, $B_4$, large amounts of $B_5$, $B_6$, $B_7$, and higher branched-oligosaccharides (probably multiply branched). Action of beta amylase on Fraction I gave D-glucose, $G_2$, and some high-molecular-weight, chromatographically immobile, beta amylase-resistant material. On degradation by pullulanase, Fraction I gave $G_2$ and all higher, chromatographically mobile malto-oligosaccharides.

Fraction II of waxy-maize amylodextrin was converted by porcine-pancreatic alpha amylase into D-glucose, $G_2$, and $G_3$, a trace of $B_3$, and large amounts of $B_4$, $B_5$, $B_6$, and $B_7$. In comparison with Fraction I, smaller amounts of higher multiply-branched dextrins were formed. Action of beta amylase on Fraction II gave D-glucose, $G_2$, $B_3$, and large amounts of higher branched-oligosaccharides in the chromatographic range $B_4$–$B_{10}$. Still higher, chromatographically-immobile branched dextrins were also produced in fairly large amounts. Pullulanase gave $G_2$ and higher oligosaccharides in equal or larger amounts as compared with Fraction I.

Fraction III of waxy-maize amylodextrin was converted by porcine-pancreatic alpha amylase into $G_2$, small amounts of $G_3$, and only traces of $B_3$–$B_7$ and higher branched-oligosaccharides in comparison with Fractions I and II. Action of beta amylase on Fraction III gave D-glucose, $G_2$, small amounts of $G_3$, traces of $B_3$–$B_8$, and almost no chromatographically immobile products. Degradation of Fraction III by pullulanase gave only small increases in the amounts of chromatographically detectable oligosaccharides over those in the control.

*Rechromatography of Fraction II of waxy-maize amylodextrin on Sephadex G-50.* — Fraction II (tubes 41–52 from a separate run) was collected, evaporated



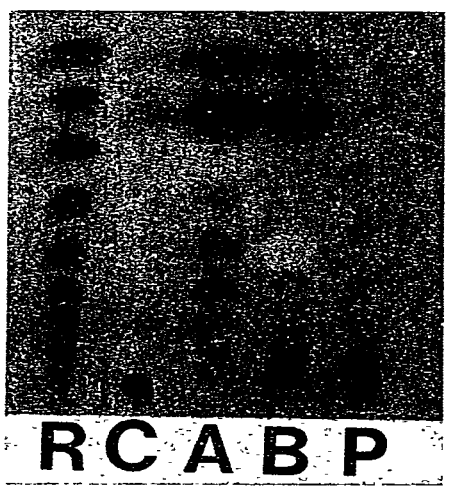




Fig. 6. Rechromatography of Fraction II of Fig. 1 on Sephadex G-50.

Fig. 7. Action of porcine-pancreatic alpha amylase, beta amylase, and pullulanase on pooled fractions 43–50 of Fig. 6.

*Abbreviations: $G_2$, $G_3$, etc., maltose, maltotriose, etc.; $B_3$, $B_4$, etc., starch-type trisaccharide, tetrasaccharide, etc., containing one α-(1→6) linkage.

to dryness, and dissolved in 0.4 mL of water by boiling. After cooling, it was rechromatographed on the column of Sephadex G-50 as with the original amylodextrin. The elution diagram is shown in Fig. 6.

*Action of porcine-pancreatic alpha amylase, beta amylase, and pullulanase on rechromatographed Fraction II.* — Rechromatographed Fraction II (tubes 43–50) of waxy-maize amylodextrin (10 mg) was collected and divided into three equal parts, and then evaporated. The material was treated with pancreatic alpha amylase, beta amylase, and pullulanase as with the original, crude fractions. The results are shown in Fig. 7. The main difference from the crude Fraction II is that the purified material gives, with pancreatic amylase, oligosaccharides much less multiply branched.

*Elution diagram of potato amylodextrin on Sephadex G-50.* — Potato-starch amylodextrin (5 mg) was chromatographed on the Sephadex G-50 column as with the waxy maize-starch amylodextrin. The elution diagram is shown in Fig. 2. The eluate was arbitrarily separated into three fractions: I (tubes 26–40), II (tubes 41–51), and III (tubes 52–64). Structures of the individual fractions were tested by examining products of the action of porcine-pancreatic alpha amylase, beta amylase, and pullulanase. The results (not shown) were essentially identical to those from waxy-maize amylodextrin.

*Significance of the enzymic degradations.* — Under the conditions used, porcine-pancreatic alpha amylase converts the linear portions of starch into D-glucose, $G_2$, and $G_3$. Well-separated branching points are converted[1,10] into a set of singly branched oligosaccharides $B_4$–$B_7$. If the branch point is less than two D-glucose-residues removed from the reducing group of the molecule, the branched, limit oligosaccharides are correspondingly smaller. Formation of isomaltose or panose, for example, clearly indicates that the branch is on the reducing group or on the second D-glucose residue of the starch chain.

Formation of multiply branched, limit dextrins simply means that branches are located so close to each other that pancreatic alpha amylase is unable to cleave between them. We have determined that this means that the branch points (isomaltose units) can be separated by no more than one D-glucose unit[1,8].

With beta amylase, a high degree of conversion into maltose requires that branches, if any, be located close to the reducing group of the molecule. Whereas native waxy-maize starch gives only about 55% conversion to maltose, waxy maize-starch amylodextrin gives more than 80% conversion. A further point of significance is the formation and character of branched oligosaccharides or limit dextrins. With Fraction I, these branched dextrins are largely chromatographically immobile, of high molecular weight, and, presumably, multiply branched. With Fraction II, the dextrins have higher chromatographic mobility and include[11] oligosaccharides as small as $B_3$ and $B_4$. The relative absence of branched dextrins from the beta amylase digest of Fraction III indicates its essentially linear character.

Although pullulanase acts slowly and incompletely on starch, it readily hydrolyzes $\alpha$-(1→6) links in starch dextrins and oligosaccharides, provided that the $\alpha$-(1→6) bond is flanked by $\alpha$-(1→4) linkages[12,13]. Pullulanase-catalyzed hydrolysis of

α-(1→6) bonds in the amylodextrin fractions is indicated by the formation of chromatographically visible products extending from $C_2$ to the origin of the paper chromatograms. Single-glucose-unit side-branches ("stubs") are immune to pullulanase attack[12]; hence, D-glucose is not seen as a product. The results with pullulanase indicate that both Fractions I and II are branched. A very few of the branches are as short as two or three glucose units, but most of the branches are in the chromatographically immobile range. Fraction III undergoes no detectable hydrolysis with pullulanase, in agreement with its essentially unbranched character.

The results of this study indicate that starch granules contain regions of acid-resistant material, which, though highly crystalline, still contain significant branching.

Although we recognize that each of the fractions is heterogeneous, we suggest that the best interpretation of the enzymic results is that Fraction I is multiply branched, with a significant degree of clustering of branches near the reducing group; Fraction II, the major product, is singly branched, with the branch point near the reducing group, and Fraction III is mostly linear. These structures might reasonably be expected to arise from the "cluster" formula for amylopectin[1], and they are depicted in a schematic helical representation in Fig. 8.

Packing of starch chains into a crystalline lattice should protect linear regions against heterogeneous acid hydrolysis. This type of protection is very common, as, for example, with cellulose, in which heterogeneous acid hydrolysis preferentially attacks the amorphous, intercrystalline regions[14,15]. In this respect, one might expect the linear outer chains of amylopectin, and possibly some longer interbranch inner chains, to participate in a crystalline organization and be resistant to attack by acid. It is not so obvious that structures in the neighborhood of branches could have the structural regularity that would permit them to fit into a crystal lattice.

Even though α-(1→6) linkage are known to be 4–10 times more resistant to acid hydrolysis than α-(1→4) linkages[16,17], if the α-(1→6) linkages were actually in contact with the acidic medium, they could hardly survive the months of exposure under the experimental conditions used. Moreover, α-(1→4) linkages immediately

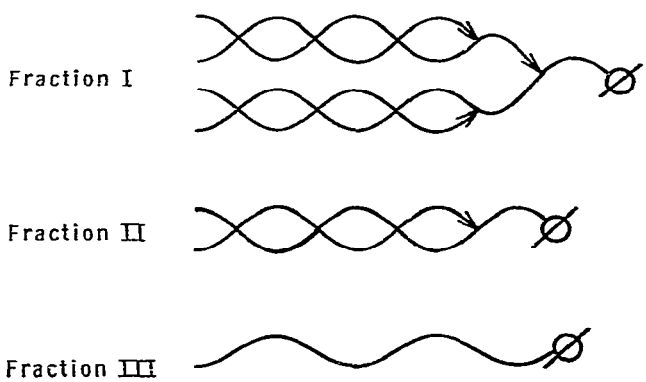



Fig. 8. Schematic structures of Fractions I, II, and III. Ø, Reducing group; ↓, branch point; wavy line, starch chain of ~12 D-glucose residues in extended or double-helical conformation.

adjacent to the branch points could likewise be expected to be especially vulnerable. Our experimental results, however, indicate that at least some of the branch points in starch granules are essentially immune to hydrolysis. This discrepancy can be resolved by having the starch chains in double helices. Models of double helices[1,18] show that interchain branches are very readily formed and that all glycosidic-bond oxygen atoms, including those of branch points, are in highly protected, rigid environments where they might be expected to be substantially resistant to attack by aqueous acid.

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