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STRUCTURE STUDIES OF AMYLOSE-V COMPLEXES AND RETROGRADED AMYLOSE BY ACTION OF ALPHA AMYLASES, AND A NEW METHOD FOR PREPARING AMYLODEXTRINS*

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ABSTRACT

Human-salivary, porcine-pancreatic, and *Bacillus subtilis* alpha amylases were used to study the structure of amylose-V complexes with butyl alcohol, *tert*-butyl alcohol, 1,1,2,2-tetrachloroethane, and 1-naphthol, and of retrograded amylose. Alpha amylase hydrolyzes the amorphous, folding areas on the surfaces of the lamella of packed helices, with the formation of resistant, amylodextrin fragments. Their degree of polymerization (d.p.) corresponds to the diameter of the helices and the folding length of the chain. The resistant fragments were fractionated on a column of Bio-Gel A-0.5m. Gel filtration of human-salivary and porcine-pancreatic alpha amylase hydrolyzates gave resistant fragments whose peak fractions, *i.e.*, the three pooled fractions from the gel-filtration column with the highest amount of carbohydrate, had a d.p. of 75 ±4 for the amylose complex with butyl alcohol, 90 ±3 for those with *tert*-butyl alcohol and tetrachloroethane, and 123 ±2 for that with 1-naphthol. These d.p. values correspond to helices of six residues per turn with a folding length of 10 nm, seven residues per turn with a folding length of 10 nm, and eight residues per turn with a folding length of 12 nm (or nine residues per turn with a folding length of 10 nm), respectively. Acid hydrolysis of retrograded amylose gave a resistant fragment having an average d.p. of 32, human-salivary and porcine-pancreatic alpha amylases gave a resistant fragment of d.p. 43, and *Bacillus subtilis* alpha amylase gave a resistant fragment of d.p. 50. A structure for retrograded amylose is proposed in which there are crystalline, double-helical regions that are 10 nm long, interspersed with amorphous regions. The amorphous regions are hydrolyzed by acid and by alpha amylases, leaving the crystalline regions intact. The differences in the sizes of the resistant amylodextrins depend on the differences in the specificities of the hydrolyzing agents: acid hydrolyzes right up to the edge of the crystalline region, whereas the alpha amylases hydrolyze up

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to some point several D-glucosyl residues away from the crystalline region, leaving "stubs" on the ends of the amylodextrins whose sizes are dependent on the sizes of the binding sites of the individual alpha amylases. The amylodextrins resulting from hydrolysis of the different amylose complexes by the different alpha amylases, and from hydrolysis of retrograded amylose by acid and by alpha amylases indicate a new method of preparing amylodextrins of different average chain-lengths, having a relatively narrow, molecular-weight distribution, in yields of 45–65%.

INTRODUCTION

It has been known for over a century and a half that amylose interacts with iodine–iodide to give a blue complex[1]. Amylose also forms helical complexes with many other organic and inorganic chemicals, with the complexing agent in the center of the the amylose helix; these are known as amylose-V complexes[2–4]. The formation of an amylose-V complex with butyl alcohol or other complexing agents is the most commonly used method of separating amylose from amylopectin[5,6].

Most of the structural studies of amylose-V complexes have been conducted by using X-ray crystallography[2–4,7–13], and/or electron microscopy[7,8,12,13]. X-Ray diffraction data indicated that open-chain complexing agents (*e.g.*, butyl alcohol) form helices having 6 D-glucosyl residues per turn[2–4,7,8], branched-chain or halogenated alkyl compounds (*e.g.*, *tert*-butyl alcohol and tetrachloroethane) form helices of 7 D-glucosyl residues per turn[9–12], and even bulkier molecules (*e.g.*, 1-naphthol) form helices having 8 D-glucosyl residues per turn[13]. Electron-microscope studies indicated that these amylose-V complexes form lamella-like crystals, with lamellar folding lengths of[9,12,13] ~10 nm for all three of the amylose helices.

We have studied the action of various alpha amylases on several amylose-V complexes, and on retrograded amylose, and report on the nature of the products, the use of alpha amylase hydrolysis to analyze the structures of the complexes, and a new method for obtaining amylodextrins of different sizes having a relatively narrow, molecular-weight distribution.

MATERIALS AND METHODS

Enzymes. — Porcine-pancreatic alpha amylase was purchased from Boehringer–Mannheim (Los Angeles, CA) and used without treatment. Human salivary and *Bacillus subtilis* var. *amyloliquefaciens* (hereinafter designated *Bacillus subtilis*) alpha amylases were crystallized according to the methods of Fischer and Stein[14].

Enzyme assay. — The amylases were assayed by measuring the increase in the reducing value of a starch digest. The amount of reducing sugar was determined with an alkaline ferricyanide–cyanide reagent[15], prepared from potassium ferricyanide (0.5 g), potassium cyanide (5 g), and sodium carbonate (20 g) dis-

solved in water (1 L), by measuring the decrease in the ferricyanide color at 420 nm. Maltose at various concentrations (10–200 μg/mL) was used as the standard. Enzyme (5 mL) was added to 5 mL of buffered, soluble starch (5 mg/mL, 20 mM buffer), and 1-mL aliquots, taken at various times, were added to ferricyanide reagent (5 mL), which was kept in a boiling-water bath for 10 min, and cooled; the decrease in the ferricyanide color was measured. One unit of enzyme was defined as the amount of enzyme needed to hydrolyze 1 μmol of glycosidic bonds per min.

Determination of the degree of polymerization (d.p.) of amylodextrin. — Total carbohydrate was determined by the orcinol–sulfuric acid method[16]. Reducing value was determined by the alkaline ferricyanide method[15]. Maltose was used as the standard in both analyses. The d.p. was calculated as

$$\text{d.p.} = \frac{\text{total carbohydrate } (\mu\text{g})}{\text{reducing sugar (as } \mu\text{g of maltose)}} \times 2.$$

Thin-layer chromatography. — Ascending thin-layer chromatography (t.l.c.) was conducted on Whatman K5F silica gel plates or Analtech HETLC-GHL plates by using one to three ascents of 4:1 (v/v) acetonitrile–water.

Column chromatography of reaction digests. — A column (1.5 × 30 cm or 2 × 100 cm) of Bio-Gel A-0.5m was used to resolve products of high and low molecular weight by using 0.02% sodium azide as the eluant. The fractions were analyzed for carbohydrate by the orcinol–sulfuric acid method. The amylose-complex hydrolyzates were analyzed in two ways: (*a*) the peak fractions, *i.e.*, the three fractions having the maximum amount of carbohydrate in the amylodextrin peak, were pooled, and the d.p. determined and designated *peak d.p.*, and (*b*) all of the fractions containing carbohydrate in the amylodextrin peak were pooled, and the d.p. determined. The amylodextrin fractions of the retrograded-amylose hydrolyzates were pooled, and the d.p. determined.

Preparation of amylose. — Native amylose (d.p. ~1000) was prepared from potato starch by the method of Schoch[5], and crystallized three times before use.

Preparation of amylose-V complexes. — Amylose (300 mg) was dissolved in dimethyl sulfoxide (10 mL), and diluted to 300 mL with distilled water. The solutions were heated to 95°, and one of the following complexing agents was added: butyl alcohol, to 15% (v/v); *tert*-butyl alcohol, to 30% (v/v); 1,1,2,2-tetrachloroethane, 1.5 g per 300 mL of amylose solution; and 1-naphthol, 1 g per 300 mL of amylose solution. Each mixture in a stoppered container was then placed in a Dewar flask filled with boiling water, and the flask was stoppered. After ~36 h, the solution had cooled to room temperature, and during the cooling, the complex precipitated. The mixture was kept at room temperature for a further 48 h, and then centrifuged at 9000*g* for 1 h at 5°.

Enzyme digests of amylose-V complexes. — The complex was weighed, and divided into three equal parts for digestion with alpha amylase. Suspensions of each complex, containing ~100 mg of amylose, were prepared in 5 mL of buffer,

which had been premixed with the appropriate complexing agent: 5% (v/v) butyl alcohol, 10% (v/v) *tert*-butyl alcohol, and saturated tetrachloroethane and 1-naphthol.

Five units of one of the three alpha amylases was added to each amylose-V complex suspension. Enzyme reactions were conducted at 25°, with constant stirring for 5–10 conversion periods (one conversion period equals the amount of time required to convert amylose completely into maltodextrin products). Aliquots were analyzed by t.l.c. and column chromatography at various times, to determine the extent of hydrolysis.

When hydrolysis was complete, ethanol (8 vol.) was added to the digests to stop the enzyme reaction and precipitate the amylase-resistant fragments. The suspension was centrifuged, and the pellet was suspended in dimethyl sulfoxide, and heated for 10 min in an oil bath at 110–120°, to ensure complete denaturation of the amylase and to dissolve the amylase-resistant fraction.

Preparation of retrograded amylose. — Amylose solution (3.5 mg/mL) was kept in a refrigerator for one to two weeks until retrogradation and precipitation were complete. The suspension of retrograded amylose was centrifuged at 9000*g* for 1 h at 5°, and the supernatant liquor was discarded.

Enzyme digests of retrograded amylose. — The reaction conditions were similar to those used for the enzymic digestion of the amylose-V complexes. The reaction was stopped by heating for 10 min in an oil bath at 110–120°. The mixture was then evaporated to dryness, the residue dissolved in dimethyl sulfoxide (100–500 μL), and the solution diluted with water to a final concentration of 1% of carbohydrate.

Acid hydrolysis of retrograded amylose. — Retrograded amylose was suspended in 16% (v/v) sulfuric acid at 25°, and the mixture was shaken by hand every day, aliquots being taken at various times and centrifuged. The pellet of the resistant fraction was collected and washed with water until it was free of acid. The pellet was dissolved in the minimal volume of dimethyl sulfoxide, the solution diluted with water and the d.p. of the resistant fraction determined by the method already described.

X-Ray diffraction studies. — Amylose-V complexes were deposited onto Whatman No. 52 filter paper (150 mg of amylose per 1.5-cm circle) by gravity filtration, to yield 1-mm thick films for examination by X-ray diffraction. The films of the butyl and *tert*-butyl alcohol and tetrachloroethane complexes were rinsed with the respective liquids; that of the 1-naphthol complex was not rinsed.

RESULTS

X-Ray diffraction studies were conducted, in order to examine the helical structure of the amylose complexes. $\sin^2 \theta$ was calculated from each of the lines (data not shown). The results were in agreement with the data of Rundle *et al.*[4], Zaslow[10], Simpson *et al.*[11], and Yamashita *et al.*[8,12,13], indicating that the com-

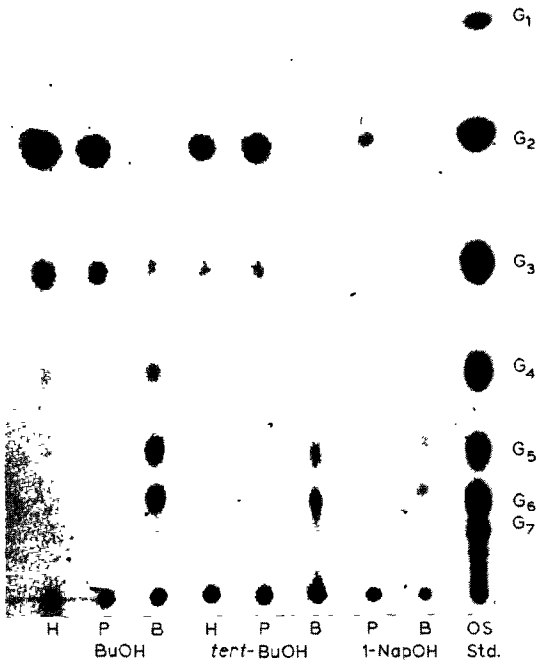

Fig. 1. Thin-layer chromatogram of products from the action of alpha amylase on amylose-V complexes. [Three ascents in 4:1 (v/v) acetonitrile–water at 25°. Malto-oligosaccharides were used as standards (OS). The amylose-V complexes used in these experiments were amylose–butyl alcohol, amylose–*tert*-butyl alcohol, and amylose–1-naphthol (1-NapOH) complexes. The amylases used included human-salivary (H), porcine-pancreatic (P), and *Bacillus subtilis* (B) alpha amylase. Enzymic digestions were conducted at room temperature, with mechanical stirring. Aliquots (10 μL) were taken at the end of 5 conversion periods.]

plexes were, indeed, helical, with the expected number of D-glucosyl residues per turn of the helix.

At the end of the enzymic hydrolyses (~5–10 conversion periods), the hydrolyzates were analyzed by t.l.c. The results, shown in Fig. 1, indicated that the major products of treatment with porcine-pancreatic alpha amylase[17] and human-salivary alpha amylase[18] were maltose, maltotriose, and a resistant amylodextrin that remained at the origin. The products in the *Bacillus subtilis* alpha amylase hydrolyzate consisted of a series of maltodextrins, including large amounts of maltotriose, maltopentaose, maltohexaose, and a resistant amylodextrin[19]. When amylose, as a

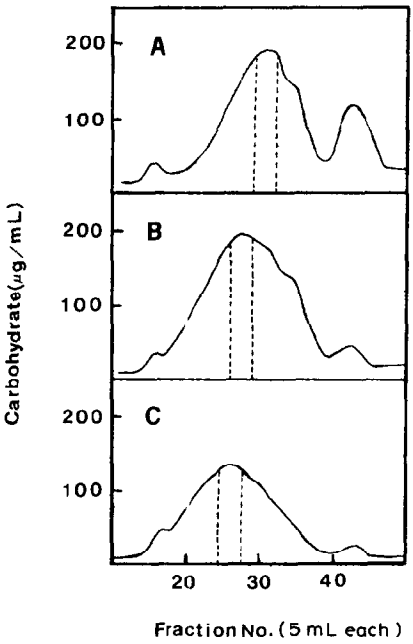

Fig. 2. Bio-Gel A-0.5m chromatography of porcine-pancreatic alpha amylase hydrolyzates of amylose-V complexes. [(A) Hydrolyzate of amylose–butyl alcohol complex; (B) hydrolyzate of amylose–*tert*-butyl alcohol complex; and (C) hydrolyzate of amylose–1-naphthol complex. Enzyme activity was stopped after 10 conversion periods by adding ethanol (8 vol.). The column was 2.5 × 90 cm, with 0.02% sodium azide eluant at 25°; void volume, 80 mL; included volume, 215 mL. The broken lines indicate the fractions that were pooled for measurement of the peak d.p. values.]

control, was subjected to hydrolysis with alpha amylase under the same conditions, all of the amylose was converted into maltodextrins long before 5 conversion periods had elapsed (data not shown).

The Bio-Gel A-0.5m elution profiles (see Fig. 2) of the ethanol-precipitated products gave three peaks: one small peak at the void volume, a large peak of amylodextrins, and a medium-sized peak of maltodextrins near the included volume. The supernatant liquor from the alcohol-digest mixture was also examined by column chromatography; no polysaccharide or amylodextrin was found.

When enzymic hydrolyzates were heated directly in a boiling-water bath (98°), the enzymic activity was not immediately stopped. The elution profiles of these samples showed that the peak of resistant amylodextrin was shifted toward lower molecular weights for the porcine-pancreatic and human-salivary alpha amylase hydrolyzates, and the peak of the resistant fraction merged into that of the maltodextrins of the *Bacillus subtilis* alpha amylase hydrolyzate (see Fig. 3).

In another experiment, hydrolysis of the amylose complex by alpha amylases was immediately stopped by addition of ethanol (8 vol.) after different lengths of time. It was found that the yield of resistant fragments diminished with time, but

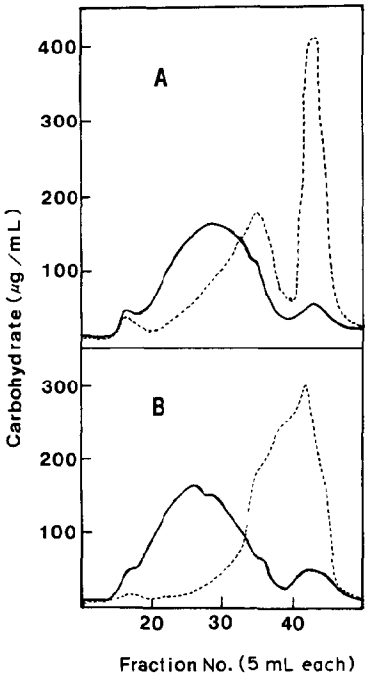

Fig. 3. Bio-Gel A-0.5m chromatography of alpha amylase hydrolyzates of amylose-V complexes. [(A) Chromatography of porcine-pancreatic alpha amylase hydrolyzates of amylose–*tert*-butyl alcohol complex. The solid line is the elution profile when the enzyme action was stopped, after 10 conversion periods, by adding ethanol (8 vol.). The broken line is the elution profile when the enzyme action was stopped, after 10 conversion periods, by heating in a boiling-water bath for 15 min. (B) Chromatography of *Bacillus subtilis* alpha amylase hydrolyzates of amylose–*tert*-butyl alcohol complex. Solid line and broken line, the same as in (A). Column and elution conditions, the same as in Fig. 2.]

that the size of the resistant fragments did not change. The amylodextrin peak remained at the same place on the Bio-Gel A-0.5m elution profile (see Fig. 4).

Molecular sizes of the resistant fractions were determined by measurement of total carbohydrate and reducing value, and by calibrated, gel-permeation column-chromatography. Three fractions (5 mL each) containing the maximum amount of carbohydrate in the peak were combined, and concentrated to 3 mL, and the amylodextrin was precipitated with an excess of ethanol (see Fig. 2). The precipitated amylodextrin was then dissolved in the minimal volume of dimethyl sulfoxide, and the solution diluted with water. The peak d.p. values of the resistant fractions from the different amylose complexes and enzymes are given in Table I. The average d.p., and the distribution of the pooled amylodextrin fractions, were determined; these are given in Table II.

The results given in Table I show that *B. subtilis* alpha amylase gives resistant fragments having a d.p. higher than the d.p. values obtained with the two other enzymes after the same number of conversion periods.

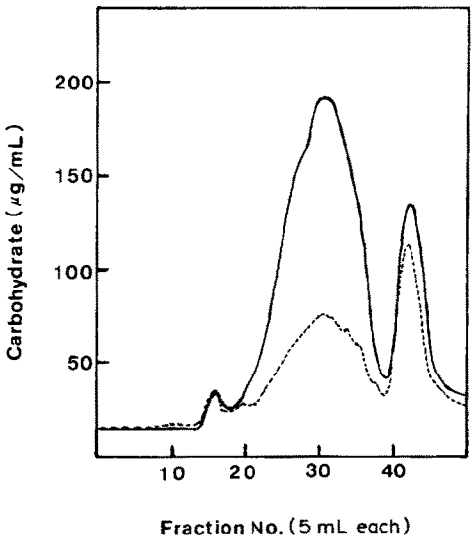

Fig. 4. Bio-Gel A-0.5m chromatography of porcine-pancreatic alpha amylase hydrolyzates of amylose–butyl alcohol complex at different conversion periods. [Enzyme activity was stopped by addition of alcohol: ———, 8 conversion periods; ------, 16 conversion periods. Column and elution conditions, the same as in Fig. 2.]

TABLE I

DEGREE OF POLYMERIZATION OF PEAK FRACTIONS OF RESISTANT FRAGMENTS OF VARIOUS AMYLOSE-V COMPLEXES HYDROLYZED BY DIFFERENT ALPHA AMYLASES

Complexing agent	D-*Glucosyl units per turn*	*Degree of polymerization*		
		Human-salivary	*Porcine-pancreatic*	Bacillus subtilis
Butyl alcohol	6	78	71	105
tert-Butyl alcohol	7	90	95	110
1,1,2,2-Tetrachloroethane	7	90	—	—
1-Naphthol	8	122	125	140

TABLE II

AVERAGE DEGREE OF POLYMERIZATION ($\overline{d.p.}$) OF THE POOLED A-0.5m CHROMATOGRAPHED, RESISTANT FRAGMENTS OF VARIOUS AMYLOSE-V COMPLEXES HYDROLYZED BY HUMAN-SALIVARY AND PORCINE-PANCREATIC ALPHA AMYLASES

Complexing agent	*Human-salivary alpha amylase*		*Porcine-pancreatic alpha amylase*	
	$\overline{d.p.}$	*Distribution*	$\overline{d.p.}$	*Distribution*
Butyl alcohol	108	30–140	—	—
tert-Butyl alcohol	122	45–170	129	45–170
1-Naphthol	169	60–230	174	60–230

TABLE III

AVERAGE DEGREE OF POLYMERIZATION ($\overline{d.p.}$) OF RESISTANT FRAGMENTS FROM RETROGRADED AMYLOSE HYDROLYZED BY PROLONGED TREATMENT WITH ACID AND WITH DIFFERENT ALPHA AMYLASES

Treatment	$\overline{d.p.}$	*Distribution*
Alpha amylase (10 conversion periods)		
human-salivary	42	30–55
porcine-pancreatic	44	30–55
Bacillus subtilis	50	45–65
Sulfuric acid (16%, 25°)		
20 days	33	25–50
40 days	31	25–50

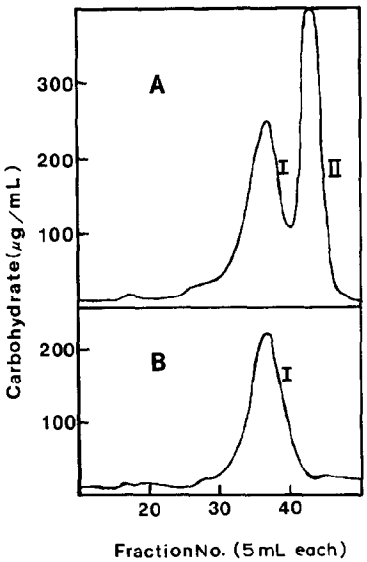

Fig. 5. Bio-Gel A-0.5m chromatography of human-salivary alpha amylase hydrolyzates of retrograded amylose. [(A) Hydrolyzate heated to 110–120°, evaporated to dryness, the residue dissolved in dimethyl sulfoxide, and the solution diluted with water, and chromatographed. (B) Hydrolyzate heated to 110–120°, and centrifuged, the pellet washed twice with water, dissolved in dimethyl sulfoxide, diluted with water, and chromatographed. I is amylodextrin, and II is soluble maltodextrins. Column and elution conditions were the same as in Fig. 2.]

Retrograded amylose was also subjected to alpha amylase hydrolysis and to prolonged acid hydrolysis[20]; the results are given in Table III. The molecular-size distribution of the products of human-salivary alpha amylase hydrolysis was determined by chromatography on a column of Bio-Gel A-0.5m (see Fig. 5A). In a second experiment, after the enzymic reaction had been stopped by heating, the insoluble material was centrifuged off, and washed twice with water (to remove maltodextrins). The pellet was dissolved in dimethyl sulfoxide, and the solution was di-

luted with water, and chromatographed on Bio-Gel A-0.5m (see Fig. 5B). Stopping the enzyme reactions by heating did not lower the average d.p. of the resulting amylodextrins from retrograded amylose (as it did for the amylose-V complexes). This indicated that the resistant amylodextrins from retrograded amylose are much more stable than the resistant amylodextrins from the amylose-V complexes.

DISCUSSION

The peak degree of polymerization, *i.e.*, the d.p. of the three pooled fractions (from the gel-filtration column) having the highest amount of carbohydrate (see Fig. 2), of the resistant fragments from the amylose–butyl alcohol, amylose–*tert*-butyl alcohol, and amylose–tetrachloroethane complexes hydrolyzed by human-salivary and porcine-pancreatic alpha amylases were determined to be 75, 92, and 90, respectively, by both the total-carbohydrate–reducing-value and gel-permeation column-chromatographic methods. These peak d.p. values correspond to a lamellar thickness of 10 nm when there are 6 and 7 D-glucosyl units respectively, per turn. These values agree with the data obtained from both X-ray crystal-

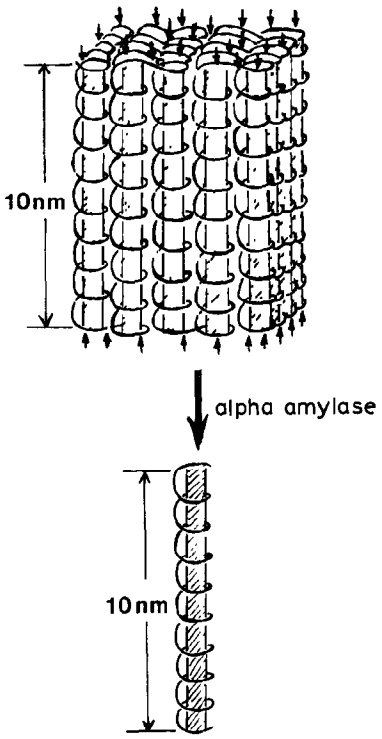

Fig. 6. Alpha amylase hydrolysis of the proposed structure of lamella-like, crystalline sheets of amylose–alcohol complex. [The arrows on the top and bottom of the sheets are the proposed sites of alpha amylase hydrolysis to give resistant amylose fragments.]

lography[7,8,10,12] and electron microscopy[7,8,12]. This constitutes further evidence for the proposed lamellar-crystal structures of amylose–alcohol complexes, shown in Fig. 6. In the lamellar structure, the amylose helices are folded back and forth in the crystal, and the folding points, the amorphous parts on the surface of the crystal, are accessible to attack by alpha amylase. We hypothesize that the alpha amylases hydrolyze these amorphous areas, leaving intact the relatively regular chains in the helical, crystalline regions. This then gives resistant amylodextrins whose d.p. values are consistent with a lamellar length of 10 nm and 6 or 7 D-glucosyl residues per turn of the helix. Extensive hydrolysis of the complex, beyond that needed to cleave the folding points, leads to a lessened yield of resistant fragments but no diminution in their size (see Fig. 4). These observations suggest that a subpopulation of helices, those at the edge of the lamella, unfold and dissociate from the lamella, thereby becoming available for rapid, enzymic degradation to maltodextrins.

The d.p. of the resistant fragments from the amylose–1-naphthol hydrolyzate is 123, which gives a lamellar thickness of 12 nm, assuming 8 residues per turn. A thickness of 12 nm is larger than that reported (~10 nm) by Yamashita and Monobe[13] from electron microscopy. If 10 nm is the actual thickness, then 123 residues corresponds to a helix having 9 D-glucosyl residues per turn, instead of 8, but we cannot judge from our data which structure is correct.

When enzymic hydrolysis is used for structural analysis, the ability to terminate the enzyme reaction immediately is crucial. We found that immersion of the digest in a boiling-water bath (98°) or hot-oil bath (110–120°) was insufficient for completely stopping the activity of any of the three alpha amylases used: *Bacillus subtilis* alpha amylase was able to continue the degradation to maltodextrins of all of the resistant fragments during a 15- to 20-min immersion in hot oil or boiling water, and the other two amylases retained sufficient activity to lower the fragment size after immersion. One factor in the continued hydrolysis by alpha amylase, after heat treatment, is the heat-mediated disruption of the lamellar structure, which makes much more of the amylodextrin chain available for enzymic hydrolysis. Another factor is the stability and enhanced activity of the enzymes during heating; *B. subtilis* alpha amylase is known to be stable and active at high temperatures[21]. The addition of eight volumes of ethanol, on the other hand, appeared to terminate the action of all three amylases instantly. This effect is due to precipitation of the substrate and to denaturation of the amylases.

Table I shows that the peak d.p. values of the resistant fragments from *B. subtilis* alpha amylase digests of amylose-V complexes are consistently greater than those d.p. values from digests with human-salivary and porcine-pancreatic alpha amylases after an equal number of conversion periods. When the substrate is retrograded amylose, *B. subtilis* alpha amylase also gives a larger, resistant fragment (see Table III). These differences are probably due to the larger binding-site (9 D-glucosyl units) of *B. subtilis* alpha amylase[19] compared with the binding sites (5 D-glucosyl units) of human-salivary and porcine-pancreatic alpha amylases[17,18]. We

thus attribute the higher d.p. values of the *B. subtilis* alpha amylase-resistant fragments to the larger binding-site of this alpha amylase, and its consequent inability to bind and hydrolyze the amorphous areas as close to the folding points of the helical chains as can human-salivary and porcine-pancreatic alpha amylases bind and hydrolyze (with their smaller binding-sites).

Furthermore, with the amylose–butyl alcohol complex, *B. subtilis* alpha amylase gives a resistant fragment much larger than that given by human-salivary and porcine-pancreatic alpha amylases (d.p. 105, compared with d.p. 71 and 78). Because the six-membered helix is the smallest, the number of D-glucosyl residues in the amorphous regions between the helical chains is also smaller than with the seven- and eight-membered helices. *B. subtilis* alpha amylase may thus not be able to hydrolyze between all of the helical segments and may therefore leave a certain number of resistant fragments that have two or more helical segments connected by D-glucosyl residues from the amorphous region. These relatively longer, resistant fragments then contribute to the larger peak d.p. values for the resistant fragments obtained by *B. subtilis* alpha amylase hydrolysis. With the larger helices containing seven and eight D-glucosyl residues per turn, *B. subtilis* alpha amylase still produced resistant fragments that were larger than the resistant fragments produced by the other two alpha amylases. The differences between the sizes were less (d.p. 110 *vs.* 90 and 95, and d.p. 140 *vs.* 122 and 125), however, because the number of D-glucosyl residues between the helical segments in the amorphous region is greater than the number for the six-membered helix. This greater number of D-glucosyl residues between the helical segments thus allows *B. subtilis* alpha amylase to hydrolyze more of the chains between the helical segments (refer to Table I and Fig. 6).

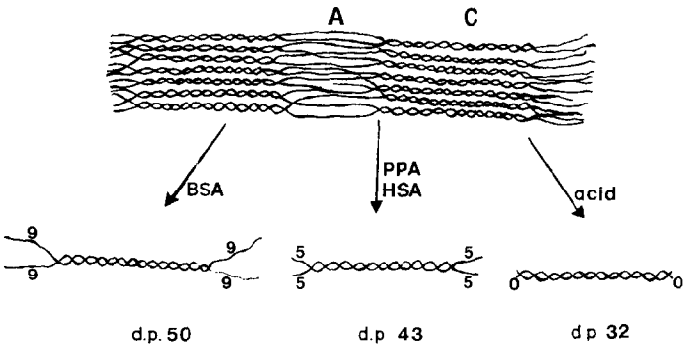

Fig. 7. Proposed structure and mechanism of hydrolysis of retrograded amylose by acid and by alpha amylases. [A is the amorphous area, and C is the crystalline area. Hydrolysis with 16% sulfuric acid for 20–40 days at 25° gave an amylodextrin having a d.p. of 32 D-glucosyl residues; hydrolysis with porcine-pancreatic alpha amylase (PPA) and with human-salivary alpha amylase (HSA) for 10 conversion periods at 25° gave an amylodextrin having a d.p. of 43 D-glucosyl residues; hydrolysis with *Bacillus subtilis* alpha amylase (BSA) for 10 conversion periods gave an amylodextrin having a d.p. of 50 D-glucosyl residues.]

The formation of resistant fragments from alpha amylase and acid hydrolysis of retrograded amylose (see Table III) may be explained by the presence in retrograded amylose both of crystalline and amorphous regions. Acid hydrolysis shows that the resistant, crystalline regions have a size of ~32 D-glycosyl residues and a length[22] of ~10 nm. We propose the structure of retrograded amylose given in Fig. 7, in which the crystalline regions are double helices interspersed with amorphous regions. This structure is similar to that proposed for starch chains in the starch granule, in which acid erodes the amorphous parts, leaving a highly crystalline amylodextrin having a double-helical structure[20,22,23]. The proposed double-helical structure of the crystalline regions, and the resulting resistant fragments, further explain the stability of the fragments toward the action of alpha amylase when the reactions are stopped by heating instead of by addition of ethanol. When the retrograded amylose is subjected to prolonged acid hydrolysis, all of the D-glucosidic bonds in the amorphous regions are hydrolyzed, leaving the double-helical, crystalline regions intact. However, when the retrograded amylose is hydrolyzed by alpha amylases, they cannot hydrolyze the D-glucosidic bonds close to the crystalline regions, because of the sizes of their binding sites. Stubs of unhydrolyzed D-glucosyl residues then result at each end of the resistant fragments (see Fig. 7).

The size of the stubs is dependent on the nature of the particular alpha amylase. With human-salivary and porcine-pancreatic alpha amylases, the stubs are 5 D-glucosyl residues long, giving a chain that is 10 D-glucosyl residues longer than that of the resistant fragments from acid hydrolysis (42 *vs.* 32), and, with *B. subtilis* alpha amylase, the stubs are each 9 D-glucosyl residues long, giving a chain that is 18 D-glucosyl residues longer than that of the resistant fragments from acid hydrolysis (50 *vs.* 32). The average lengths of the stubs correspond exactly to the number of binding subsites of the amylases: 5 for[17] porcine-pancreatic and human-salivary alpha amylases, and 9 for[19] *B. subtilis* alpha amylase.

A further feature is the relative constancy of the 10-nm length of the repeating fold of the helices, regardless of whether the size of the helix is 6 or 7 D-glucosyl residues per turn, or whether it is the double helix of retrograded amylose.

A practical result of the present work is that the hydrolysis of the amylose complexes, or the hydrolysis of retrograded amylose, provides a means of preparing amylodextrins of various sizes, having a relatively narrow, d.p. distribution. Hydrolysis, with human-salivary and porcine-pancreatic alpha amylases of the amylose complexes having 6 D-glucosyl residues per turn gave an amylodextrin of average d.p. 108, with a distribution of 30–140 D-glucosyl residues; hydrolysis of amylose complexes having 7 D-glucosyl residues per turn gave an amylodextrin of average d.p. 108, with a distribution of 30–140 D-glucosyl residues; hydrolysis of amylose complexes having 7 D-glucosyl residues per turn gave an amylodextrin of a distribution of 60–230.

Hydrolysis of retrograded amylose with alpha amylases and with acid gave a resistant amylodextrin having a narrower distribution. Acid gave an average d.p. of 32 D-glucosyl residues, having a distribution of 25–50 D-glucosyl residues; human-

salivary and porcine-pancreatic alpha amylases gave an average d.p. of 43 D-glucosyl residues, with a distribution of 30–55 D-glucosyl residues; and *B. subtilis* alpha amylase gave an average d.p. of 50 D-glucosyl residues, with a distribution of 30–80 D-glucosyl residues. The yields of these amylodextrins were 45–65%.

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