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INVESTIGATION OF THE FINE STRUCTURE OF AMYLOPECTIN USING ALPHA- AND BETA-AMYLASE

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ABSTRACT

The hydrolysis of waxy-maize amylopectin and its beta-amylolysis limit dextrin by the alpha-amylase of *Bacillus subtilis* was followed by gel filtration on Sepharose CL-6B. The mixture of alpha-dextrins was also treated with beta-amylase. The d.p. of these β-limit dextrins was similar to those of the products obtained when the amylopectin β-limit dextrin was the substrate. It is suggested that amylopectin and its β-limit dextrin are hydrolysed by the alpha-amylase by fission of longer internal chains between unit clusters. A model is presented which shows how these unit clusters can be connected to build up the amylopectin molecule. Maltohexaose was produced from the amylopectin, but not from the amylopectin β-limit dextrin, and it is concluded that the latter is produced solely from the external chains.

INTRODUCTION

The hydrolysis of waxy-maize amylopectin by the alpha-amylase of *Bacillus subtilis* has been suggested to involve two independent processes[1]. In one process, the internal chains are cleaved to give alpha-dextrins which have a defined number of chains. The molecular weights of the dextrins are not constant during the alpha-amylolysis, because of the other process in which maltohexaose is produced continuously by fission of the external chains.

The formation of specific products from amylopectin indicates that the substrate has a defined structure[2]. Many studies with debranching enzymes have shown that the chain lengths of amylopectins of various origins are slightly different[3–8]. The cluster model for the structure[9–11] is now widely accepted, but only a few reports on the actual size of the clusters have appeared[2,12,13]. In a densely branched model structure, one cluster contains, on average, only 4.22 chains[14], whereas, in a more tightly branched model, some 22–25 chains were suggested to be involved[8]. Very little is known about how individual clusters are inter-linked, although it has been suggesed that the longer chains with at least 40 D-glucosyl residues connect two or more clusters[8,10,14].

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A comparison is now reported of the products of alpha-amylolysis of amylopectin and its β-limit dextrin.

EXPERIMENTAL

Waxy-maize starch granules (amylopectin, Sigma) were deproteinised and defatted as described[15]. The activity[15] of alpha-amylase [(1→4)-α-D-glucan glucanohydrolase, EC 3.2.1.1, *Bacillus subtilis*; Koch–Light] was 6.25 U/mg. Beta-amylase [(1→4)-α-D-glucan maltohydrolase, EC 3.2.1.2, sweet potato; Sigma], purified[15] as described by Marshall and Whelan[16], had an activity of 5000 U/mL.

Alpha-amylolysis of amylopectin. — An aqueous suspension of waxy-maize starch granules (20 mg/mL) was gelatinised by boiling for 1 h. To a portion (50 mL) of this solution was added an equal volume of alpha-amylase (6.67 μg/mL) in 0.05M sodium acetate buffer (pH 5.5). The mixture contained 2 mU of enzyme/mg of amylopectin and the reaction took place at 23°. At intervals, aliquots (5 mL) were treated with 5M KOH (0.125 mL) to stop the reaction; 24 h before gel chromatography, 5M KOH (0.45 mL) was added to a portion (2.05 mL) of the aliquot, which was diluted to 5 mL with M KOH, and a 2.5-mL portion was applied to the column.

Beta-amylolysis of alpha-amylase-treated samples. — The pH of each aliquot (2.05 mL), taken at intervals of the alpha-amylolysis, was adjusted to 4.8 with M acetic acid (~0.3 mL), the solution was diluted to 3.5 mL with 0.1M sodium acetate buffer (pH 4.8), and beta-amylase (0.5 mL, 4 U) diluted in the acetate buffer was added. After 3 h at room temperature, the reaction was stopped with 5M KOH (1 mL). Within 2 h, 2.5 mL of this sample was applied to the gel chromatography column.

Preparation of amylopectin beta-amylase limit dextrin. — Waxy-maize starch granules (1.2 g) were gelatinised in boiling water (60 mL) for 1 h. A solution of beta-amylase (4 U/mL in 0.05M acetate buffer, pH 4.8) was added. The mixture was stirred for 3 h at 35°, then treated in a boiling water bath for 10 min. The degree of beta-amylolysis (β-limit) was 58% (not increased by beta-amylolysis for 24 h) based on the reducing power[17] before and after acid hydrolysis. A β-limit of 55% was determined enzymically using $NADP^+$ coupled reactions[1].

The sample was dialysed overnight against 0.05M acetate buffer (pH 5.5), then precipitated with 5 vol. of methanol. The β-limit dextrin was collected by centrifugation, washed with acetone, and dried in ether.

Alpha-amylolysis of amylopectin β-limit dextrin. — An aqueous solution (50 mL, 8 mg/mL) of the β-limit dextrin was treated with the alpha-amylase as described for the alpha-amylolysis of the amylopectin.

Gel chromatography[15]. — Columns (2.5 × 90 cm) of Sepharose CL-6B (Pharmacia) were eluted with 0.5M KOH (2-mL fractions) and calibrated with alpha-dextrins. The void volume (V_o) of the gel as determined with amylopectin was 136 mL (fraction 68), and the total volume (V_t) was 362 mL (fraction 181) as determined[18] with KCl.

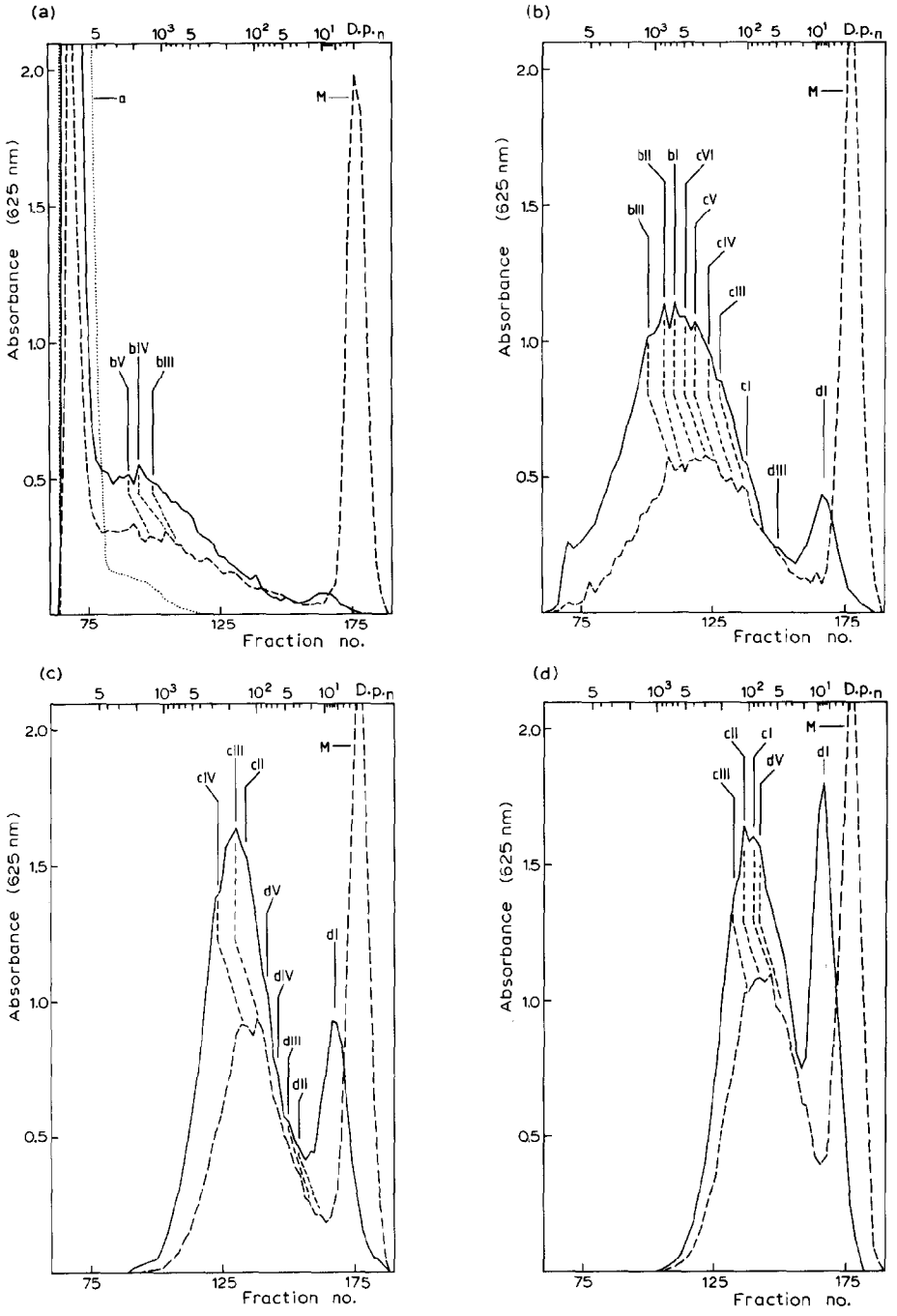

Fig. 1. Fractionation on Sepharose CL-6B of the hydrolysates of waxy-maize amylopectin after the action of alpha-amylase (——) and the successive treatment with beta-amylase (— — —): (*a*) before (·····) and after hydrolysis for 10 min, and hydrolysis for (*b*) 40 min, (*c*) 90 min, and (*d*) 150 min; dI-bV indicates characteristic intermediate alpha-dextrins, and the dotted lines trace their position after the treatment with beta-amylase (giving α,β-dextrins), a indicates large molecules excluded from the gel, and M is maltose.

RESULTS

The compositions of the hydrolysates at different times during alpha-amylolysis are shown in Fig. 1. The intermediate products have been characterised[1,2,15] and are designated bV-cI; dI was maltohexaose[15] and was produced simultaneously with the higher-molecular-weight dextrins.

In the early stages of the reaction, the material of high molecular weight excluded from the gel (fraction a) was hydrolysed to dextrins (bV-bI) with a high d.p. There were also dextrins larger than bV, which were eluted close to the void volume of the gel (Fig. 1a).

As the reaction proceeded, the dextrins of fraction c preponderated; after 90 min, cIII and cII were the main dextrins (Fig. 1c). The rate of the reaction then decreased, so that, after a further 60 min, cII still preponderated together with cI (Fig. 1d), and there was only a small decrease in the amount of cIII. Material (dV-dII) smaller than cI appeared as shoulders on the chromatograms and there was a clear increase in the amount of dI.

Addition of beta-amylase to the reaction mixture at different stages of alpha-amylolysis changed the molecular-weight distribution (Fig. 1). The maltohexaose (dI) was converted completely into maltose. The other intermediate alpha-dextrins were not completely hydrolysed, showing that they contained branch points. Thus, the new peaks in the chromatograms represented β-limit dextrins formed from the intermediate alpha-dextrins. They are named α,β-dextrins and designated bVβ-dIIβ, indicating the alpha-dextrin of origin.

The α,β-dextrins had similar elution volumes and were not clearly visible in all chromatograms (Fig. 1). Regardless of the duration of alpha-amylolysis, the α,β-dextrins were eluted in the same volume, showing that the d.p. was constant, as would be expected for a true β-limit dextrin. This finding was in contrast with that for the alpha-dextrins shown in Fig. 1. The alpha-dextrin peaks bV-cI moved slightly towards lower d.p. as the alpha-amylolysis proceeded. Thus, the β-limit, which can be estimated from the difference in d.p. between the alpha-dextrins and the respective α,β-dextrins, decreased with time in accord with earlier findings[2,15].

The smaller alpha-dextrins dV-dIV showed[1] higher β-limits with smaller d.p. after beta-amylolysis for 210 min. The time-dependence of the beta-amylolysis limits for these small dextrins is difficult to determine, since they are not detected easily in the early stages of reaction (Fig. 1). The d.p. values for the α,β-dextrins, as estimated from the gel filtration results, are summarised in Table I.

The gel chromatogram (Fig. 2a) shows amylopectin β-limit dextrin to be high-molecular-weight material eluted at the void volume. This material was rapidly degraded by alpha-amylase. As with amylopectin, the hydrolysate of its β-limit dextrin gave more or less clear peaks on gel filtration (Fig. 2), indicating that the products had defined molecular weights. These intermediate dextrins are named β,α-dextrins because they were produced from the β-limit dextrins. The positions of the peaks in the gel chromatograms were close to those of the α,β-dextrins and therefore the peaks are numbered in the same way as shown in Fig. 1.

TABLE I

D.P. OF α,β- AND β,α-DEXTRINS AND THEIR CHAIN NUMBERS

Dextrin	*D.p. of α,β-dextrin*[a]	*D.p. of β,α-dextrin*[a]	*Chain number*[b]
bV	1280		153
bIV	920		111
bIII	660		80
bII	475		58
bI	370	390	45
cVI	295	300	36
cV	211	230	26
cIV	151	160	19
cIII	106	117	14
cII	82	88	11
cI	58	68	8
dV	45	51	7
dIV	35	40	5
dIII	23	27	4
dII	17	16	3

[a]Determined from gel filtration. [b]Calculated from the standard curve in Fig. 6.

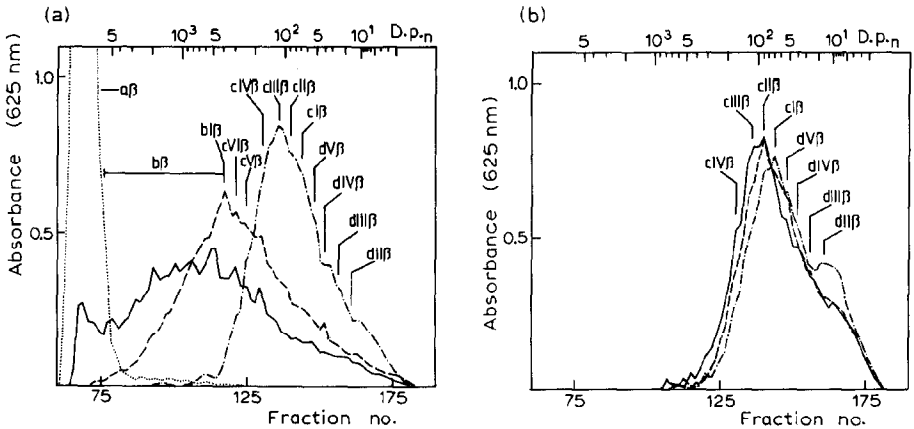

Fig. 2. Fractionation on Sepharose CL-6B of the hydrolysates of waxy-maize amylopectin β-limit dextrin after the action of alpha-amylase: (*a*) before (·····) and after hydrolysis for 5 (——), 10 (— — —), and 40 min (—·—); (*b*) after hydrolysis for 90 (——), 120 (— — —), and 210 min (—·—). The position of the characteristic intermediate β,α-dextrins is indicated.

Because of the rapid degradation of the amylopectin β-limit dextrin in the early stages of the reaction, it was difficult to get a clear picture of the higher-molecular-weight β,α-dextrins formed. After 40 min (Fig. 2a), only dextrins in fractions c and d remained, and cIVβ-cIIβ were the main products. Further reaction was slower so that, after 90 min, cIIIβ and cIIβ still preponderated (Fig. 2b) and there was a larger amount of cIβ. In the later stages of the reaction (Fig. 2b), the most noticeable changes were a slow decrease in the concentration of β,α-

dextrins larger than cIIβ and an increase in the concentration of dVβ-dIIβ. Material smaller in size than dIIβ was also produced, but clear peaks in this area were not observed.

Despite the higher rate of reaction in the early stages, the alpha-amylolysis of amylopectin β-limit dextrin was similar to the hydrolysis of amylopectin. The most obvious difference, however, was the absence of a peak corresponding to maltohexaose (dI).

Like the α,β-dextrins, the β,α-dextrins seemed to have a constant molecular size regardless of the duration of alpha-amylolysis. The estimated d.p. was slightly higher than that of the α,β-dextrins (Table I).

DISCUSSION

The β-limit of the original amylopectin[1] was 55%. If the intermediate alpha-dextrins contain clusters of branch points and the production of new external chains by fission of internal chains is negligible, then the addition of beta-amylase to the hydrolysis mixture at any time during alpha-amylolysis should give a total β-limit similar to that of the original amylopectin.

Since maltose was not detected in the alpha-amylase reaction, the maltose present in the α,β-dextrin mixtures was derived from the beta-amylase reaction and can be used to calculate the total β-limit. However, small carbohydrates are susceptible to alkaline degradation, to give products[19] that do not react with the anthrone–sulphuric acid reagent. The absorbance in the anthrone reaction of a solution of maltose in M KOH decreased by 50% in 32 h. Storing of solutions of alpha-amylase hydrolysis mixtures in M KOH for up to 5 days did not affect the absorbance.

The use of 0.5M KOH as an eluant in gel filtration, therefore, is not suitable for maltose. Elution of starch with water has been severally described[20–22] but, in our experience, an alkaline eluant is necessary for the high-molecular-weight dextrins, which are not eluted with water. The alkaline medium is also a good solvent for high-molecular-weight dextrins[23].

Because of the alkaline degradation, the maltose peaks in Fig. 1a-1d are low and required correction in order to obtain the true total β-limits. The amount of maltose degraded was taken as the difference between the mean peak areas of the alpha-dextrins and α,β-dextrins. The results are shown in Table II and, on the average, the maltose degraded in each sample was 22%. The corrected total β-limits are also shown in Table II and the average value (54%) was close to that of the original sample.

The mean β-limit of the branched alpha-dextrins a-dII can be calculated from the relevant peak areas before and after treatment with beta-amylase. As shown in Table II, the β-limit decreased linearly with time, indicating that the external chains of the alpha-dextrins were shortened progressively.

It has been suggested that maltohexaose is produced from the external chains

TABLE II

BETA-AMYLOLYSIS LIMIT OF ALPHA-DEXTRINS IN THE AMYLOPECTIN HYDROLYSATE AFTER THE ACTION OF ALPHA-AMYLASE

Duration of α-amylolysis (min)	*Maltose degraded by alkali[a] (%)*	*Total β-limit[b] (%)*	*β-Limit of alpha-dextrins a-dII[c] (%)*
10	28	54	53
30	23	54	50
40	22	54	48
50	23	55	47
60	15	54	44
90	25	56	42
120	18	54	36
150	19	53	28

[a]The maltose degraded in the sample when exposed to 0.5M KOH during gel filtration, calculated as the difference between the mean peak areas in the alpha- and α,β-dextrin chromatograms in Fig. 1. [b]The β-limit of all the alpha-dextrins in the hydrolysate, calculated from the area of the peak for maltose in Fig. 1, corrected for the maltose degraded by alkali. [c]Calculated from the difference between the peak areas of alpha-dextrins a-dII in Fig. 1 before and after treatment with beta-amylase.

of amylopectin[2,24,25] by an independent process[1]. In the alpha-amylolysis of the amylopectin β-limit dextrin, maltohexaose was not obtained (Fig. 2) in accord with the results with waxy-maize starch[24]. The action of the alpha-amylase of *B. subtilis* on potato amylopectin β-limit dextrin produced glucose, maltose, and maltotriose from the internal chains[25], thus indicating a clear difference in the fine structures of potato and waxy-maize amylopectin. It is known that potato amylopectin contains more long B-chains than the cereal starches[26].

The production of branched alpha-dextrins from amylopectin has been proposed to proceed independently of the production of maltohexaose by fission of internal B-chains[1]. Thus, the amylopectin β-limit dextrin, in which the external chains are too short to be attacked by the alpha-amylase, should be degraded solely in this way. Fig. 2 shows that the β,α-dextrins had molecular weights almost identical to those of the α,β-dextrins in Fig. 1 and therefore were probably identical with the α,β-dextrins. An explanation of the somewhat higher d.p. for most of the β,α-dextrins, as compared to the α,β-dextrins (Table I), is suggested in Fig.3. The fission of an internal chain by alpha-amylase before the action of beta-amylase results in a smaller product than if the attack occurs afterwards.

In an earlier report[2], the molar concentration of the alpha-dextrins was calculated after different times of hydrolysis, but a decrease in the molecular weights of the intermediate products was not observed. Moreover, the standard polysaccharides [dextrans and *O*-(2-hydroxyethyl)starch][2] used to calibrate the gel did not give a correct estimate of the molecular weight of the amylopectin alpha-dextrins[15]. As the molecular weight of the alpha-dextrins changed with time, it is more convenient to use the constant molecular weight (d.p. × 162) of the α,β-

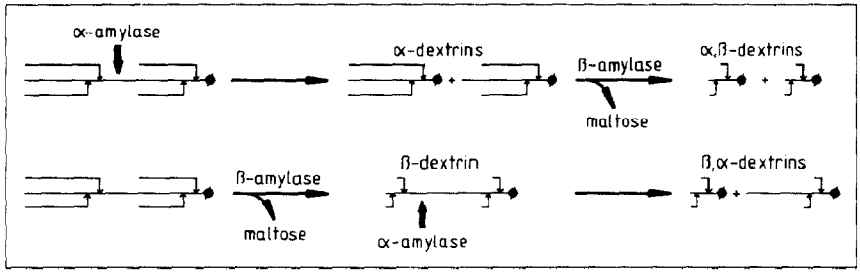

Fig. 3. Schematic presentation of the production of α,β- and β,α-dextrins, showing the difference in the structure of the products: ——, (1→4)-linked chains; ↓, (1→6) linkages; ●, reducing units.

dextrins in these calculations. The peak height in Fig. 1 was used as a measure of the arbitrary concentration of dextrin.

The arbitrary molarity of the α,β-dextrins as a function of time is seen in Fig. 4. The production of the intermediate products in group c and in group b (not shown) reflected consecutive reactions. The concentrations of cIβ and the lower-molecular-weight group d increased throughout the reaction; cIβ, dVβ, and dIVβ seemed to be produced in approximately equimolar amounts at least up to 120 min of hydrolysis. The small dextrins dIIIβ and dIIβ were produced in similar amounts,

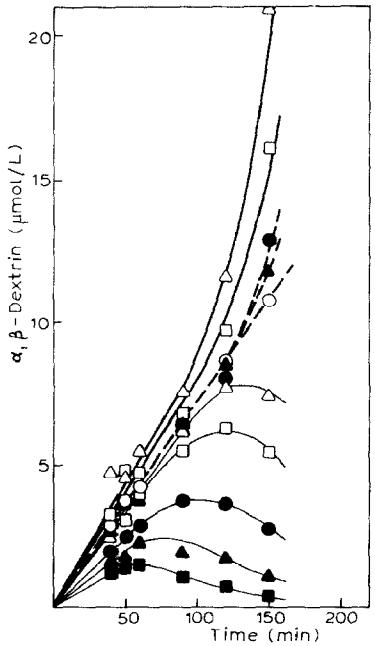

Fig. 4. Molar concentration of α,β-dextrins obtained by alpha-amylolysis and successive beta-amylolysis of amylopectin as a function of time. The concentration was calculated arbitrarily from the peak height in Fig. 1, assuming that an absorbance value of 1.0 equals 0.1 mg of carbohydrate/mL. Peak numbers are symbolised as follows: I, ○; II, △; III, □; IV, ●; V, ▲; and VI, ■; ——, cVIβ-cIIβ; – – –, cIβ-dIVβ; ——, dIIIβ and dIIβ.

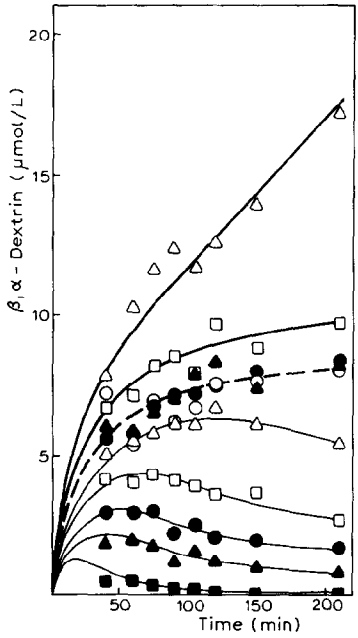

Fig. 5. Molar concentration of β,α-dextrins obtained by alpha-amylolysis of amylopectin β-limit dextrin as a function of time. The concentration was calculated arbitrarily from the peak height in Fig. 2, assuming that an absorbance value of 1.0 equals 0.1 mg of carbohydrate/mL. The symbols are as in Fig. 4.

but, after 90 min of hydrolysis, the recovery of these dextrins increased markedly, which was probably artificial due to overlap of the other large peaks at this stage of the reaction (Fig. 1). Fig. 4 shows that the rate of the alpha-amylase action did not decrease in the later stages of the hydrolysis, but the change in molecular weight distribution was smaller (Fig. 1). This result could be due to the comparatively larger number of (1→4) linkages that had to be hydrolysed in order to produce the small alpha-dextrins.

The molarities of the β,α-dextrins as a function of time are illustrated in Fig. 5. In this reaction, the production of the dextrins was much faster. Probably, more enzyme was available for the hydrolysis of the internal chains in the absence of external chains.

The production of β,α-dextrins and α,β-dextrins was similar. The dextrins larger than cIβ were formed consecutively and the amount of smaller dextrins increased continuously though slowly in the later stages of the hydrolysis. The dextrins cIβ, dVβ, and dIVβ were produced in approximately equimolar amounts, and the smaller dextrins dIIIβ and dIIβ in somewhat higher yields. This similarity indicates that the hydrolysis of amylopectin β-limit dextrin occurred by the same process as the proposed production of branched dextrins from amylopectin.

In the alpha-amylolysis of amylopectin β-limit dextrin, the production of

dextrin dIIIβ resembled more the formation of dextrins cIβ-dIVβ, whereas the production of dIIIβ from the intermediate alpha-dextrins was similar to that of dextrin dIIβ. Fig. 2 shows that there was carbohydrate material smaller than dIIβ. It is possible that this material was produced preferentially from dIIIβ, thus decreasing the concentration of this dextrin. Whether such material also was produced in the hydrolysis of amylopectin was difficult to see because of the large peak for maltohexaose (Fig. 1).

It was suggested[2] that the molecular weights of the intermediate alpha-dextrins can be combined to give the molecular weights of larger intermediate fractions. As the molecular weight of the alpha-dextrins is not constant, it is preferable to use the mol. wt. (or d.p.) of the dextrins after the treatment with beta-amylase. From Fig. 3, it is clear that addition of the d.p. of the α,β-dextrins will result in an underestimate of the value of the d.p. for larger dextrins, whereas addition of the d.p. of β,α-dextrins will be more useful.

Another way to trace the origin of the dextrin products is by comparing the chain numbers. This number will be constant throughout the reaction. From the data given by Bertoft[1] on the β-limit and chain number in samples isolated from amylopectin hydrolysis mixtures, it is possible to construct a "standard" curve for the average d.p. of the α,β-dextrin samples *versus* chain number as shown in Fig. 6. Using the d.p. of the individual α,β-dextrins, their theoretical chain number can be estimated (Table I). The chain numbers of the small dextrins in fraction d are

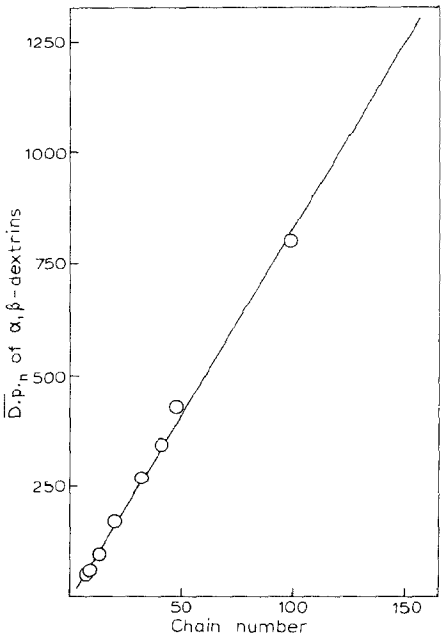

Fig. 6. "Standard" curve on the average d.p. of α,β-dextrins *versus* chain number (based on data given in ref. 1).

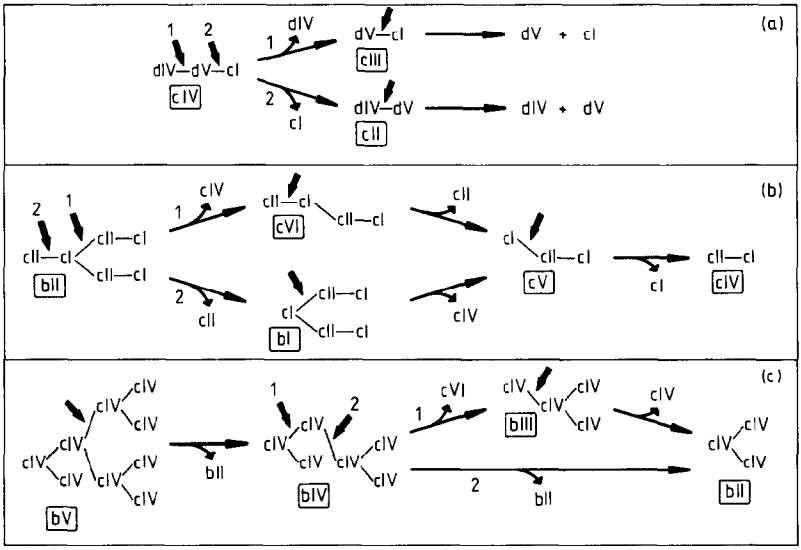

Fig. 7. Proposed cluster structure for waxy-maize amylopectin: (*a*) intermediate product cIV is a unit cluster built up of sub-cluster structures (attack by the alpha-amylase at certain internal chains is indicated by thick arrows, and produces cI, dV, and dIV): (*b*) dextrin bII is a larger structural unit composed of three unit clusters (attack at certain internal chains yields alpha-dextrins intermediate in size between the larger structural unit and the unit cluster; (*c*) more unit clusters can be added to bII, giving the larger alpha-dextrin products.

uncertain, because it is not clear if the standard curve in Fig. 6 is linear in this region.

On the basis of the enzymic analysis of alpha-dextrins, it was suggested[1] that the size of a unit cluster in the amylopectin probably can be found among the dextrin products that are slightly larger or smaller than cI. Dextrins dIV, dV, and cI were produced in approximately equimolar amounts and were also very slowly hydrolysed during alpha-amylolysis (Figs. 1 and 2). This result indicates that readily hydrolysable internal chains, which are presumed to be longer B-chains, are absent from these dextrins.

Fig. 7 shows a model of the amylopectin molecule in which dIV, dV, and cI represent parts of a unit cluster, or unit clusters of different sizes. The combination of these clusters is based on the chain numbers in Table I. In order to obtain the chain number of a larger dextrin, the sum of the chain numbers of two combined smaller dextrins has to be reduced by one.

The structure of amylopectin shown in Fig. 7 is regular. The products of hydrolysis of such macromolecules should have defined structures and molecular weights, as the existence of the intermediate dextrins in this work indicates. The combination of dIV, dV, and cI gives the alpha-dextrin cIV (Fig. 7a), which is attacked preferentially by the alpha-amylase at two sites, giving either cIII or cII. Bender *et al.*[12] studied the action of cyclodextrin glycosyltransferase (CGT) on

waxy-maize starch. This enzyme seems to be very similar to the alpha-amylase of *B. subtilis* in its action pattern on amylopectin, because it preferentially attacks the external chains and the longer internal B-chains[12,21]. CGT produces mainly three intermediate products from the amylopectin, which were suggested to represent unit clusters of three different sizes[12]. The beta-amylolysis limit dextrins of these products (MIβ, MIIβ, and MIIIβ) correspond closely to the d.p. of the α,β-dextrins cIVβ, cIIβ, and cIβ, respectively. According to Fig. 7a, the CGT would attack site 2 preferentially in cIV, whereas the alpha-amylase attacks both sites.

The size of a unit cluster is a matter of definition. Using different enzymes, such as the alpha-amylase of *B. subtilis* and CGT, a slightly different picture emerges, probably because of differences in the action patterns of the enzymes. Bender *et al.*[12] showed that the three suggested unit clusters obtained from the digest after the action of CGT did not contain any longer B-chains. Possibly, therefore, this is true also for the alpha-dextrin cIV. If the long B-chains characteristically connect unit clusters[8], the dextrin cIV is the unit cluster structure, whereas dIV, dV, and cI could be defined as sub-cluster structures connected by short B-chains.

The cluster structure cIV can be connected by long B-chains to give the larger alpha-dextrins, as shown in Figs. 7b and 7c. Each cIV binds two other cIV units. Thus, two of the 19 chains in cIV are long B-chains, which will give a ratio of short A- and B-chains to long B-chains of 8.5, which corresponds well with the value of 9 reported by Robin *et al.*[10]. Hizukuri[7] and Biliaderis *et al.*[4] found molar ratios of 11 and 10.7, respectively, whereas Baba and Arai[27] reported the ratio to be 6.4 in waxy-maize starch. In Fig. 7, the dextrin bII constitutes a larger structural unit, eventually a "super cluster" as was suggested earlier[2,13].

The structure drawn in Fig. 7 is one of several possibilities. Future work on the fine structure of amylopectin will be focussed on the smaller dextrins in fraction d and an examination of the individual chains in the alpha-dextrins.

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