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FRACTIONAL PRECIPITATION OF AMYLOPECTIN ALPHA-DEXTRINS USING METHANOL

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ABSTRACT

The oligosaccharides produced on hydrolysis of waxy-maize amylopectin by the alpha-amylase of *Bacillus subtilis* for 60 and 210 min were fractionated by precipitation with methanol and the fractions were analysed on Sepharose CL-6B. The smaller dextrins were comparatively well separated and the larger dextrins were obtained as mixtures. The d.p. of the products ranged from 67 to 1550. Maltohexaose was formed in addition to the precipitable dextrins.

INTRODUCTION

Amyloses and amylodextrins readily form complexes with a wide variety of compounds. The helical structures of these complexes are different[1–6]. Whereas amylose is soluble in dimethyl sulfoxide or potassium hydroxide, the complexes between amylose and alcohols precipitate from solution. Molecules as small as methyl α-maltoside form a complex with dimethyl sulfoxide[6].

The most common method used to separate the amylose and amylopectin components of starch is by precipitation of the former with 1-butanol[7]. Ethanol has also been used for this purpose[8], for the precipitation of amylodextrins[4], and for the fractionation of alpha-amylase digests of amylose[9]. Precipitation with methanol has been used[10] to fractionate the products obtained by the action of cyclodextrin glycosyltransferase on amylopectin.

We now describe a methanol precipitation method for the fractionation of the dextrins produced[11] by the action of the alpha-amylase of *Bacillus subtilis* on amylopectin.

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EXPERIMENTAL

Waxy-maize starch granules (amylopectin, Sigma) were deproteinised by stirring overnight with chloroform–1-butanol (5:1) and defatted by extraction (Soxhlet) with hot aqueous 85% methanol overnight. The granules were dried with acetone and then ether.

The activity (6.25 U/mg) of the alpha-amylase of *B. subtilis* [(1→4)-α-D-glucan glucanohydrolase, EC 3.2.1.1; Koch–Light] was measured in 0.05M sodium acetate buffer (pH 5.5) at 23° and that (19,000 U/mL) of sweet-potato beta-amylase [(1→4)-α-D-glucan maltohydrolase, EC 3.2.1.2; Sigma] in acetate buffer (pH 4.8), using soluble starch (Merck) as the substrate at 5 mg/mL. Reducing power was determined with the Nelson reagent[12], using maltose as the standard. One unit of enzyme activity is defined as that producing 1 μmol of reducing groups per min per mL.

Purification of beta-amylase. — Commercial beta-amylase was purified twice by ion-exchange chromatography[13] and stored at 4° as a precipitate (activity, 5,000 U/mL) in 3.5M ammonium sulphate. The purification was checked using gelatinised amylopectin: 0.1 U/mg of amylopectin gave a maximum beta-amylolysis limit of 54.6% within 2 h, indicating the absence of other amylolytic activities.

Alpha-amylolysis of amylopectin. — Waxy-maize starch granules (12 g) were suspended in deionised water (1140 mL) and gelatinised for 1 h at 100° with continuous stirring. The mixture was cooled to 25°, and a solution (60 mL; 0.133 mg/mL) of alpha-amylase in 0.5M sodium acetate buffer (pH 5.5) was added. At intervals, aliquots (1 mL) were treated with 5M KOH (25 μL) to stop the reaction, to a portion (0.5 mL) was added 5M KOH (0.225 mL), and the volume was made up to 2.5 mL with 0.5M KOH before gel chromatography. The main part (1196 mL) of the mixture was treated with 5M KOH (29.9 mL) after hydrolysis for 60 min and was used for methanol precipitation.

In a second experiment, alpha-amylolysis was continued for 210 min.

Methanol precipitation. — The pH of each amylopectin hydrolysate was adjusted to 11 with 1.5M HCl (~80 mL), methanol (5 vol.) was added, each mixture was left overnight at 23°, then decanted, and the residual precipitates were collected by centrifugation, washed with acetone, and air-dried. The products I and II were obtained after alpha-amylolysis for 60 and 210 min, respectively.

The decanted supernatant solution from the 210-min hydrolysis was vacuum-dried and the residual gel-like substance was washed with acetone to give product III.

To a solution of each product in deionised water (10 mg/mL) at 23° was added methanol intially to a methanol–water ratio of 0.5:1. Each mixture was stored for 30 min at 23°, then centrifuged for 10–15 min, and the precipitate was washed twice with acetone and air-dried. More methanol was then added to the supernatant solution and the procedure was repeated until no more precipitate was obtained. The details are given in Fig. 1. Those precipitates obtained in sufficient amounts were fractionated further as described above.

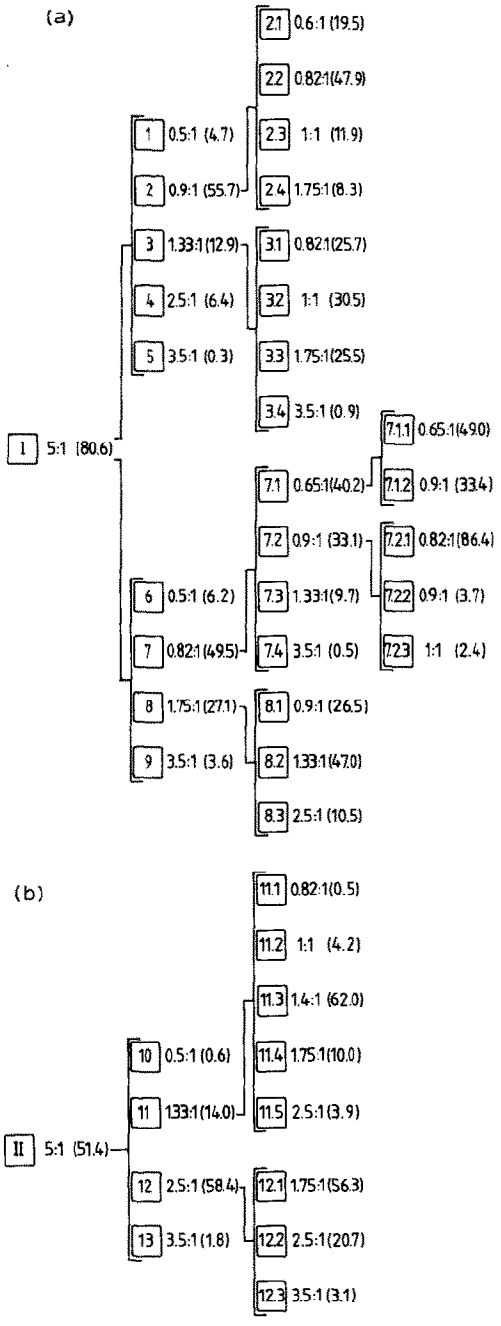

Fig. 1. Fractional precipitation from aqueous solution with methanol of (*a*) I and (*b*) II. The fractions, designated in the boxes, were obtained with the methanol–water ratios shown, and percentage yields are shown in parenthesis.

Gel chromatography. — Solutions of samples I and II (5 mg of each) and the other methanol-precipitated products (2 mg) each in 0.5M KOH (2.5 mL) were eluted from columns (2.5 × 90 cm) of Sepharose CL-6B (Pharmacia) with 0.5M KOH at 1 mL/min. Fractions (2 mL) were analysed for carbohydrates, using the anthrone–sulphuric acid reagent[14]. The void volume (V_0) of the gel was taken as the elution volume of amylopectin (fraction 73), and the total volume (V_t) was determined[15] with KCl (fraction 199).

Beta-amylolysis of methanol-precipitated products. — To portions (0.5 mL) of aqueous solutions (4 mg/mL, 16 mg/mL for III) of alpha-dextrins was added M KOH (0.5 mL), and the volume was made up to 2.5 mL with 0.5M KOH before gel chromatography.

A small part of the sample was diluted with water (× 25) and used for the enzymic determination[16] of the total carbohydrate content, using amyloglucosidase [(1→4)-α-D-glucan glucohydrolase, EC 3.2.1.3, *Aspergillus niger*], hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1, yeast), and glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate:NADP oxidoreductase, EC 1.1.1.49, yeast), obtained from Boehringer–Mannheim.

To a third part (0.75 mL) of the alpha-dextrin solution was added an equal volume of 0.1M acetate buffer (pH 4.8) followed by purified beta-amylase (3 μL, diluted to 0.1 U/μL). The mixture was kept overnight at 35° and then boiled for 3 min, the pH of a portion (0.5 mL) was adjusted to 6.6 with 0.5M KOH, and the solution was diluted to 12.5 mL with 0.1M citrate buffer (pH 6.6) containing 1.35M EDTA and 20mM *N*-acetylcysteine. The maltose was then hydrolysed with α-D-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20, yeast; Boehringer–Mannheim), and the D-glucose formed was determined with the hexokinase/glucose 6-phosphate dehydrogenase system[17]. The beta-amylolysis limit is expressed as maltose liberated/total carbohydrate (as maltose equivalents).

The other aliquot (~1.0 mL) of the beta-amylolysis mixture was treated with M KOH (1 mL) and diluted to 2.5 mL with 0.5M KOH before gel chromatography.

Analysis of reducing-end residues. — Solutions containing 0.02–0.04 μmol of reducing-end D-glucosyl residues per mL were analysed by the method of Park and Johnson[18] except that the carbonate–cyanide solution was replaced by the cyanide-containing carbonate buffer[19].

The average degree of polymerization (d.p.) was calculated as the molar concentration of total carbohydrate/reducing-end residues. The total carbohydrate concentration was determined as described for beta-amylolysis.

RESULTS

The intermediate alpha-dextrins obtained by hydrolysis of waxy-maize starch with the alpha-amylase of *B. subtilis* are shown in the gel-filtration chromatograms in Fig. 2. The macrodextrins obtained in early stages of the reaction (Fig. 2a) are designated[11] bV-cI. As the reaction proceeded, bV-bI disappeared and new

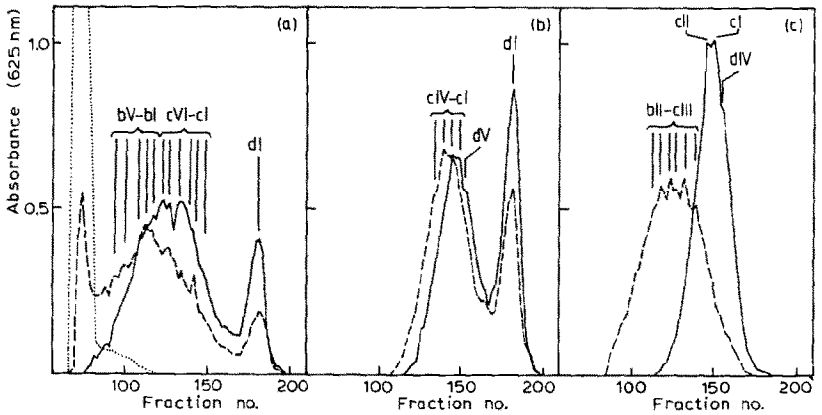

Fig. 2. Fractionation on Sepharose CL-6B of the hydrolysates of waxy-maize amylopectin obtained after the action of alpha-amylase. (*a*) Amylopectin before (·····) and after the hydrolysis for 30 (— — —) and 60 min (——); (*b*) amylopectin after hydrolysis for 90 (– — –) and 210 min (——); (*c*) the first methanol precipitate of the amylopectin hydrolysate after alpha-amylolysis for 60 (— — —, I) and 210 min (——, II). dI-bV indicates characteristic intermediate alpha-dextrin products.

products (cVI-cI) were produced together with a product (dI) of low molecular weight. After alpha-amylolysis for 60 min, the intermediate products bII-cIII preponderated and dI constituted ~15% of the total carbohydrate.

The molecular weight distribution of the product (I) precipitated from this mixture by 5 vol. of methanol is shown in Fig. 2c and contained no dI. The yield of I was 80.6% (Fig. 1).

When alpha-amylolysis was allowed to proceed for 210 min (Fig. 2b), both the apparent rate of the reaction and the number of intermediate products were reduced[11]. The main products were cII and cI, products (dV) smaller in molecular weight than cI were formed, and dI constituted ~35% of the carbohydrate content. Gel filtration of the material (II) precipitated by the addition of 5 vol. of methanol is shown in Fig. 2c. No dI was precipitated and the recovery of material of higher molecular weight was 51.4%. Fig. 2c indicates that material (dIV) smaller in molecular weight than dV was present.

Gel filtration (Fig. 6c) revealed the non-precipitated material (III) to be almost exclusively dI with ~10% of material having slightly higher molecular weight.

Fractionation of I by methanol precipitation, using two series of methanol–water ratios, gave fractions 1–5 and 6–9, respectively; the molecular weight distribution curves of the latter are shown in Fig. 3a. Precipitation with a 0.5:1 methanol–water ratio gave a variable but low yield of material which was eluted as a broad peak, sometimes beginning at the void volume of the gel (as sample 6 in Fig. 3a) but sometimes covering the whole elution volume. This material possibly represented retrograded alpha-dextrins and was not studied further.

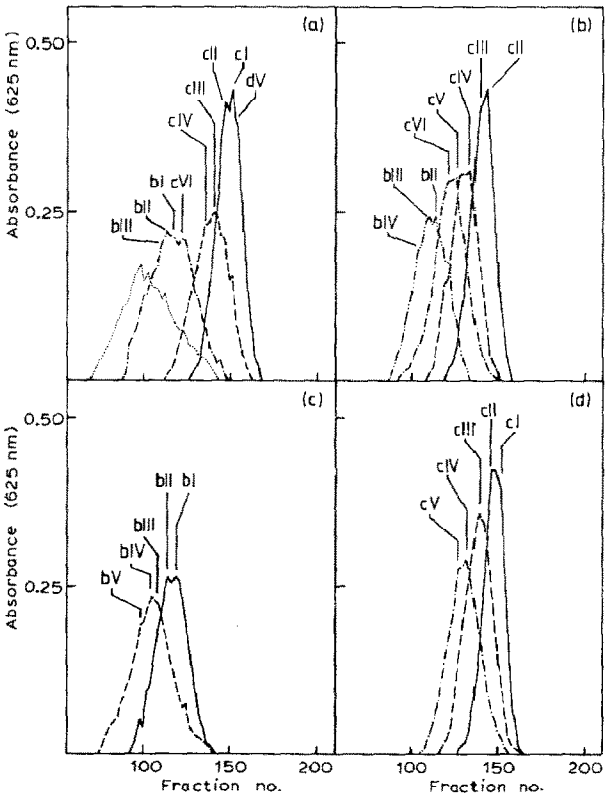

Fig. 3. Gel filtration of (*a*) I to give 6 (·····), 7 (— · —), 8 (— — —), and 9 (——); (*b*) 7 from (*a*) to give 7.1 (—··—), 7.2 (—·—), 7.3 (— — —), and 7.4 (——); (*c*) 7.1 from (*b*) to give 7.1.1. (— — —) and 7.1.2 (——); (*d*) 8 from (a) to give 8.1 (— ·—), 8.2 (— — —), and 8.3 (——).

Further stepwise addition of methanol gave a series of precipitates (fractions 7–9) in which the intermediate alpha-dextrins were seen as peaks or shoulders on the chromatograms (Fig. 3a). Samples recovered in sufficient amounts (at least 500 mg) were fractionated further, and the highest yields were obtained with slightly smaller volumes of methanol because of the higher initial concentration (Fig. 1a); no material was precipitated by a 0.5:1 methanol–water ratio.

Fig. 3b shows the sub-fractions (7.1–7.4) obtained from fraction 7, which consisted mainly of dextrins bIII-cVI. Sub-fraction 7.1 (40.2%) contained mostly the high-molecular-weight alpha-dextrins bIV-bI, 7.2 (33.1%) contained mostly cVI and cV, and 7.3 and 7.4 contained small amounts of cV-cII. Further fractionation of 7.1 gave 7.1.1 and 7.1.2, the molecular weight distributions of which are shown in Fig. 3c, indicating them to contain mostly bV-bIII and bII-bI, respectively.

Further fractionation of fraction 8 (Fig. 3d) gave 8.1 in which cV and cIV preponderated. The recovery of 8.2 was 47.0% and it contained mostly cIII, as

TABLE I

COMPOSITION AND D.P. OF THE PRODUCTS OBTAINED BY FRACTIONATION OF THE MIXTURE I FORMED BY ALPHA-AMYLOLYSIS OF AMYLOPECTIN FOR 60 MIN

Fraction	*Composition*[a]	*D.p.*[b]
I	bII-cIII	
2	bIII-cV	610
2.1	bIV-bII	1210
2.2	bII-cVI	612
2.3	cV	420
2.4	cIV	257
3	cV-cIII	305
3.1	cV-cIV	351
3.2	cIV-cIII	265
3.3	cIII	195
3.4	cIII	195
4	cIII-cII	135
5	dV	84
7	bIII-cVI	510
7.1	bIV-bI	1040
7.1.1	bV-bIII	1550
7.1.2	bII-bI	810
7.2	cVI-cV	515
7.2.1	bI-cVI	625
7.2.2	cVI-cV	480
7.2.3	cV	440
7.3	cV-cIV	325
7.4	cIII	170
8	cIV-cII	187
8.1	cV-cIV	330
8.2	cIII	191
8.3	cII-cI	127
9	cII-dV	95

[a]Intermediate alpha-dextrins constituting the main part of the sample. [b]Mean value determined by gel filtration.

expected from the molecular weight distribution of fraction 8 (Fig. 3a). Sub-fraction 8.3 contained mainly cII and cI. Further fractionations did not result in further purification of the individual alpha-dextrins.

The sub-fractionation of the fractions 1–5 (Fig. 1a) gave results (data not shown) similar to those presented in Fig. 3. Table I summarises the composition of the fractions obtained after alpha-amylolysis for 60 min. The intermediate alpha-dextrins constitute the main products, and it is clear from Fig. 3 that each fraction contains a range of alpha-dextrins.

The 210-min-alpha-amylolysis product (II) was fractionated with methanol as for I (Fig. 1b). A 0.5:1 methanol–water ratio gave <1% of a precipitate (10) of possibly retrograded material (Fig. 4a). As expected from the composition of II (Fig. 2c), the bulk of the material (12) was precipitated with a 2.5:1 methanol–water ratio (Fig. 4a). Further fractionations of 11 and 12 are shown in Figs. 4b and 4c, respectively, and the compositions are summarised in Table II.

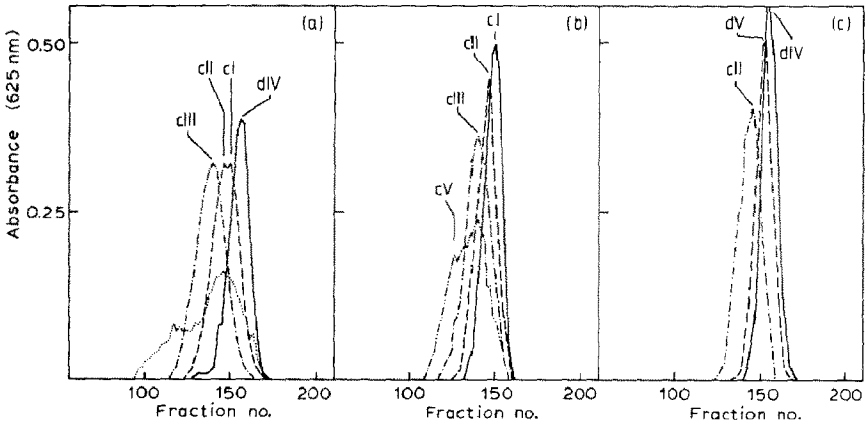

Fig. 4. Gel filtration of (*a*) II to give 10 (·····), 11 (—·—), 12 (— — —), and 13 (——); (*b*) 11 from (*a*) to give 11.2 (—··—), 11.3 (—·—), 11.4 (— — —), and 11.5 (——); (*c*) 12 from (*a*) to give 12.1 (—·—), 12.2 (— — —), and 12.3 (——).

The d.p. values (accurate to ±7%) of the alpha-dextrins (Table III) varied from 6 (III) to 1562 (7.1.1) and were used to construct the standard curve in Fig. 5 for calibration of the Sepharose CL-6B. There was a linear relation between the log d.p. or mol. wt. and the K_{av} down to d.p. 40 or K_{av} 0.75. As shown in the standard curve, maltohexaose and maltose can be distinguished, as could maltose and glucose, the latter being eluted at or immediately before the total volume of the column (data not shown).

TABLE II

COMPOSITION AND D.P. OF THE PRODUCTS OBTAINED BY FRACTIONATION OF THE MIXTURE II (FORMED BY ALPHA-AMYLOLYSIS OF AMYLOPECTIN FOR 210 MIN)

Fraction	*Composition*[a]	*D.p.*[b]
II	cII-dV	
11	cIII	165
11.1	cIV-cII	165
11.2	cV-cII	193
11.3	cIII	165
11.4	cII	115
11.5	cI	92
12	cII-cI	104
12.1	cII	117
12.2	dV	75
12.3	dIV	67
13	dIV	67
III	dI	6

[a]Intermediate alpha-dextrins constituting the main part of the sample. [b]Mean value determined by gel filtration.

TABLE III

D.P. OF FRACTIONATED ALPHA-AMYLOLYSIS PRODUCTS AND THEIR β-LIMIT DEXTRINS

Fraction	*D.p.*[a]	*β-D.p.*[b]	*β-limit (%)*
7.1.1	1562	808	48
7.1.2	802	425	47
7.2.1	641	354	45
7.2	510	267	48
2.3	462	244	47
7.3	414	218	47
8.1	310	169	46
3.2	252	137	46
11.3	197	137	30
4	133	66	50
9	81	40	51
12.3	67	42	38
III	6		87
Maltose[c]	2		

[a]Determined by reducing-end group analysis. [b]Mean value calculated from: β-d.p. = (% β-limit/100) × d.p. [c]Commercial sample.

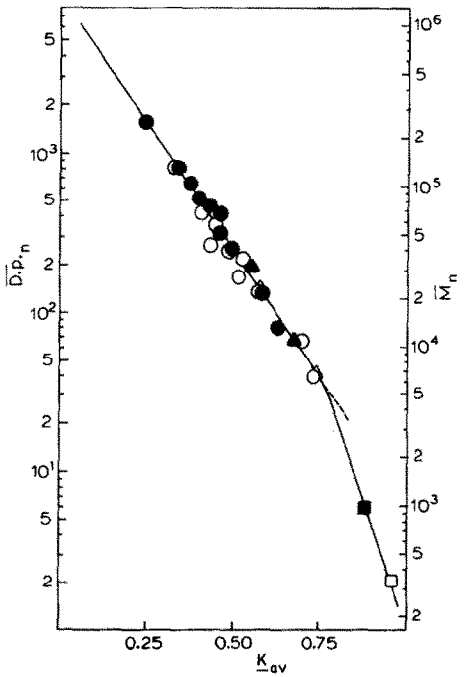

Fig. 5. Calibration curve for gel filtration of alpha-dextrins on Sepharose CL-6B. The mean d.p. was determined by reducing-end group analysis. The average mol. wt. = d.p. × 162. K_{av} is the partition coefficient and defined as $V_e - V_o/V_t - V_o$, where V_e is the elution volume of the sample, V_o is the void volume, and V_t is the total volume. Filled symbols are the alpha-dextrin samples, and open symbols are their beta-amylolysis products: ●, fractions from I; ▲, fractions from II; ■, III (maltohexaose); □, maltose.

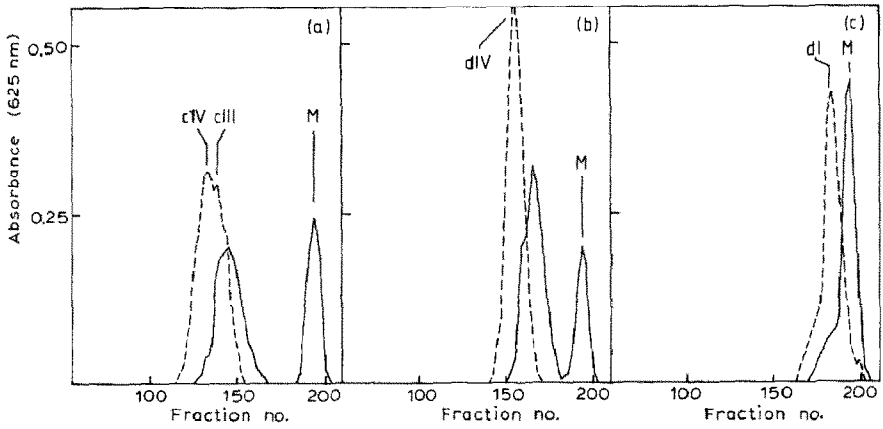

Fig. 6. Molecular weight distribution curves from gel filtration of alpha-dextrins before (— — —) and after (——) beta-amylolysis: (*a*) 3.2, (*b*) 12.3, (*c*) III; M, maltose.

The alpha-dextrins in Table III, when hydrolysed with sweet-potato beta-amylase, had β-limits of 45–50%, with the exception of samples 11.3 and 12.3 which had lower β-limits. Product III had a β-limit of 87%.

From the β-limit values, the d.p. of the residual β-limit dextrin (β-d.p. in Table III) can be calculated. The K_{av} values for these dextrins were obtained by gel filtration. As shown in Fig. 6, the original alpha-dextrins were hydrolysed to maltose and β-limit dextrins, and dI in III was hydrolysed completely to maltose, consistent with its being maltohexaose. However, the small amount of material of higher molecular weight in III was partly resistant to beta-amylolysis, as also suggested by the β-limit value of 87% in Table III.

The elution volumes of the β-limit dextrins and alpha-dextrins are compared in Fig. 5. The d.p. of each methanol-precipitated fraction was determined from gel filtration, using the standard curve in Fig. 5, and the results are presented in Tables I and II.

DISCUSSION

The use of methanol precipitation[10] to fractionate the products of hydrolysis of amylopectin is simple and the recovery is good, typically >80% for the higher-molecular-weight alpha-dextrins (Fig. 1a), and 74.8–80.6% for the lower-molecular-weight alpha-dextrins (Fig. 1b). The carbohydrate content, measured enzymically as glucose, of the precipitable dextrins was at least 90%, whereas that of the non-precipitated material (fraction III) was 22.5%.

The formation of complexes between methanol and the alpha-dextrins is strongly dependent on temperature[6]. The precipitation and centrifugation steps were carried out at 24–26°. At temperatures slightly higher than 30°, the precipitates dissolved almost completely, whereas there was almost complete precipitation of all the dextrins at 4°.

The behaviour of a polymer in gel filtration is dependent on its effective hydrodynamic volume[20] and therefore on its conformation[21,22]. Hizukuri and Takagi[22] have shown that different glucans having the same d.p. are eluted with different volumes in gel filtration. The d.p. values of some of the alpha-dextrins were determined in order to obtain suitable standards (Table III). The alpha-dextrins in Table III had beta-amylolysis limits in the range 30–51%, showing that they had external chains of different lengths. Hydrolysis with beta-amylase reduces the external chains to 1–3 D-glucosyl residues[23]. As seen in Fig. 5, there was a linear relation between the d.p. and the partition coefficient among the alpha-dextrins and their β-limit dextrin products down to K_{av} 0.75. Thus, the dextrins obtained by alpha- or beta-amylolysis of amylopectin behave similarly during the gel filtration regardless of their composition.

The use of reducing-end group analysis to measure the d.p. of amylose[4,19,22,24] and smaller dextrins[25–27] is generally accepted. A d.p. of 1649 was obtained (modified Park–Johnson method[19]) for the original amylopectin sample, and a value of 718 was obtained for the β-limit dextrin[28] which accords with the beta-amylolysis limit[28] of 54%. The facts that the higher-molecular-weight alpha-dextrins had d.p. values of at least the same order, and that both the amylopectin and its β-limit dextrin were eluted at the void volume of the Sepharose gel, suggest that the above d.p. values are erroneous. Earlier reports on the d.p. of various amylopectins determined using alkaline dinitrosalicylic acid[29–31] are in agreement with our results. However, the latter reagent is subject to alkaline degradation[32], so that the apparent agreement may be fortuitous.

The molecular weights of the alpha-dextrins estimated[11] by gel filtration, using standard samples of hydroxyethylstarch and dextrans, are probably incorrect because of the variation in types of polymer. Tables I and II give the d.p. of the samples obtained after comparison with the standard curve in Fig. 5, and these values should be more reliable.

The molecular-weight distribution curves in Fig. 2 indicate that the peaks representing individual alpha-dextrins moved to lower d.p. as the hydrolysis proceeded. Indeed, comparison of the alpha-dextrins in Tables I and II indicates that the d.p. values of the individual intermediate products were not constant during the hydrolysis. Hence, the intermediate alpha-dextrin products listed in Tables I and II have different molecular weights. The definition of an intermediate product should therefore be that it contains a specific number of (1→6)-D-glucosidic linkages, probably grouped into clusters, and a specific number of chains. On this definition, a decrease in d.p. would be caused by a shortening of the external chains in the molecule.

A more detailed analysis of the methanol-precipitated fractions from this work is presented in the following paper[33].

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