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A comparison of the structures of the fractions of normal and high-amylose pea-seed starches prepared by precipitation with concanavalin A

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ABSTRACT

A method of separating amylose and amylopectin is based on proteolysis of the precipitated concanavalin A–amylopectin complex. The molecular-size distributions, together with the λ_{max} and A_{max} of the iodine complexes of fractions obtained by gel chromatography, indicated efficient separation of amylose and amylopectin. The amylose from the normal (*n*) genotype of pea seeds had a $\overline{M}_w$ higher than that from the high-amylose (HA) genotype. Hydrolysis by the debranching enzyme indicated a slight degree of branching in each amylose and that the branches associated with HA-amylose were shorter. The $\overline{M}_w$ of HA-amylopectin was lower and the column fractions gave a stronger colour with iodine and higher λ_{max} than for the *n*-amylopectin. The total average chain-length and internal and external average chain-lengths were all higher for the HA-amylopectin. There was no evidence of a significant quantity of an intermediate fraction.

INTRODUCTION

The starches of normal (*n*) and high-amylose (HA) peas differ in their isopotential iodine absorption and colour with iodine[1], apparently reflecting differing amounts of unbranched fraction. Fractionation of HA-starch from both peas[2] and maize[3,4], by complexing with such agents as 1-butanol and thymol, gave amylopectin (soluble) which showed a longer average chain-length and beta-amylolysis limit for the HA-amylopectin than for the *n*-amylopectin[2,4–13]. Ultracentrifugation indicated that amylopectin from HA-starches, prepared by complexing with 1-butanol, contained amylose of low molecular weight. Some measurements of average chain-length and limits of beta-amylolysis on the purified amylopectin gave values similar to those of *n*-starches[6–13], whereas others indicated that the average chain-length was still long[5,6]. When waxy, normal, and high-amylose corn starches were converted into the beta-limit dextrins, gel chromatography of the products on Sephadex G-50 indicated that the HA-material had a higher average chain-length, leading to the observation[14] that the inner-chain-length of HA-amylopectin was higher than that of *n*-amylopectin.

Starch may be regarded as a spectrum of structures with various molecular sizes, degrees of branching, and average chain-lengths. An increase in molecular size and decreases in the λ_{max} (wavelength of maximum absorption) and A_{max} (maximum absorption) values of the iodine–glucan complex reflect an increase in the degree of branching and a decrease in the average chain-length. The number of fractions and their properties

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depend on the method of fractionation. Starches from different sources and stages of physiological development have various proportions of particular structural types. The properties of the fractions reflect the nature and effectiveness of the method used. As well as the complexing of amylose with such agents as 1-butanol, the fractionation of starch has been achieved by ultracentrifugation[5-7,9,10], gel chromatography[15-19], selective aqueous leaching[20], retrogradation[21], preferential precipitation with iodine[12], and complexing with concanavalin A[22]. The effectiveness of the separation also depends on the source of the starch. Mature *n*-starches, particularly potato, are fractionated into amylose and amylopectin on complexing with 1-butanol. However, for some starch fractions, amylopectin is present in the amylose preparations and *vice versa*[15,22,23]. The centrifugal force applied to the 1-butanol complex may be critical. Gel chromatography and ultracentrifugation depend on an absence of overlap of molecular sizes between the fractions, and this does not occur often. Mature-wheat starch can be fractionated effectively by chromatography on Sepharose CL-2B. Leaching and retrogradation techniques gave partial separation, and amylopectin was not recovered quantitatively from the concanavalin A complex, either as a precipitate or on columns[22]. Combinations of fractionation procedures have also been used[6-9,24,25].

A preparative method is now reported for the quantitative recovery of amylopectin from the concanavalin A–amylopectin complex, and some properties of amylose and amylopectin of pea-seed starches, prepared by complexing with this lectin are described.

RESULTS AND DISCUSSION

The preparative procedure[22] was modified in two ways. Because a slight translucency remained in the HA-starch solution after centrifugation of the concanavalin A–glucan complex at 14 000*g*, the centrifugal force was increased to 20 000*g* in order to give a supernatant solution as clear as that obtained with *n*-starch. With the procedure described previously, there was incomplete recovery of the branched fraction, due to the difficulty of decomposing the lectin–glucan complex. A modification, involving in-

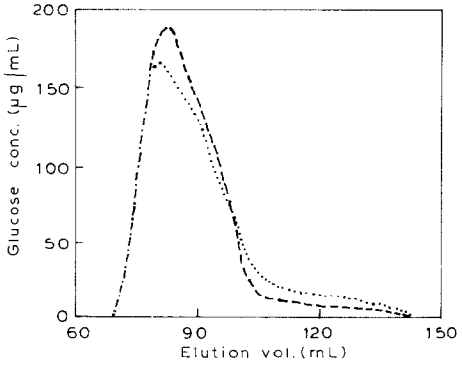

Fig. 1. Gel chromatography on Fractogel TSK 75 HW(S) of waxy rice starch (------) and waxy rice starch precipitated by concanavalin A, treated with Proteinase K, and isolated as the iodine complex (·····).

cubation with Proteinase K (Boehringer) to decompose the lectin, gave quantitative recovery of glucan. When a solution of potato starch was incubated with Proteinase K for 7 days, no change in the iodine absorption spectrum was detected (*cf.* ref. 26). Gel chromatography on Sepharose CL-2B of waxy rice starch, before and after precipitation by concanavalin A and recovery by iodine precipitation, with removal of the lectin with Proteinase K, revealed no significant difference. However, the use of Fractogel TSK HW-75(S) (Fig. 1) revealed a slight decrease in molecular size.

Gel chromatography on Sepharose CL-2B of the fractions (amylose) from normal and high-amylose pea-seed starches that were not precipitated by concanavalin A gave broad peaks with similar elution volumes (V_{ep}), but the $\overline{M}_w$ of the *n*-amylose was higher (Table I, Figs. 2a and 2b). Amylopectin was not detected: the ratios of A_{max} per unit weight of glucan ($100\,A_{max}/c$) and the λ_{max} values of each fraction from the column were characteristic of amylose. $-\log K_{wav} = -\log (V_{ew}-V_o)/(V_t-V_o)$ = molecular size/a constant, where V_t is the total volume of the column, V_{ew} the weighted-average elution volume, and V_o the void volume. The constant is specific for each matrix. Provided a significant amount of material at the void or total volume is not present, $-\log K_{wav}$ is approximately linearly related to molecular size. The elution profiles on CL-4B (Table I, Figs. 2c and 2d) also indicated a lower $\overline{M}_w$ for HA-amylose, although, on this matrix, there was a large excluded fraction. A similar molecular-size relationship

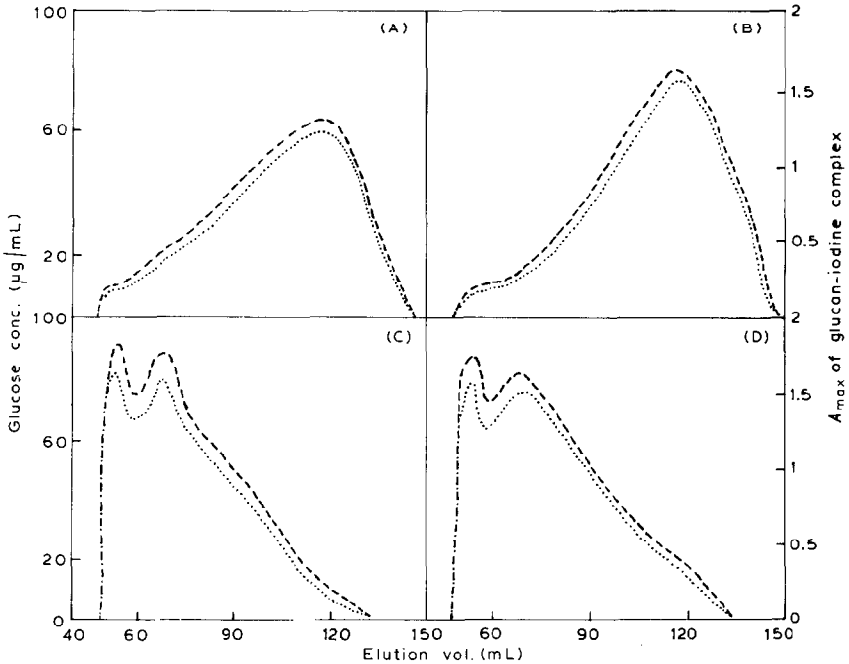

Fig. 2. Gel chromatography of the soluble (amylose) fractions from precipitation by concanavalin A: A, *n*-pea seeds on Sepharose CL-2B; B, HA-pea seeds on CL-2B; C, *n*-pea seeds on CL-4B; D, HA-pea seeds on CL-4B; ------, glucose concentration; ·····, A_{max} of glucan–iodine complex.

TABLE I

Behaviour of amylose fractions on gel chromatography

Source of amylose	*Column matrix*	*100* A_{max}/c	λ_{max} *(nm)*	*log* K_{WAV}[a]	$-log\ K_{PAV}$[a]
n-Pea seeds	CL-2B	2.0	620–630	0.27	0.16
n-Pea seeds	CL-4B	1.9	620–630	0.59	V_o, 0.77
n-Pea seeds ppted by 1-BuOH	CL-2B	1.9	(1) 550–570 (2) 620	0.27	V_o, 0.16
HA-Pea seeds	CL-2B	2.0	620–630	0.23	0.17
HA-Pea seeds	CL-4B	1.9	620–630	0.56	V_o, 0.77
n-Pea seeds debranched	CL-2B	2.1	620–630	0.22	0.16
n-Pea seeds debranched	CL-4B	2.1	620–630	0.55	V_o, 0.75
n-Pea seeds ppted by 1-BuOH debranched	CL-2B	1.9	620–630	0.22	0.16, V_t
HA-Pea seeds debranched	CL-2B	2.1	620–630	0.20	0.16
HA-Pea seeds debranched	CL-4B	2.0	620–630	0.46	V_o, 0.63

[a] $K_{WAV} = (V_{EW} - V_o)/(V_t - V_o)$ and $K_{PAV} = (K_{ep} - V_o)/(V_t - V_o)$; V_{ew}, weighted mean elution volume; V_t, total volume; V_{ep}, peak elution volume; V_o, void volume; c, glucan concentration in μg/mL.

was found[27] by viscometry after fractionation by complexing with 1-butanol combined with differential ultracentrifugation.

Both amylose fractions were treated with debranching enzyme (isoamylase EC, 3.2.1.68), and the products were chromatographed on Sepharose CL-2B (Table I, and Figs. 3a and 3b) and CL-4B (Table I, and Figs. 3c and 3d). Recoveries were always >90%, and $\overline{M}_w$ had decreased but with no significant change in the peak elution volume. This result is consistent with the model of amylose that has few branches but with a considerably higher d.p. than those of amylopectin[22,28]. However, there was evidence that the chains of HA-amylose were shorter than those of the *n* genotype (Fig. 3). The elution pattern of the former on Sepharose CL-2B (Fig. 3b) has an inflexion point near 130 mL [$-\log K_{PAV} = 0.09$] and on CL-4B at 120 mL ($-\log K_{PAV} = 0.14$), which are not present in that of the debranched *n*-amylose (Figs. 3b and 3c). The debranched HA-fraction still gave an iodine colour with a high A_{max} and λ_{max}, indicating that the average chain-length was higher than for chains from debranched amylopectin.

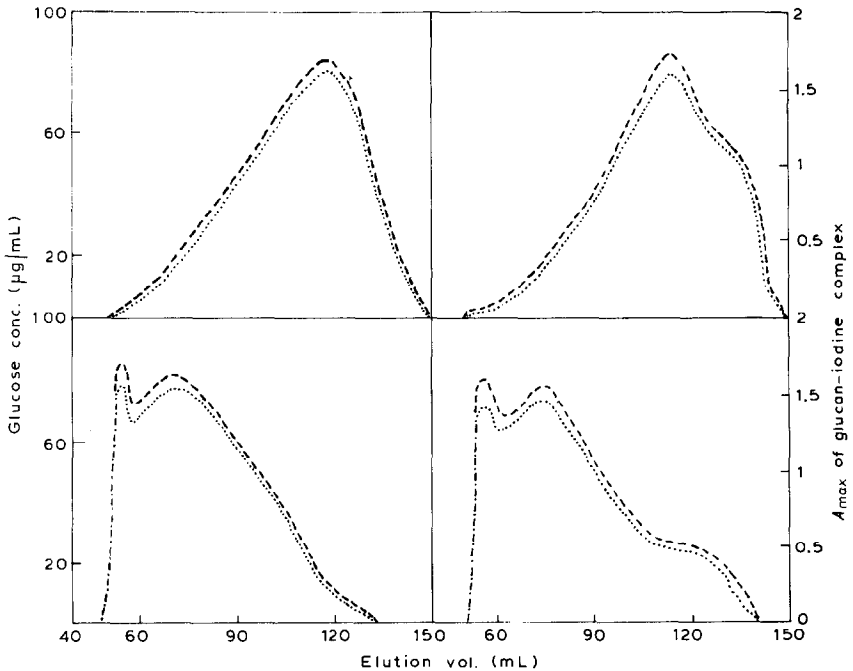

Fig. 3. Gel chromatography of soluble (amylose) fractions from precipitation by concanavalin A, after treatment with isoamylase: A, *n*-pea seeds on Sepharose CL-2B; B, HA-pea seeds on CL-2B; C, *n*-pea seeds on CL-4B; D, HA-pea seeds on CL-4B; ------, glucose conc.; ·····, A_{max} of glucan–iodine complex.

The elution volume can be compared with that of the debranched *n*-amylose fraction, prepared by complexing with 1-butanol (Fig. 4a), which has a peak at the total volume with an iodine colour having low A_{max}. In the elution pattern of this *n*-amylose fraction prior to debranching (Fig. 4b), there was a peak at the void volume with an iodine colour having low A_{max} and λ_{max}, characteristic of contaminating amylopectin.

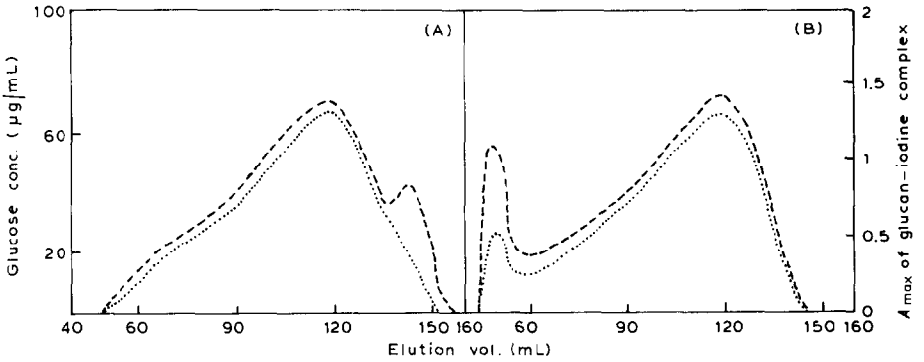

Fig. 4. Gel chromatography on Sepharose CL-2B: A, debranched pea amylose prepared by complexing with 1-butanol; B, pea amylose prepared by complexing with 1-butanol; ------, glucose concentration; ·····, A_{max} of glucan–iodine complex.

TABLE II

Behaviour of amylopectin fractions on gel chromatography

Source of amylopectin	*Column matrix*	*100* A_{max}/c	λ_{max} *(nm)*	$-log\ K_{W:AV}$[a]	$-log\ K_{PAV}$[a]
n-Pea seeds	CL-2B	0.93	550	1.04	V_o
HA-Pea seeds	CL-2B	1.2	570–580	0.69	V_o
HA-Pea seeds 650*g* ppt.	CL-2B	1.2	570–580	0.68	V_o
HA-Pea seeds 20 000*g* ppt.	CL-2B	1.2	570–580	0.70	V_o
n-Pea seeds	TSK 75(S)	0.92	550	0.69	V_o
HA-Pea seeds	TSK 75(S)	1.2	570–580	0.47	V_o
Waxy rice grain	TSK 75(S)	0.86	530	0.79	V_o

[a] See footnotes to Table I for abbreviations.

Gel chromatography of the fractions (amylopectin) of normal and high-amylose pea-seed starches precipitated by concanavalin A and waxy rice starch (Table II, Figs. 1 and 5) exhibited significant differences in λ_{max} and A_{max} per unit weight of glucan of the iodine complexes, and also in the molecular sizes. The patterns on Sepharose CL-2B (Table II, Figs. 5a and 5b) show that most of the polysaccharide was eluted at the void volume, but that more material in the HA-sample was included. The higher molecular size of the *n*-amylopectin was indicated by the higher weighted-average elution-volume and by chromatography on Fractogel TSK HW 75(S) (Table II, Figs. 5c and 5d). The molecular size of waxy rice starch was even higher (Table II, Fig. 1). HA-Pea amylopectin, prepared by complexing with 1-butanol and differential ultracentrifugation[27], had a higher molecular size than *n*-amylopectin when these were determined by light scattering and gel chromatography.

The A_{max} per unit weight of glucan and λ_{max} of HA-amylopectin on both Sepharose CL-2B and Fractogel TSK HW-75(S) (Table II) were higher (1.2 and 570–580 nm, respectively) than for *n*-amylopectin (0.92 and 550 nm, respectively). These properties were essentially constant over the whole elution range, indicating, along with the distribution of molecular sizes, that these fractions were free of amylose and that the different values were an inherent property of the HA-amylopectin molecule (probably higher average chain-length). The λ_{max} of waxy rice starch (530 nm) was lower than that of *n*-pea starch, consistent with its lower average chain-length. The relative molecular homogeneity of the HA-amylopectin was illustrated by gel chromatography on Sepharose CL-2B of sub-fractions of the concanavalin A–amylopectin complex, obtained by centrifugation at 650*g* and then at 20 000*g* (the ratio produced was ~ 1:2). The elution

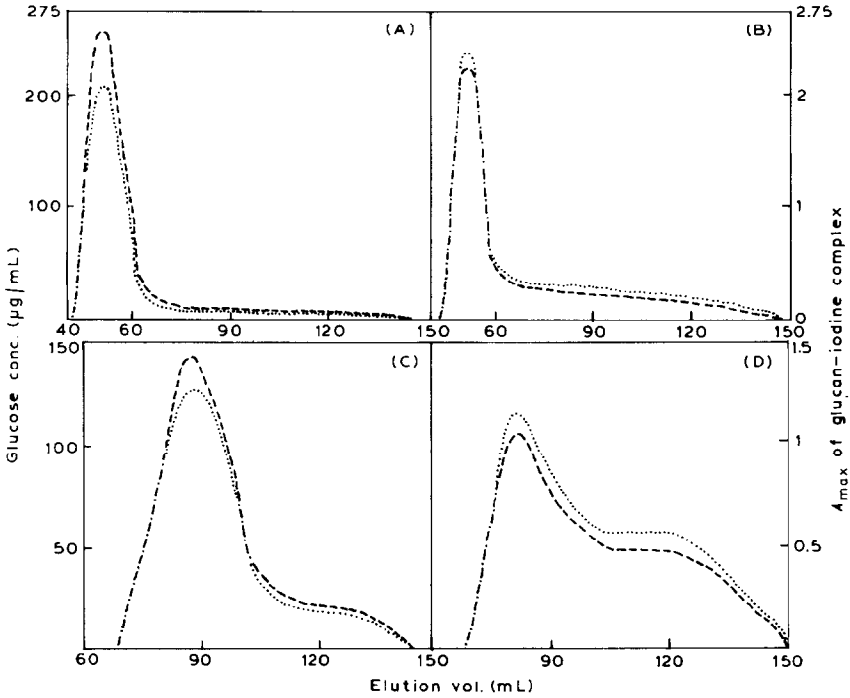

Fig. 5. Gel chromatography of fractions (amylopectin) precipitated by convanavalin A: A, *n*-pea seeds on Sepharose CL-2B; B, HA-pea seeds on Sepharose CL-2B; C, *n*-pea seeds on Fractogel TSK HW 75(S); D, HA-pea seeds on TSK HW 75(S): ------, glucose concentration; ·····, A_{max} of glucan–iodine complex.

TABLE III

Beta-amylolysis and chain lengths of fractions of pea starch

Source	*Average chain length*	*Beta-amylolysis limit*	*Chain length*	
			External	*Internal*
HA-Pea seeds amylopectin	38	59	24	13
n-Pea seeds amylopectin	26	55	16	9
HA-Pea seeds amylose	-	83	-	-
n-Pea seeds amylose	-	69	-	-
HA-Pea seeds starch	-	72 (calc. 73[a])	-	-
n-Pea seeds starch	-	60 (calc. 59)	-	-

[a] Calculated from amylopectin and amylose values, and amylose content

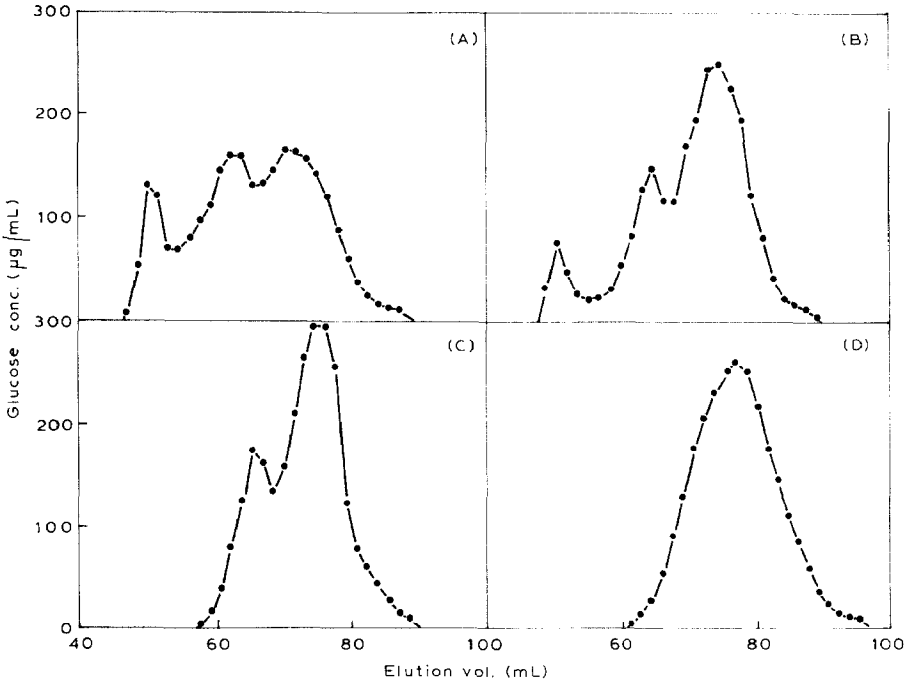

Fig. 6. Gel chromatography of debranched fractions of branched (1→4)(1→6)-α-D-glucans on Fractogel TSK HW 50(S): A, HA-pea seeds; B, *n*-pea seeds; C, waxy rice starch; D, phytoglycogen.

TABLE IV

Behaviour of debranched amylopectins on gel chromatography (Fractogel TSK 50HW(S)

Source	$-log\ K_{WAV}$[a]	$-log\ K_{PAV}$[a]	*Chains (%)*		
			L[b]	M	S
HA-Pea seeds	0.52	V_o, 0.55, 0.40	16	35	49
HA-Pea seeds 650*g* ppt.	0.52	V_o, 0.58, 0.40	16	34	50
HA-Pea seeds 20000*g* ppt.	0.53	V_o, 0.59, 0.39	16	36	48
n-Pea seeds	0.40	V_o, 0.55, 0.33	8	28	64
Waxy rice grain	0.36	0.52, 0.31	0	26	74
Phytoglycogen	0.29	0.29	0	0	100

[a] See footnotes to Table I for abbreviations. [b] L, Long; M, medium; S, short.

patterns (Table II) were similar. There was a tendency for λ_{max} of the material of lower molecular size in both centrifuged fractions, as well as in the whole precipitate, to be 580 nm. The beta-amylolysis limits and average chain-lengths (estimated by copper reduction after debranching) of HA- and *n*-amylopectins (Table III) gave higher average, external, and internal chain-lengths for the former (*cf.* refs. 2, 4, 5, and 13).

Debranching by isoamylase, followed by chromatography on Fractogel TSK50-HW(S) (Table IV, Fig. 6), confirmed that the average chain-length of HA-pea amylopectin, as fractionated by concanavalin A, was higher than that of *n*-amylopectin, with the elution pattern showing significantly higher proportions of long and medium chains. The amylopectins derived from the precipitates of the concanavalin A complex from HA-starch, centrifuged at 650*g* and than at 20 000*g*, were similar to the whole starch. The elution patterns on this matrix of waxy rice starch and sweet-corn phytoglycogen, with shorter average chain-lengths, revealed no long chains in the former and neither long nor medium chains in the latter. Recoveries were all >90%. On chromatography[32] on Biogel P-6, after debranching with isoamylase, of waxy and normal barley amylopectins (the latter prepared by complexing with 1-butanol), the *n*-amylopectin had a higher proportion of longer branches than the waxy amylopectin. Chromatography on Sepharose 4B of debranched maize and waxy maize HA-amylopectins[24] revealed the chains of the former to be longer. This HA-amylopectin was prepared by complexing with 1-butanol followed by gel chromatography on Sepharose CL-2B.

The differences in the structures of HA- and *n*-starches reflect the activity of branching enzyme [(1→4)-α-D-glucan, (1→4)-α-D-glucan-6-D-glucosyl transferase; EC 2.4.1.8)]. Differences result from the levels of the two forms with differing branching capacities[29–32] that produce different average chain-lengths of the branches. In mature normal starches, almost all of the molecules may be designated as amylopectin (high average molecular weight, average chain-lengths of ~25 D-glucosyl residues, high degree of branching) and amylose (with a lower average molecular weight, a slight degree of branching, long average chain-length). It has been suggested that HA-starches may contain a significant level of a fraction with an intermediate average chain-length and molecular size. If any genotype would be expected to have two types of branched fractions, it would be the *n*, which has two forms of branching enzyme. However, these two forms may need to be compartmentalised within the plastid for the synthesis of different structures. An intermediate fraction may be a consequence of a method of fractionation.

Thus, with concanavalin A, HA-pea starch can be fractionated into two parts, each of which has uniform properties for the iodine complexes of column fractions. The branched component of HA-pea starch has a higher average chain-length, leading to a higher λ_{max} and A_{max} per unit weight of glucan for the iodine complex, than the branched component of *n*-starch. There is somewhat more polymer of low molecular size than in *n*-amylopectin. Both the HA- and *n*-amylose fractions show limited branching, but with chains of shorter average length in HA. Provided the wider range of molecular size is recognized, it is not necessary to propose a large intermediate fraction in HA-pea starch. The atypical, isopotential iodine-titration curves of HA-starches (with a curved plateau

region) can be explained by the absorption characteristics of the amylopectin fraction, which result from the higher average chain-length.

Complexing with concanavalin A provides another method of fractionation of starch, which precipitates highly branched chains. It is additional to those, such as gel chromatography and ultracentrifugation, which separate on the basis of molecular size, and complexing with 1-butanol, which precipitates long (1→4)-α-D-glucan chains that have limited or no branching.

EXPERIMENTAL

Fractionation of starches with concanavalin A. — Amylose fractions were prepared as described[22], except that the centrifugal force was increased to 20 000*g*. For the isolation of the amylopectin fraction, the precipitate obtained after centrifugation was dissolved by stirring with a solution of methyl α-D-mannopyranoside (3.2 g) in 0.2M sodium acetate and 0.01M calcium chloride (40 mL).

The solution was boiled in order to denature protein, then cooled, and incubated overnight with Proteinase K (2 mL; Boehringer, 5 mg.mL^{-1}, in 0.2M Tris hydrochloride, and 0.1M calcium chloride, pH 8) at 30°, with one drop of added toluene. This mixture was cooled to 4°, 2M acetic acid (10 mL) was added, followed by 2% iodine in aqueous 20% potassium iodide (10 mL), and the mixture was stored at 4°. As soon as the starch–iodine precipitate had formed, it was separated by centrifugation (14 000*g*, 15 min, 4°), decolorized, and dissolved in 0.1M sodium arsenite in 0.2M phosphate buffer (pH 5, 1 mL) plus 0.1M sodium chloride, to give a concentration of glucan of ~2 mg.mL^{-1}. Ethanol (3 vol.) was added, the mixture was stored at 4° overnight and then centrifuged, and the residue was washed with ethanol–water (3:1) and re-dissolved in 0.1M sodium chloride to 2 mg.mL^{-1}.

Chromatography. — Aliquots (2.0 mL) were mixed with 2.5M potassium hydroxide (0.20 mL), and 2.0 mL was chromatographed[22] on Sepharose CL-2B, Sepharose CL-4B (for amylose), and Fractogel TSK HW-75(S) (for amylopectin). The material in aliquots was debranched[22] and the products were chromatographed on TSK HW-50(S).

Fractionation of n-*pea starch with 1-butanol.* — Starch (300 mg) was dispersed in methyl sulphoxide (5.0 mL), dissolved in 0.1M sodium chloride (45 mL), and heated to 60°, 1-butanol (2.5 mL) was added, and the mixture was cooled slowly. After 48 h, the precipitate was collected by centrifugation (14 000*g*, 10 min, 20°), washed with 1-butanol (1 mL) in 0.1M sodium chloride, then with ethanol, and dried in a vacuum for several hours. This product was dispersed in methyl sulphoxide (2.0 mL), dissolved in 0.1M sodium chloride (20 ml), and heated to 60°, 1-butanol (1.1 mL) was added, and the mixture was cooled slowly. After 48 h, the precipitate was collected by centrifugation, washed with ethanol, and dried. The residue was dispersed in methyl sulphoxide (1.0 mL) and dissolved in 0.1M sodium chloride to 2 mg.mL^{-1}. Aliquots (2.0 mL), after treatment with 2.5M potassium hydroxide (0.2 mL) and also after debranching with isoamylase, were chromatographed on Sepharose CL-2B.

Determination by Cu^{2+} reduction of beta-amylolysis limits and of average chain-

length after debranching. — A solution of glucan (12–16 mg) in 0.05M acetate buffer (pH 5.0) was incubated with sweet-potato beta-amylase (250 Boehringer units) at 30°, and a similar amount of enzyme was added after 30 and 60 min. After 90 min, the solution was diluted and aliquots were assayed for reducing power by the Nelson–Somogyi method[34].

A solution of amylopectin (8–10 mg) in 0.05M acetate buffer (pH 5.0) was incubated for 18 h with isoamylase (6500 Hayashibara units), and the reducing power was estimated by the Nelson–Somogyi method.

Total glucan content was estimated with phenol–sulphuric acid[35].

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