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ESTIMATION AND FRACTIONATION OF THE ESSENTIALLY UNBRANCHED (AMYLOSE) AND BRANCHED (AMYLOPECTIN) COMPONENTS OF STARCHES WITH CONCANAVALIN A

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ABSTRACT

Conditions have been devised for the quantitative precipitation of the branched fraction in starches by concanavalin A and the enzymic estimation of the α-D-glucan in this and the soluble components. For some starches, such as those from cereal and high-amylose pea seeds, the percentage of branched fraction determined by this procedure was higher than that calculated by deducting from one hundred the apparent amylose content as measured by potentiometric iodine titration, suggesting a method for estimating the content of material having an atypical structure in the whole starch. The procedure has been adapted to provide a preparative method of separating amylose and amylopectin fractions. It efficiently separated these in starches that cannot be fully fractionated by complexing with 1-butanol, such as those from high-amylose pea seeds and tobacco leaves.

INTRODUCTION

A number of methods have been described[1,2] for estimating the ratio of amylose to amylopectin in starches; potentiometric or amperometric titration of bound iodine, complexing with such polar organic reagents as 1-butanol, and spectrophotometric measurement of the colour with iodine. The last method is not applicable to amylomaize starches[3] and the second is not readily applicable to some starches. Another approach has been to use debranching enzymes to convert the whole starch into unbranched (1→4)-linked chains and separate these by gel chromatography[4], when the amount of the longest chains in the trimodal elution pattern is an estimate of the amylose. Alternatively, these chains can be precipitated with 1-butanol[5].

Concanavalin A interacts with non-reducing terminal α-D-glucosyl groups[6]. Reaction with amylopectin[7,8] is not as strong as with glycogen, and amylose produces no turbidity, since the single (or few) non-reducing end group per molecule does not allow multivalent association. Dextrans also react if there is sufficient branching[9,10].

Preparative fractionation of starch has involved the selective leaching of

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amylose from starch granules, which preferentially releases molecules of low molecular size but does not give a separate amylopectin fraction, or dispersion of the granule followed by complexing of the amylose with a partially water-soluble agent such as 1-butanol, thymol, or nitromethane. However, complexation methods are unsuitable for a number of starches, such as those from immature cereal seeds[11], leaf starches, and high-amylose starches[1–3]. In each of these starches, the amylose fraction also contains amylopectin. In the second and third starches, the amylopectin fraction includes amylose of low molecular size. Gel chromatography on agarose gels[11,12] has also been used but can lead to mixed fractions. Amylopectin has been purified and a mixture of amylose and amylopectin separated by affinity chromatography on Sepharose–concanavalin A, when the amylose was unbound[13].

Although starch is generally described as a mixture of two components, there appears to be a range of structures with overlapping molecular sizes and also possibly some that are intermediate between the two models of unbranched chains and branched chains with a particular average chain-length and degree of branching. The proportions of particular structural types vary with the source of the starch. Potato starch is near to the ideal model of mainly amylopectin of high molecular size, with an average chain-length of 25 glucosyl residues, and essentially unbranched amylose of lower molecular size. However, high-amylose starches are reported to contain substantial amounts of a branched fraction with a low molecular weight and a longer average chain-length, as well as amylose of quite low molecular weight.

Therefore, any method that applies a different principle of separation may be of value. We now report on the use of the lectin concanavalin A in solution, for the estimation and separation of starch fractions.

RESULTS AND DISCUSSION

In the estimation of the amount of branched fraction, precipitation with concanavalin A separated a portion of a number of starches. Examination of different concentrations of reactants showed that a concentration of concanavalin A of 3 mg/mL and of waxy rice starch of 250–300 μg/mL gave essentially quantitative precipitation. When the precipitation of potato tuber starch was measured at final concentrations of concanavalin A of 1, 2, 3, and 4 mg/mL, 1 mg/mL gave low amounts of precipitate (94% of the maximum value) and concentrations of 2, 3, and 4 mg/mL gave higher, similar amounts. Concentrations, after mixing, of 250–300 μg/mL of branched fractions and of 3 mg/mL of concanavalin A were adopted. Studies of time *versus* amount of precipitate indicated that reaction was complete after 2 h at room temperature. Washing of the precipitates, which were gums that adhered to the glass wall, from both potato and high-amylose pea-seed starches, if well drained, made no significant difference. Although the precipitates from waxy rice and potato tuber starches could be precipitated at a low centrifugal force

(1,000*g* × 15 min), those from other starches (*e.g.*, high-amylose pea seeds and tobacco leaves) required a high force (14,000*g* × 15 min) to produce a clear supernatant solution, so the latter conditions were adopted. Re-dissolving the precipitate in 0.2M acetic acid needed up to 1 h with occasional mixing. If 0.2M sodium acetate was added before dissolution was complete, it became difficult to dissolve the residue. If the concanavalin A was not denatured by heating, amyloglucosidase and alpha-amylase were precipitated on addition. At this stage, samples of both the original total-starch solution and the re-dissolved precipitate were hydrolysed enzymically, the glucose contents were measured colorimetrically with D-glucose oxidase, and the contents of branched fraction, calculated from the ratio of absorbances, were expressed as percentages.

Estimation of precipitated, branched α-D-glucan from starches of three different types of plant tissues (seeds, tubers, and leaves) from granules that ranged from high-amylose to waxy showed (Table I) that the method gave significantly different values and the order of the values was as expected. Two rice starches, whose amylose contents are within the normal range but differ slightly, were also differentiated. However, when compared to the apparent amylopectin content determined by subtracting the apparent amylose content, measured by potentiometric iodine titration, from 100, the values were higher and the difference increased as the apparent content of amylopectin decreased. The only exception was waxy rice starch, for which the value was lower but just significant. The complex of concanavalin A with the branched fraction may have a slight solubility (*cf.* ref. 10) or the waxy starch from the whole grain may have contained a little amylose. The difference in values between the apparent amylopectin determined by iodine titration and the branched fraction precipitated by concanavalin A was particularly noticeable for high-amylose pea-seed starch. The values for potato tuber starch were very close, suggesting that it is composed almost completely of a branched fraction of higher molecular weight (amylopectin) and an unbranched fraction of lower molecular weight (amylose), but that high-amylose pea-seed starch, and the cereal starches to a lesser degree, have a significant content of atypical material. A possible reason for the differences is that concanavalin A precipitates essentially all, or most, of the material with (1→6) branches, including any intermediate fraction, and that the iodine titration value contains a component due to intermediate material or material with an atypical structure.

Estimation of the unbranched fraction in the supernatant solution remaining after centrifugation of the concanavalin A complex was also investigated. Protein was denatured by heating and the mixture incubated with amyloglucosidase–alpha-amylase. The high-amylose pea starch gave satisfactory readings, but the low concentration of glucan in the supernatant solutions of normal starches produced final A_{max} readings at the lower end (0.3) of the desirable range, even with minimal dilution. However, values of 57 and 22% were obtained for high-amylose pea-seed and potato tuber starches, respectively.

Thus, precipitation of the branched fraction of starch, under appropriate con-

TABLE I

CONTENTS (%) OF BRANCHED FRACTION OF VARIOUS STARCHES

Method of analysis	*Source of starch*						
	Waxy rice grain	*Potato tubers*	*Rice grain (cv Caloro)*	*Rice grain (cv Dular)*	*Mature tobacco leaves*	*Wheat grain (cv Cook)*	*Wrinkled seeded (high amylose) pea seeds*
Precipitation by con A (amylopectin % ±1.5%)	97.6	77.6	79.0	75.2	76.7	72.7	41.8
Potentiometric iodine titration (amylopectin[a])	~99	76.3	77.1	71.5	72.2		24.3
Amylopectin (by con A) minus amylopectin (by iodine titration)	~ −1.4	+1.3	+1.9	+3.7	+4.5		+17.5

[a]100 − apparent amylose content.

ditions, with concanavalin A, and determination (D-glucose oxidase) of the D-glucose content of the precipitate, as well as total starch after hydrolysis with amyloglucosidase–alpha-amylase, provides a method of estimating an apparent content of amylopectin using 1–2 mg of sample. The method uses simple apparatus and procedures; it does not require a separate determination of glucose content with a glucose standard or an iodine absorption value for a sample of pure amylose, and a set of multiple analyses can be performed in one day. The value for the branched fraction determined by this method was generally higher than the value for apparent amylopectin, calculated by subtracting from 100 the apparent amylose measured by potentiometric iodine titration. This increase probably provides an estimate of the amount of atypical material.

The procedure was adapted to a preparative scale, using ~30 mg of normal or 60–70 mg of high-amylose starch. After centrifugation of the concanavalin A complex, the supernatant solution was warmed in a steam bath to denature the protein. When this was removed by centrifugation, the glucan remaining in solution was precipitated as the iodine complex, collected by centrifugation, and decolourised with arsenite, giving essentially quantitative recovery. However, if the precipitated complex of concanavalin A and branched fraction was re-dissolved in dilute acetic acid, the pH brought to 5 with sodium acetate, and protein denatured by heating, the recovery of the glucan as the iodine complex was low and unrepresentative, containing elevated levels of polymer of lower molecular size. Modification of the procedure, so that protein was denatured after treatment with methyl α-D-mannopyranoside to decompose the glucan–concanavalin A complex, gave an increased, but still incomplete, recovery: about half the expected amount was obtained. Further extraction by dispersing in 0.1M alkali and then acidifying with dilute acetic acid produced progressively decreasing amounts of glucan. The final procedure involved three extractions with alkali. A similar effect was found on attempts at preparative chromatography of starches on concanavalin A–Sepharose, when an unbound fraction with the properties of amylose was recovered in high yield, but the bound fraction, which was eluted with methyl α-D-mannopyranoside, was recovered in low amounts, even on elution with saturated aqueous methyl α-D-mannopyranoside solution. Furthermore, this eluted material was not representative of the whole amylopectin, since it contained an increased proportion of polymer of lower molecular size. This irreversible binding of part of the amylopectin was consistent with the limited life of the column (at best 5–6 runs).

Examination of the fractions not precipitated by concanavalin A of starches from potato tubers, rice grains, tobacco leaves, and high-amylose pea seeds showed that the structures corresponded to those of amylose, *i.e.*, a polymer with a range of lower molecular sizes, without or with limited branching. The elution profiles obtained by chromatography on Sepharose CL-2B of these fractions are shown in Fig. 1. The glucose content and colour with iodine of column fractions were measured. The molecular size range (mostly included), the λ_{max} values of the iodine complex (620 nm), and the high ratio of the A_{max} of the iodine complex to the

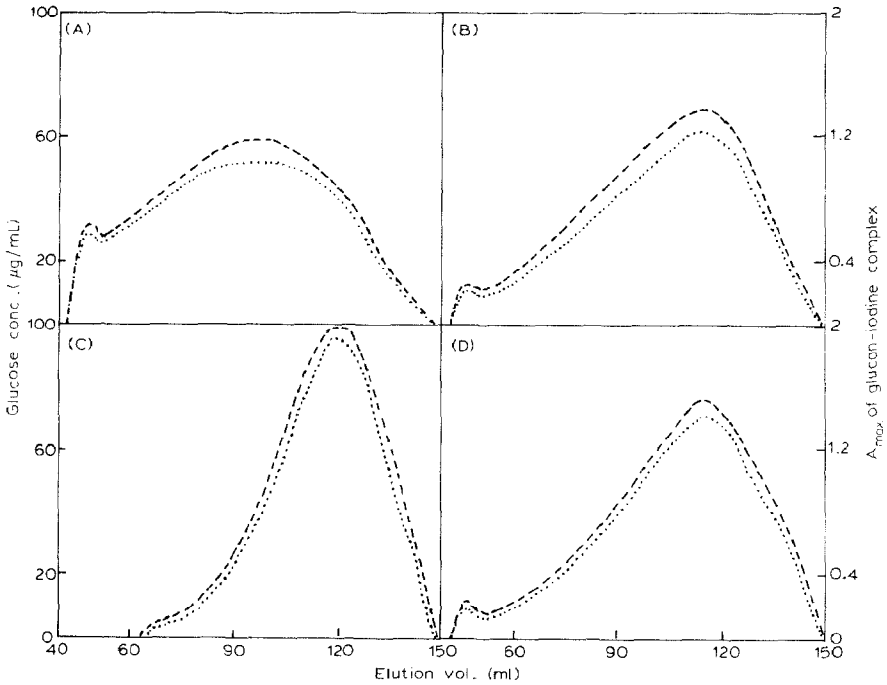

Fig. 1. Gel chromatography on Sepharose CL-2B of starch fractions soluble in concanavalin A: A, potato tubers; B, rice grains; C, tobacco leaves; D, high-amylose pea seed; -----, glucose concentration; ·····, A_{max} of glucan–iodine complex.

glucose content (~1.8) for the whole elution range were consistent with the fractions being described as amylose. Elution profiles of fractions from the starches of tobacco leaves and high-amylose pea seeds, obtained by complexing with 1-butanol, are shown in Fig. 2. In those fractions that precipitated on treatment with 1-butanol (amylose) (Figs. 2A and 2B), the presence of amylopectin is indicated by the material of high molecular size at the void volume, the λ_{max} of the iodine colour, and the ratio of A_{max} to glucose content (Table II). The results show a more effective fractionation of these starches by concanavalin A than by complexing with 1-butanol.

The soluble fraction obtained by concanavalin A from potato tuber starch was treated with the debranching enzyme, isoamylase, and the results are shown in Fig. 3A. Incubation changed the elution profile on Sepharose CL-2B to lower molecular sizes, indicating that the debranching involved amylose and not the contaminating amylopectin. That part of the elution curve of untreated polysaccharide that disappeared after enzymic reaction (from the void volume to near 85 mL) accounted for 22% of the total. The amount of increase in polymer of lower molecular size in the elution profile of the debranched material–after the crossover point (near 85 mL) of the untreated and the enzymically treated polysaccharides,

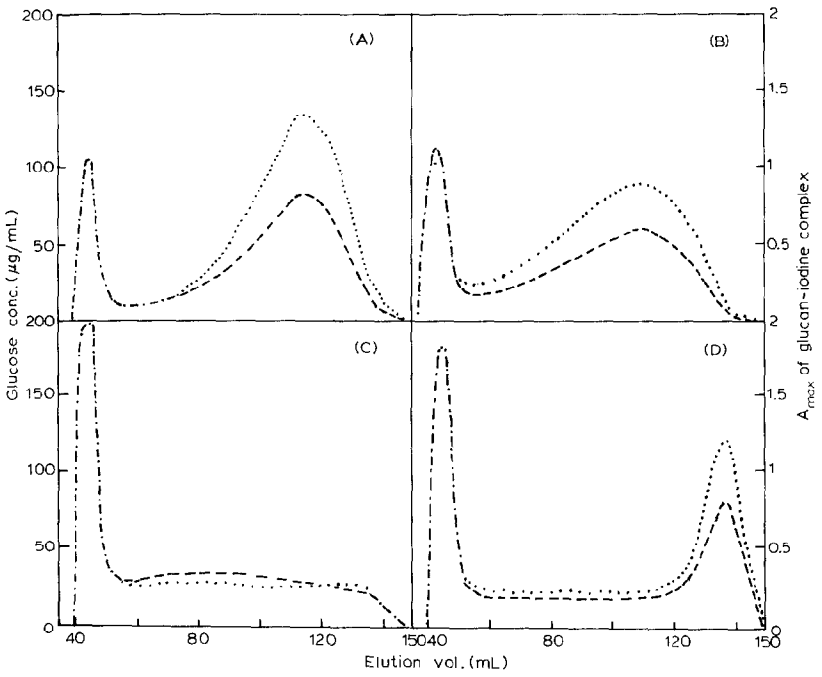

Fig. 2. Gel chromatography on Sepharose CL-2B of fractions obtained using 1-butanol: A, tobacco leaf complexed with 1-butanol; B, high-amylose pea seed complexed with 1-butanol; C, tobacco leaf soluble in 1-butanol solution; D, high-amylose pea seed soluble in 1-butanol solution; ------, glucose concentration; ·····, A_{max} of glucan–iodine complex.

and before the elution volume (near 125 mL) at which point a similar amount of debranched amylopectin to the lost soluble fraction started to elute on Sepharose CL-2B—was 21%. This similarity, 22% and 21%, indicated that the polymer of higher molecular size in the soluble fraction, that disappeared on debranching, was not amylopectin. A number of studies have described the occurrence of branching in amylose fractions prepared by complexing with compounds like 1-butanol[2]. Debranching of wheat amylose with pullulanase[14] has indicated that the branches released are long chain.

The soluble fractions prepared using concanavalin A each showed a wide range of molecular sizes with differences between the plant species. Potato tuber amylose had more material of larger molecular size than rice grain amylose which, in turn, had more than tobacco leaf amylose. The high-amylose pea-seed fraction consisted of a wide range of sizes, including polymer of low molecular size but no discretely defined sub-fraction of amylose of low molecular size.

The elution profiles of the fractions precipitated by concanavalin A (Fig. 4) had the properties of amylopectin. In the potato tuber and rice grain samples (Figs. 4A and 4B, and Table II), most of the polymer was excluded (79 and 76%, respectively), the λ_{max} of the iodine colour was at 560 nm, and the ratios of A_{max} of the

TABLE II

ELUTION PATTERNS FROM GEL CHROMATOGRAPHY OF STARCH FRACTIONS ON SEPHAROSE CL-2B

Starch source	*Fractionation procedure*	λ_{max} *(nm)*	*Mean of 100* A_{max}*/glucose conc. (μg)*	K_{av} *for amylose (maximum)*	*Amylose (%)*		*Amylopectin (%)* K_{av} *<0.2*
					K_{av} *<0.44*	K_{av} *>0.7*	
Potato tubers	Pptd. by con A	560 (540)	0.90 ±0.07				79
Rice seed		560 (540)	0.83 ±0.06				76
Tobacco leaves		560	0.90 ±0.05				45
HA Pea seeds		580	1.08 ±0.08				62
Tobacco leaves	Soluble on	560 (580)	0.90 ±0.08				45
HA Pea seeds	*n*-BuOH fractionation	560 (580)	1.22 ±0.13				37
Potato tubers	Soluble on	620 (640)	1.75 ±0.06	0.55	41	18	
Rice seeds	con A fractionation	620 (640)	1.71 ±0.09	0.67	25	28	
Tobacco leaves		620–640	1.79 ±0.11	0.73	6	54	
HA Pea seeds		620	1.82 ±0.08	0.70	22	34	
Tobacco leaves	Pptd. by *n*-BuOH	560–640	1.41 ±0.33	void and 0.68	31	24	
HA Pea seeds		580–620	1.44 ±0.20	void and 0.65	45	16	

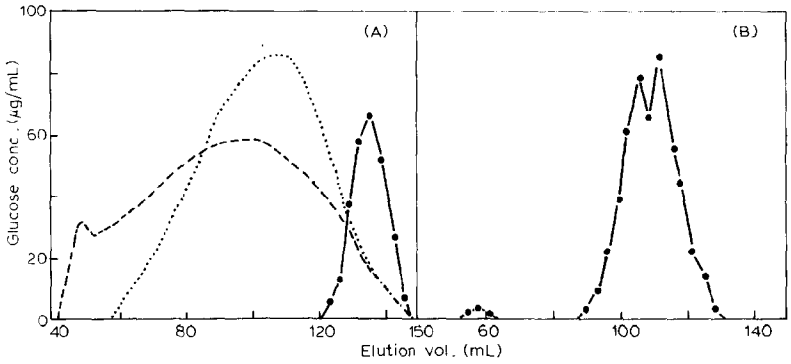

Fig. 3. Gel chromatography of starch fractions and debranched starch fractions: A, on Sepharose CL-2B; B, on Superose 6B ------, potato tuber fraction soluble in concanavalin A; ·····, debranched potato-tuber fraction soluble in concanavalin A, ——, debranched potato-tuber fraction precipitated by concanavalin A.

iodine colour to the glucose content of column fractions over the whole elution range were low (~0.8). The tobacco leaf fraction contained much more included fraction (55%), but the λ_{max} values of the iodine complex (560 nm) and the ratios of iodine colour to glucose content were low for all column fractions. The elution profile of the fraction of tobacco leaf starch not complexing with 1-butanol (Fig. 2C and Table II) indicated that it was mainly amylopectin with a small amount of amylose of lower molecular size: the ratio of A_{max} to glucose content increased in the later fractions and λ_{max} increased to 580 nm.

The elution profile of the fraction from pea seeds precipitated by concanavalin A contained 38% of polymer of lower molecular size, (Fig. 4D, Table II) and showed further differences to the other amylopectins prepared by this method. The λ_{max} of the iodine colour was higher (580 nm) and the ratios of iodine–glucan colour to the glucose content of column fractions were significantly higher (1.08). These differences were apparent over the whole range of elution, unlike the fraction not precipitated by 1-butanol (Fig. 2D, Table II) which, as has been observed previously, contained amylose of low molecular size, as judged by the λ_{max} (600–620) and A_{max} to glucose ratio (1.5). The data from concanavalin A precipitation suggest that the amylopectin fraction of the starch of high-amylose pea seeds consists of, or contains, a high proportion of a highly branched component with distinct structural characteristics that lead to an iodine absorption higher than usually found. This high absorption may contribute to the high apparent amylose content of the whole starch, as detemined by isopotential iodine absorption, providing a reason for the difference in amylopectin content determined by precipitation with concanavalin A and by isopotential iodine binding (Table I). It may also be the reason for the less definite inflection point obtained on titration with iodine[1,2].

Debranching with isoamylase of potato amylopectin prepared by precipitation with concanavalin A, followed by chromatography on Superose 6B (Fig. 3B),

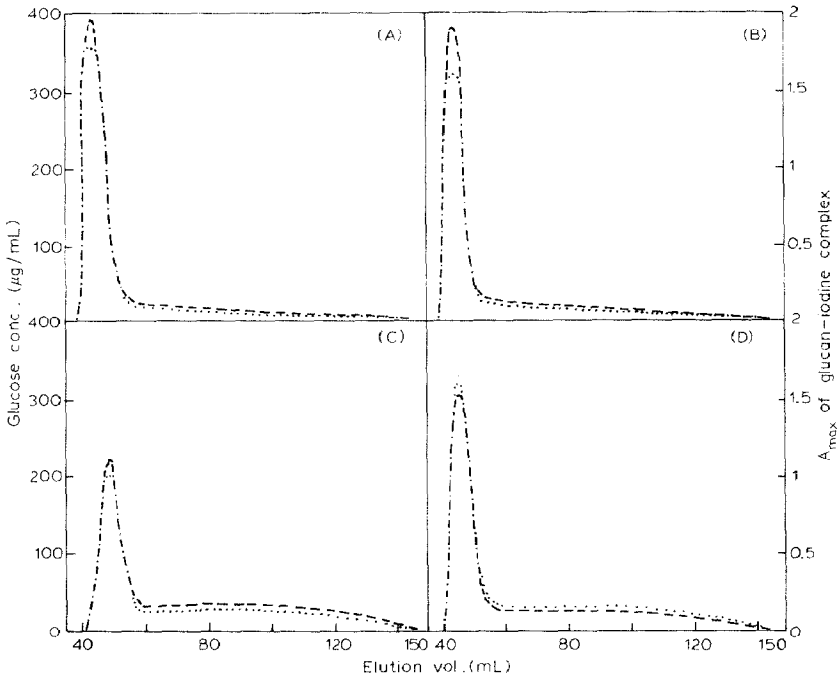

Fig. 4. Gel chromatography on Sepharose CL-2B of starch fractions precipitated by concanavalin A; A, potato tuber; B, rice grains; C, tobacco leaves; D, high-amylose pea seeds; ------, glucose concentration; ·····, A_{max} of glucan–iodine complex.

showed that only 1% of a fraction eluting at the void volume was present, indicating the fraction to be essentially pure amylopectin.

Thus, precipitation with concanavalin A provides an additional method of fractionating starches, which, applied analytically, estimates the highly branched (amylopectin) fraction. It gives a different value for high-amylose pea-seed starch than that derived from iodine titration, and this may be due to a differing structure of the whole or almost all of the amylopectin fraction to that from normal starches. Preparatively, the method can separate starches into two fractions, highly branched (amylopectin) and unbranched or lightly branched (amylose), and it can be applied to starches that are not satisfactorily separated by complexing with 1-butanol.

EXPERIMENTAL

Starch isolation and preparation. — Starches from potato tubers[15] and tobacco leaves[16,17] were isolated by short maceration in sodium chloride–mercuric chloride solution with toluene, followed by centrifugation (600*g*). Seed starches were prepared[18,19] by soaking seeds in 0.2M ammonium hydroxide for 24–48 h, and then macerating briefly in fresh ammonium hydroxide. The resulting slurry was

passed through cheese cloth and the cellular debris was re-extracted repeatedly. The combined filtrates were allowed to settle overnight and the supernatant solution was decanted. The residue was re-suspended in, and decanted twice from, 0.2M ammonium hydroxide and then water, macerated in 0.2M acetic acid, and washed with water, ethanol, acetone, and ether. The dried starch was sieved by rubbing through a 10XX silk screen. The starch granules were swelled in dimethyl sulphoxide (1 g per 10 mL) and precipitated with 3 vol. of ethanol, washed with ethanol, acetone, and ether, and dried under vacuum.

Estimation of branched and essentially unbranched components by precipitation with concanavalin A. — Starch, precipitated from dimethyl sulphoxide, was dissolved in 0.1M sodium hydroxide and the solution was neutralised to Bromothymol Blue with 0.1M hydrochloric acid. Starch solution *A* was then prepared by dilution, as a solution containing branched fraction (500–600 μg/mL) and M Na^+, mM Mn^{2+}, Ca^{2+}, and Mg^{2+} as chlorides in 0.2M sodium acetate buffer (pH 6.4). The solution for estimation of total starch (*B*) was prepared at the same starch concentration in 0.2M sodium acetate buffer (pH 5.0). Fibrous, non-starchy polymers should not be present, as these can interfere with sedimentation of the complex. Aliquots of starch solution *A* (1.50 mL) were mixed with concanavalin A (Sigma type V, C-7275) in the same solvent (1.50 mL) containing 6 mg of protein/mL, in a 15-mL Corex glass tube and stored for 2 h. The mixture was centrifuged (14,000*g*, 15 min, 20°), the supernatant solution was discarded, the tube was drained for 30 min, and the insides of the upper section of the tubes were dried with tissue. The residue was dissolved in 0.2M acetic acid (2.0 mL). This required storage, with occasional shaking, for up to 1 h. 0.2M Sodium acetate (3.0 mL) was added and protein was denatured by warming in a steam bath. Solution *B* (1.50 mL) was mixed with 0.2M sodium acetate buffer (3.50 mL, pH 5.0). Both sets of tubes were incubated for 2 h at 37° with amyloglucosidase–alpha-amylase solution (1.0 mL) [amyloglucosidase (250 U) and porcine pancreatic alpha-amylase (50 U) in 0.2M acetate buffer (pH 5.0) to a final volume of 15 mL]. The tubes were centrifuged (3,000*g* × 15 min) and a portion of the supernatant solution was suitably diluted for estimation of D-glucose with D-glucose oxidase. The single reagent incorporating 3-hydroxybenzoic acid was used[20], except that absorbance was read at 490 nm.

To estimate the glucan content of the fraction soluble in concanavalin A, the supernatant solutions from centrifugation were poured into volumetric flasks (10 mL for high-amylose pea seeds, 5 mL for normal starches), and each precipitate was washed (2 × 1 mL and 2 × 0.4 mL, respectively) with concanavalin A reagent. The pH was reduced to 5 by the addition of the calculated amount of 2M acetic acid and protein was denatured by warming in a steam bath. Amyloglucosidase–alpha-amylase reagent (1.0 mL) was added to the cooled flask, the volume was made up with 0.2M acetate buffer (pH 5), and the released D-glucose was estimated as previously described.

Preparative fractionation of starches with concanavalin A, and chromatog-

raphy. — Starch (25 to 30 mg ÷ amylopectin proportion) was dispersed in dimethyl sulphoxide (0.5–1.0 mL), diluted to 50 mL in M Na^+, mM Mn^{2+}, Ca^{2+}, and Mg^{2+} as chlorides in 0.2M sodium acetate (pH 6.4), and mixed with concanavalin A (300 mg) in the same solvent (50 mL). After 2 h, the mixture was centrifuged (14,000*g*, 15 min, 20°). The supernatant solution was mixed with disodium EDTA (1 mL saturated at 37°) and warmed in a steam bath to precipitate protein. After centrifugation (14,000*g*, 15 min, 20°), 2M acetic acid (5.0 mL) and then 2% iodine in 20% potassium iodide (10.0 mL) were added and the mixture was stored at 4°. As soon as the iodine–glucan complex had precipitated, the mixture was centrifuged (14,000*g*, 15 min, 4°) and the supernatant solution was discarded. The residue was decolourized and dissolved by mixing with 0.1M sodium arsenite in 0.2M phosphate buffer (pH 6, 0.4 mL) and an appropriate volume of 0.1M sodium chloride. 2.5M Potassium hydroxide (0.2 mL) was added to an aliquot (2.0 mL), and a portion (2.0 mL), containing ~4 mg of glucan, was chromatographed on a column (1.6 cm × 75 cm) of Sepharose CL-2B with 0.25M potassium hydroxide as solvent, and collecting 45–48 tubes containing slightly more than 3 mL. The α-D-glucan–iodine colour of each tube was assayed by mixing an aliquot (2.0 mL) with 0.08% iodine and 0.8% potassium iodide in 0.5M sulphuric acid (1.20 mL), and the absorptions were read at 580, 600, 620, 640, and 660 nm. Aliquots of tube fractions were also taken for estimation of glucose equivalents with phenol–sulphuric acid (the phenol solution was prepared in 0.2M sulphuric acid).

The residue from the first centrifugation was drained for 30 min, a solution of methyl α-D-mannopyranoside [3.2 g in 40 mL of 0.2M acetate buffer (pH 5.8)] was added, and the mixture was kept (with occasional shaking) until all the precipitate had dissolved. A solution of disodium EDTA (saturated at 37°, 1 mL) was added, and the mixture was boiled and then cooled quickly. After centrifugation (14,000*g*, 15 min, 20°), the supernatant solution was collected, and the residue was completely dispersed in 0.1M potassium hydroxide (25 mL) and then acidified with 2M acetic acid (2.0 mL). After centrifugation (14,000*g*, 15 min, 20°), the supernatant solution was combined with the first supernatant solution. This alkaline extraction process was repeated twice. 2% Iodine in 20% potassium iodide (10 mL) was added to the combined supernatant solutions and the mixture was stored at 4° until the α-D-glucan–iodine complex precipitated. The supernatant solution from centrifugation (14,000*g*, 15 min, 4°) was discarded and the residue was decolourized with 0.1M sodium arsenite in 0.2M phosphate buffer (pH 6, 1 mL) and a suitable volume of 0.1M sodium chloride for later experiments. An aliquot (2.0 mL) was chromatographed on Sepharose CL-2B by the same procedure as that used for the fraction soluble in concanavalin A.

Debranching and chromatography of fractions. — An aliquot (1.8 mL), containing ~4 mg of glucan, was mixed with 2M acetate buffer (pH 4.8, 0.20 mL) and isoamylase (Hayashibara) from *Pseudomonas amyloderamosa* (1200 units)[21] and incubated overnight at 37°. 2.5M Potassium hydroxide (0.20 mL) was added and an aliquot (2.0 mL) was chromatographed on Sepharose CL-2B (for amylose) or Superose 6B (for amylopectin).

Fractionation of starches with 1-butanol. — Starch (300 mg), which had previously been treated with dimethyl sulphoxide and precipitated with ethanol, was dissolved by heating in a steam bath in 0.1M sodium chloride (45 mL), the solution was cooled to 60°, and 1-butanol (2.5 mL) was added. After 48 h, the mixture was centrifuged (14,000*g*, 15 min, 20°). The residue was re-dissolved in 0.1M sodium chloride (30 mL), and 1-butanol (1.7 mL) was added at 60°. After 48 h, the mixture was centrifuged, and the supernatant solution was combined with the first supernatant solution and precipitated with 3 vol. of ethanol. After centrifuging, washing with ethanol, acetone, and ether, and drying, the precipitate was dissolved in 0.25M potassium hydroxide prior to chromatography. The complex with 1-butanol was also dissolved in alkali prior to chromatography.

Potentiometric iodine titration. — The method of Bates *et al.*[22] was modified[11]. Starch solutions were prepared by dispersing, in dimethyl sulphoxide with heating, the polysaccharide that had been pre-treated with dimethyl sulphoxide. The glucan content was determined by hydrolysis with amyloglucosidase and alpha-amylase combined with the D-glucose oxidase reaction, as described above, on suitably diluted aliquots of the same starch solution. Apparent amylose content was calculated by assuming[2] that amylose absorbs 19.2 mg of iodine per 100 mg.

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