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SEPARATION OF THE COMPONENTS OF STARCH BASED ON THE ADSORPTION OF THE 1-BUTANOL–AMYLOSE COMPLEX DURING COLUMN CHROMATOGRAPHY ON CELLULOSE

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ABSTRACT

Amylose and amylopectin from defatted, dispersed starch can be separated quantitatively by column chromatography on cellulose, using 0.1M acetate buffer (pH 4.8) containing 8.5% of 1-butanol and 2M urea at 30°. The amylose–1-butanol complex is adsorbed and the amylopectin–1-butanol complex is eluted. The amylose is then eluted by decreasing the concentration of 1-butanol in the eluent. The asymmetric elution profile of amylose indicates heterogeneity as judged by the blue values, beta-amylolysis limits, and the action of pullulanase on the various fractions.

INTRODUCTION

In plants, the polysaccharide deposited in starch granules normally consists[1] of a mixture of 20–30% of amylose and 70–80% of amylopectin. The amylose is heterogeneous with respect to molecular weight (1.5×10^5–2.4×10^6) and the amylopectin is heterogeneous with respect to the degree of branching and molecular weight (1×10^6–6×10^7). Debranching enzymes, such as pullulanase and isoamylase, convert amylopectin into a mixture of long (d.p. 40–60) and short (d.p. 11–25) malto-oligosaccharides. In the cluster model[2], the shorter chains are arranged in tightly packed groups attached to a basic structure composed of long chains. This model accounts for the bimodal nature of the chains, the high crystallinity of native amylopectin, and the high viscosity of solutions of amylopectin. There is some evidence for the presence in starch granules of 4–9% of an intermediate fraction[3] which consists of amylopectin with a lower degree of branching, and some of the amylose may be slightly branched with (1→6) linkages that are resistant to beta-amylase (EC 3.2.1.2) but are hydrolysed by pullulanase (EC 3.2.1.41)[4]. The branched amylose molecules of different origins have characteristic molecular size, inner chain-length, and number of chains[5]. The molar fractions of unbranched and branched molecules vary with the origin of the amylose.

The separation of starch components is based chiefly on differences in solubility[6] and preferential adsorption of the components or their complexes. Rice

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starch has been fractionated[7] by gradient ultracentrifugation with a linear gradient of CsCl followed by gel filtration on Sepharose-2B. Normal maize starch containing ~23% of amylose showed[8] a biphasic elution profile on Sephacryl S-1000, but the amylose could not be separated clearly from the amylopectin. Amylose and amylopectin from *Amaranthus hypochandriacus* have been separated partially[9] on Sephacryl S-1000. Gel-permeation chromatography[10] on Toyopearl HW-75F is a sensitive method for determining the purity of amylose samples from different sources. High-performance size-exclusion chromatography (h.p.s.e.c.) has been used[11] for the rapid (<20 min) separation of starch components, using a two-column system and methyl sulphoxide as the mobile phase. This method is useful for monitoring the purity of preparations of amylose and amylopectin.

Adsorption[12] and affinity[13] chromatography can be used to separate the components of potato starch. Further, the amylose component is heterogeneous, as judged from the asymmetric elution profile, blue value, and beta-amylolysis limit. We now describe the use of cellulose column chromatography for the quantitative separation of starch components from defatted starch based on the specific adsorption of the amylose–1-butanol complex.

EXPERIMENTAL

Defatting of potato starch granules and cellulose powder. — A suspension of cellulose powder (100 g) in 2:1 chloroform–methanol (300 mL) was boiled under reflux for 1 h with intermittent stirring, then filtered hot. The insoluble material was washed 2–3 times with hot 2:1 chloroform–methanol followed by aqueous 95% ethanol, then air-dried at 26–28°. Starch granules were defatted in the same way.

Dispersion of starch granules. — The defatted starch granules (200 mg) were dispersed by treatment with M sodium hydroxide (5 mL) with intermittent stirring at 26–28° under nitrogen for 16–18 h followed by neutralisation with M HCl to pH 7.0 and centrifugation (2418*g*).

Determination of blue value. — The method of McCready and Hassid[14] was used. The absorbance was determined of solutions containing polysaccharide (0.1 mg), reagent (1 mL; 7.87×10^{-4}M iodine + 1.21×10^{-2}M KI), and 0.1M acetate buffer (pH 4.8, 1 mL) diluted with distilled water to 10 mL. The mixture was stored for 30 min at 30° in the dark and the absorbance then recorded at 680 nm in a 1.0-cm cuvette with a Beckman DU2 spectrophotometer. The readings were multiplied by 4 since the values reported[14] were obtained with a path length of 4.0 cm.

Beta-amylolysis limit. — The system used involved aqueous solutions of polysaccharide (0.1 mg in 1.0 mL), beta-amylase (Sigma, 20 U in 0.2 mL), 0.1M acetate buffer (pH 4.8, 0.5 mL), and water to give a total volume of 2.0 mL. The mixture was incubated for 16–18 h at 37 ±0.5°, and the maltose liberated was determined by the method of Nelson[15].

Action of pullulanase and beta-amylase. — A mixture of pullulanase (Nakarai

Chemicals, Japan) solution (1 mL, 1 U/mL), polysaccharide (0.1 mg in 1 mL of water), and 0.02M citrate–phosphate buffer (pH 5.0, 3 mL) was incubated for 30 min at 30° and then treated with beta-amylase (20 units in 0.2 mL) for 16–18 h, and the reducing sugar was determined by Nelson's method[15].

Column chromatography. — Defatted cellulose powder was stirred with solvent *A* [0.1M acetate buffer (pH 4.8) containing 8.5% (v/v) of 1-butanol and 2M urea] for 3–4 h at 26–28° and then allowed to settle under gravity, the supernatant suspension was removed by decantation, the cellulose slurry was added to a jacketed glass column (1.8 × 35 cm) in small lots, and uniform packing was obtained under gravity. There was a layer (1 cm) of acid-washed sand at the top and bottom of the column. The column was equilibrated with solvent *A* at 30°. The solutions were introduced onto the top of the column at 30 mL/h and circulated by a peristaltic pump at the bottom.

A mixture of freshly neutralised, aqueous 2% starch (10 mL after centrifugation at 2418*g*), solvent *B* [10 mL; 0.2M acetate buffer (pH 4.8) containing 8.5% (v/v) of 1-butanol and 4M urea], and 1-butanol (0.85 mL) was loaded on the column. In order to obtain complete retention of the amylose–1-butanol complex, it was necessary to recycle the solution through the column three times. The solution then emerging from the column contained only amylopectin. The column was then washed with solvent *A* [2 × bed volume (70 mL)]. The adsorbed amylose was eluted with 0.1M acetate buffer (pH 4.8) containing 2M urea by decreasing the concentration of 1-butanol, using a constant volume gradient unit[12] containing 100 mL of solvent *A* in the mixing chamber. Fractions (5.0 mL) were monitored for polysaccharide by the thymol–$FeCl_3$–HCl method[16]. The blue value[14] and beta-amylolysis limit[15] were determined for the polysaccharide in each fraction. The column was regenerated by elution with 4–5 bed volumes of solvent *A* and could be used 3–4 times.

RESULTS AND DISCUSSION

1-Butanol, 1-pentanol, cyclohexanol, and sodium dodecyl sulphate (SDS) form helical complexes with amylose, with a characteristic "V" type X-ray diffraction pattern[17]. The amylose chain is sufficiently flexible to undergo conformational changes in aqueous solution, and the helical complexes prevent retrogradation. Urea helps to stabilise the helical conformation of amylose in solution[18].

Hydrodynamic studies[19,20] have shown that amylose exists in solution as flexible chains that can form helical coils and entrap ligands. The helical domains contain 110–130 D-glucose residues and are interspersed by regions of random coils which contribute to the flexibility of the polymer chain[21]. The amylose–1-butanol complex retained on the cellulose is probably in a helical form, the adsorption is a slow process, and re-cycling is required to complete the process, although the cellulose is in large excess and the adsorption is reversible. The amylopectin obtained in the eluate was essentially free from amylose as judged from the blue

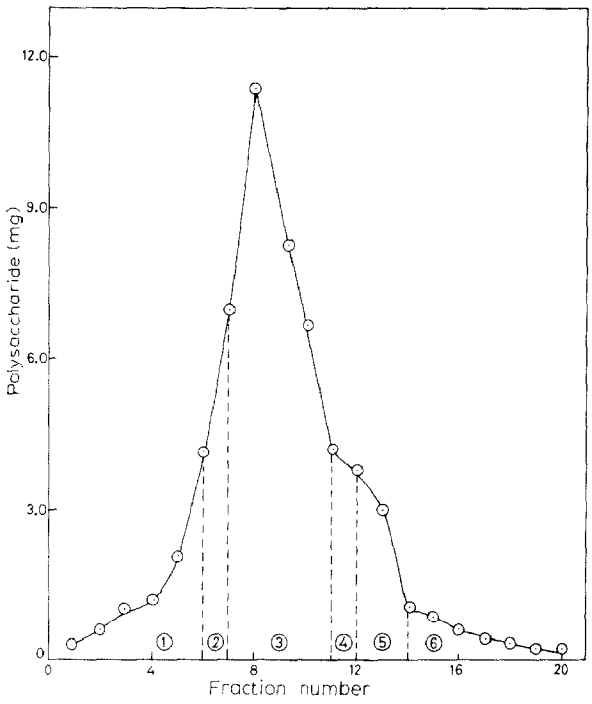

Fig. 1. Elution of amylose–1-butanol complexes from potato starch. The elution was obtained by decreasing the concentration of 1-butanol by diluting 100 mL of solvent *A* in the mixing chamber[12] with acetate buffer (pH 4.8, 0.1M) containing 2M urea.

value (0.16–0.17) and beta-amylolysis limit (48–50%). The amylose–1-butanol complex could be eluted with a gradient of decreasing 1-butanol concentration (Fig. 1). The content of each fraction was characterised by its blue value[14] and beta-amylolysis limit[15], and the results are given in Table I. The elution profile reveals that the amylose is heterogeneous. The peak fraction (3) in Fig. 1 contained

TABLE I

CHARACTERISATION OF AMYLOSE FROM POTATO STARCH

Fraction	*Blue value*	*Beta-amylolysis limit*	*Percent of the total amylose*[a]
1	1.24	90.0	2.5
2	1.28	92.0	2.5
3	1.35	98.0	11.0
4	1.30	95.0	1.5
5	1.27	92.0	1.5
6	1.25	90.0	1.0

[a]The total recovery was 96–98%.

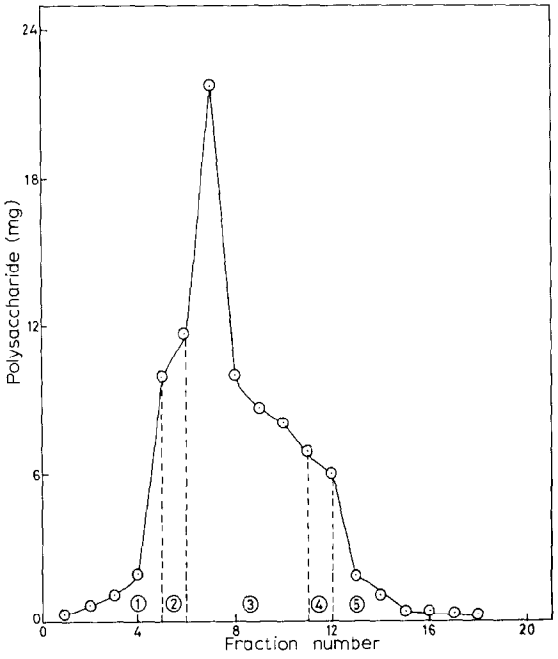

Fig. 2. Elution (see legend for Fig. 1) of amylose–1-butanol complexes from the intermediate fraction obtained[22] in the preparation of amylose and amylopectin from potato starch.

essentially linear amylose of high molecular weight [blue value 1.35; beta-amylolysis limit 98% (unaffeced by treatment with pullulanase)]. The material in the adjacent fractions (1, 2, 4–6) on treatment with pullulanase (EC 3.2.1.41) had enhanced beta-amylolysis limits of 98 ±1% (mean of 5 replicates), indicating the presence of (1→6) linkages.

Chromatography of the intermediate fraction. — The elution profile (solvent *A*) of the intermediate fraction obtained in the preparation of amylose and

TABLE II

CHARACTERISATION OF AMYLOSE FROM THE INTERMEDIATE FRACTION

Fraction	*Blue value*	*Beta-amylolysis limit*	*Percent of the total amylose*[a]
1	1.24	90.0	3.0
2	1.31	95.0	2.4
3	1.35	98.0	10.0
4	1.30	94.0	2.6
5	1.27	91.0	2.0

[a]The total recovery was 96–98%.

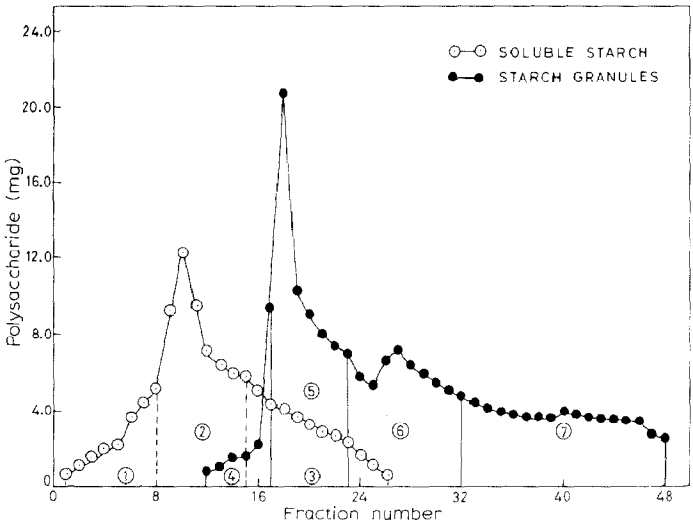

Fig. 3. Elution (see legend for Fig. 1) of potato amylose fraction from soluble starch (200 mg) and amylose (200 mg) from starch granules after adsorption of the complexes with 1-butanol on the cellulose column.

amylopectin from potato starch[22], illustrated in Fig. 2 and Table II, is similar to that (Fig. 1) of amylose from potato starch. These results indicate that the intermediate fraction is a mixture of amylose and amylopectin, which is loosely attached to cellulose.

Chromatography of soluble starch. — The experiments with soluble starch (potato) showed that, in the presence of 1-butanol, the partially degraded amylose was retained on the cellulose column, and that the partially degraded amylopectin and low-molecular-weight amylose were not adsorbed. The amylose–1-butanol complex from soluble starch was eluted before that from potato starch (Fig. 3). The characterisation of the fractions is given in Table III.

TABLE III

CHARACTERISATION OF AMYLOSE FROM SOLUBLE STARCH AND STARCH GRANULES

Fraction	*Blue value*	*Beta-amylolysis limit*
1	1.22	87.0
2	1.30	95.0
3	1.24	89.0
4	1.28	91.0
5	1.35	98.0
6	1.30	95.0
7	1.27	90.0

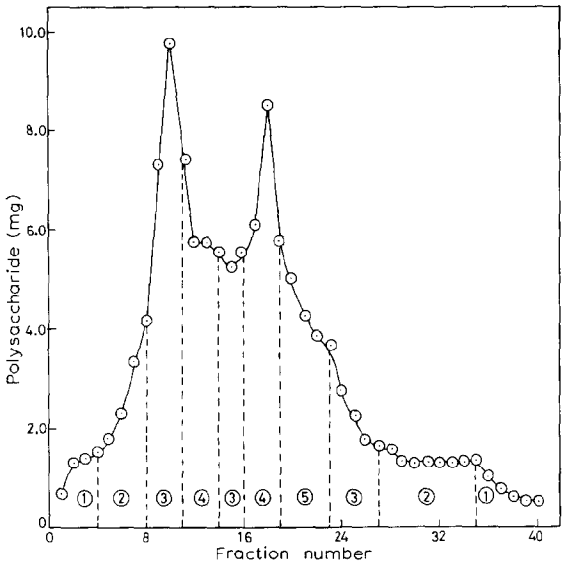

Fig. 4. Elution (see legend for Fig. 1) of potato amylose from a mixture of soluble starch (200 mg) + starch granules (500 mg) after adsorption of the complexes with 1-butanol on the cellulose column.

Elution (solvent *A*) of a mixture of soluble starch (potato) and potato starch gave two peaks (Fig. 4, Table IV) for amyloses of low and high molecular weight. A similar result was obtained with a mixture of soluble starch and potato amylose.

Treatment of potato amylopectin with pullulanase (EC 3.2.1.41) gave malto-oligosaccharides of d.p. 20–25, the 1-butanol complexes of which in solvent *A* were not retained on the column.

The results with 1-pentanol were similar to those with 1-butanol, but the amylose–cyclohexanol complex precipitated on the column. The use of sodium dodecyl sulphate (SDS) gave good separation, but it was very difficult to remove traces of SDS from amylose fractions. SDS also interfered in the determination of blue values, but the beta-amylolysis limits were not affected.

The cellulose column could adsorb the amylose–1-butanol complex almost

TABLE IV

CHARACTERISATION OF AMYLOSE FROM SOLUBLE STARCH + STARCH GRANULES

Fraction	*Blue value*	*Beta-amylolysis limit*
1	1.20	87.0
2	1.26	90.0
3	1.32	93.0
4	1.35	97.0
5	1.33	95.0

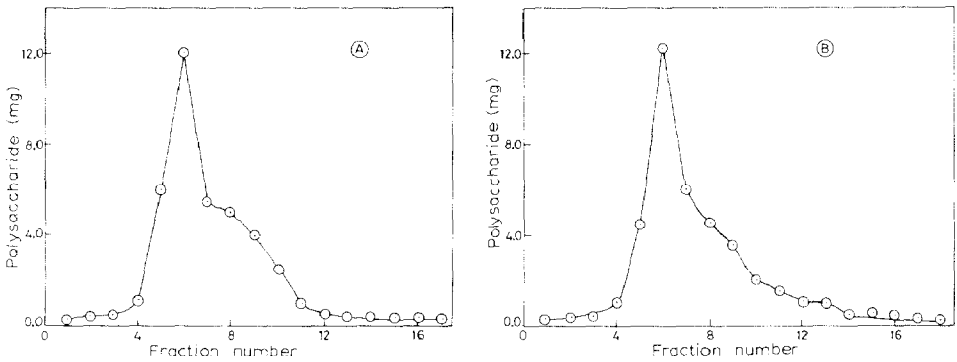

Fig. 5. Elution from cellulose column of the amylose from A, corn starch; B, tapioca starch.

quantitatively from dispersed starch solution. The elution profiles for potato amylose were asymmetrical, and the blue values and beta-amylolysis limits of the material eluted indicated the heterogeneity of native amylose. These results accord with observations on affinity chromatography of starch components[12]. The observations on the presence of branched amylose also accord with reported results[23].

Starch components can be separated rapidly[9] by h.p.s.e.c. The method described here can be modified for the rapid separation of starch components by using a single column (1.8 × 5.0 cm). The dispersed starch solution (20 mg) in solvent *A* can be recycled rapidly and the amylopectin can be separated. The

TABLE V

ADSORPTION OF AMYLOSE–1-BUTANOL COMPLEXES ON CELLULOSE

Cellulose[a]	*Amylose adsorbed (g/100 g of cellulose)*	*Amorphous regions (%)*
Cellulose (wood) (Selecta)	2.5	25.0
Whatman xt-1	2.0	20.0
Whatman xt-78	1.7	17.0
DEAE Sephacel	2.7	27.0
Carboxymethyl cellulose (Whatman CM-23)	2.87	28.7
Cotton (absorbant) Commercial	0.81	8.1
Cellulose fibers (*G. hirsutum*)	0.47	4.7
Cellulose fibers (*B. mulbericum*)	0.15	1.5
Microcrystalline	0.37	3.7
Microcrystalline cellulose (Whatman CC-31)	0.39	3.9

[a]Each sample was defatted; (100 − % amorphous region = % crystallinity).

adsorbed amylose can be eluted with 2M urea in 0.1M acetate buffer (pH 4.8) in a single step.

Amyloses from corn and tapioca starch have been examined (Fig. 5) and shown to contain 50–60% of an essentially linear fraction together with material containing some (1→6) linkages that are hydrolysed by pullulanase (EC 3.2.1.41).

The adsorption of the amylose–1-butanol complex on native cellulose, modified celluloses, and cellulose derivatives has been studied (Table V), using solvent *A* and a batchwise adsorption and elution technique. The complex was adsorbed on cellulose but not on its microcrystalline form, suggesting that the amorphous regions in cellulose have more affinity for the complex. This method can be used to characterise cellulose products for the crystalline and amorphous domains usually studied by X-ray diffraction[24]. Amylose has no affinity for cellulose.

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