Plant Physiol. (1979) 64, 833–836
0032-0889/79/64/0833/04/$00.50/0

Isolation and Purification of Leaf Starch Components

Received for publication April 23, 1979 and in revised form June 15, 1979

CHONG W. CHANG
United States Department of Agriculture, Science and Education Administration, Agricultural Research, Western Cotton Research Laboratory, 4135 E. Broadway Road, Phoenix, Arizona 85040

ABSTRACT

A procedure was developed for the separation and purification of amylose and amylopectin isolated from cotton leaves. Cotton leaves were homogenized in 0.02 molar phosphate buffer at pH 7.0 containing $HgCl_2$ plus toluene. Crude starch granules were collected by centrifugation and partially purified by treating with acetone and toluene. The starch granules were then dispersed in dimethylsulfoxide and precipitated with ethyl alcohol. The precipitate was suspended in boiling water. Amylose was separated from amylopectin and cell wall particles on a Sepharose 2B column and further purified with thymol and butanol. Amylopectin was then separated from the colloidal cell wall contaminants by its specific interaction with concanavalin A. Purities of starch components were verified by specific biochemical and enzymic tests in addition to their iodine-binding capacity. This procedure should also be suitable for purification of starch components from other plant sources.

In the purification of starch from either seeds or leaves for physicochemical studies, no specific processes have been employed to remove cell wall contaminants (1, 3, 11, 12, 15, 22, 24). In these procedures dispersed starch has been treated with various precipitants such as butanol (24), butanol plus pentasol 27 (Pennsalt Chemical Corp.)[1] (9, 15), thymol (13), and others (14, 28). The complex of amylose plus precipitant is then directly centrifuged to achieve a separation from amylopectin. The butanol and thymol procedures (13, 24) were tested with cotton leaves. Unfortunately, the complex of amylose and precipitant was heavily contaminated with cell wall fragments.

Banks *et al.* (3) used nylon mesh to screen cell wall debris from seed homogenate. A similar technique was used by Adkins and Greenwood (1), but the fate of contaminants smaller than 75 μm was unknown. Matheson and Wheatley (22) tried to purify starch granules from tobacco leaves by the procedures of Porter and Martin (26). They subjected an aqueous suspension of crude starch granules to gravity sedimentation. The sediments, when dispersed in boiling water, were visibly turbid due to cell wall contaminants.

Dispersed seed starch was fractionated into amylose and amylopectin on Sepharose 2B (21) or 4B (8). The former gel filtration, however, failed to exclude cell wall colloids from the amylopectin fraction when tested with a cotton leaf crude starch preparation.

Con A,[2] a plant metalloprotein (phytohemagglutinin), interacts specifically with branched chain polysaccharides to form an insoluble complex (16). The use of this lectin for purification of leaf starch granules rich in cell wall contaminants has not been reported.

This communication reports a method for purification of starch components from cotton leaves that avoids the use of reagents likely to cause physicochemical alteration. The method uses Sepharose 2B and thymol to separate amylose from amylopectin and associated cell wall contaminants. The latter starch component can be purified by its specific affinity for the metalloprotein, Con A. The isolation of purer glucans is necessary for more accurate determinations of their physicochemical properties.

MATERIALS AND METHODS

Isolation of Crude Starch Granules. About 20 g of fresh cotton (*Gossypium hirsutum* L.) leaves were mixed with 78 ml of cold (4 C) 0.02 M Na-phosphate buffer (pH 7.0), containing 0.01 M $HgCl_2$ and 13 ml of toluene. The mixture was homogenized for 3 min at ice bath temperature with a Sorvall Omni-Mixer (35% full speed). The homogenate was then filtered through two layers of cheesecloth and centrifuged at 1,085*g* for 3 min. The supernatant, including the surface layer of protein, green pigments, and lipids, was discarded. The surface of the starch residue was rinsed with cold water; the starch pellet was then washed twice by resuspension in about 25 ml of cold water and centrifugation at 1,085*g* for 3 min. The pellet was extracted with acetone three times, resuspended in 10 ml of cold water, and then shaken with an equal volume of toluene for 3 min. The mixture was centrifuged at 1,085*g* for 3 min and the top toluene-protein layer was subjected to a stream of N_2 gas to release the trapped starch granules in toluene. This layer was then removed with a pipette and discarded (decantation caused contamination of the starch residue with denatured protein). The process was repeated four times or until the toluene layer no longer became turbid. The starch granules were again washed twice with cold water and stored under water-toluene in the cold (4 C) until required.

Pretreatment and Dispersion of Starch. The crude starch suspension was centrifuged and the weight of the wet starch was recorded. This material was dissolved in 99.8% DMSO (0.6 g wet weight/10 ml) with stirring at about 70 C (9). After 10 min the solution was centrifuged at 1,085*g* for 20 min. The supernatant fraction was removed with a pipette without disturbing the pellets of the macrocell wall fragments at the bottom of the tubes (decantation of the supernatant revealed that no starch residue was visibly sedimented with the cell wall fragments). The supernatant was poured into 3 volumes of ethanol. The resulting starch precipitate with associated colloidal cell wall contaminants was washed thoroughly with ethanol to remove all traces of DMSO (4).

A thin slurry of ethanol-moistened starch was prepared with 50% ethanol and added to a container of vigorously boiling water under N_2 atmosphere. The water was previously bubbled with dry N_2 gas (99.9% min volume per cent in purity) for 20 min. The suspension was boiled for 10 min, when about 0.5 the initial volume remained. After cooling, the crude starch solution was

[1] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable.

[2] Abbreviations: Con A: concanavalin A; DMSO; dimethylsulfoxide; MMP: Methyl alpha-D-mannopyranoside.

diluted to 1 to 2% (w/v) with warm water and centrifuged at room temperature for 20 min at 1,085*g*. The supernatant was removed with a pipette without disturbing the sedimented cell wall particles, which were discarded. (Glucan concentrations of the samples loaded on the column in all subsequent experiments were estimated by *A* at 660 or 560 nm after treatment of separate aliquots with iodine-potassium iodide solution.)

Separation and Purification of Amylose and Amylopectin. A sample of crude dispersed starch solution was adjusted to 0.1 M NaCl and to an *A* of about 50 A_{660} units. A sample (8 ml) was applied to a column of Sepharose 2B (3 × 30 cm) as described previously (10). The column was eluted with 0.1 M NaCl and fractions 31 to 42 were pooled (Fig. 1). They contained amylopectin, cell wall fragments in a colloidal state, and possibly a trace amount of amylose. Fractions between the 50th and 95th tube were also pooled. This fraction contained amylose contaminated with a trace amount of amylopectin.

The solution of impure amylose from the Sepharose column was lyophilized and dispersed in a small volume of water as described under "Pretreatment and Dispersion of Starch." The concentration of amylose was adjusted with water to about 50 A_{660} units. A sample (6 ml) was applied to a column of Sephadex G-50 (2.5 × 25 cm) and eluted with distilled H_2O. Fractions between the 14th and 25th tube were pooled. They contained amylose with a minor contaminant of amylopectin in a salt-free medium. Fractions (between the 30th and 50th tube) which contained salts were discarded. Salts were identified as previously described (10). The concentration of the polymers was adjusted to about 1%. Powdered thymol was added to the sample solution to a final concentration of 0.13% (w/v) and the mixture was incubated at room temperature for 3 days (2). The mixture was then centrifuged at 17,300*g* for 10 min; the supernatant, which usually contained a trace amount of amylopectin contaminants, was discarded. The insoluble amylose-thymol complex was repeatedly suspended in absolute ethanol with stirring and centrifuged. The supernatant containing thymol was discarded. The residue was dispersed in water and a small aliquot of the sample was removed for a purity test. Excess butanol was added to the remainder. After 24 h the amylose-butanol complex was removed. The purification process was repeated twice or until the A_{660} in the reaction with iodine-potassium iodide solution reached a constant maximum (23).

The Sepharose 2B fractions (tubes 31–42 in Fig. 1) containing amylopectin and cell wall contaminants were lyophilized and dispersed. The mixture was cleared by centrifugation at 1,085*g* for 10 min and the supernatant was removed with a pipette without disturbing the minute sedimented pellet. After adjustment to 0.9% (w/v) NaCl, the sample was applied to a column of Con A-Sepharose 4B (an *A* of about 5.5 A_{560} units/ml of gel column material). The column was washed with 0.9% NaCl in 0.02 M Na-phosphate buffer at pH 7.2 until amylose and cell wall particles were completely eluted. Trace amounts of these contaminants were found in the earliest fractions following the void volume (amylopectin was bound to the column). The column was then washed with 0.1 M MMP to desorb the amylopectin. The fractions (21–33) containing amylopectin were identified by the *A* at 560 nm and pooled (Fig. 2). The amylopectin-MMP mixture was

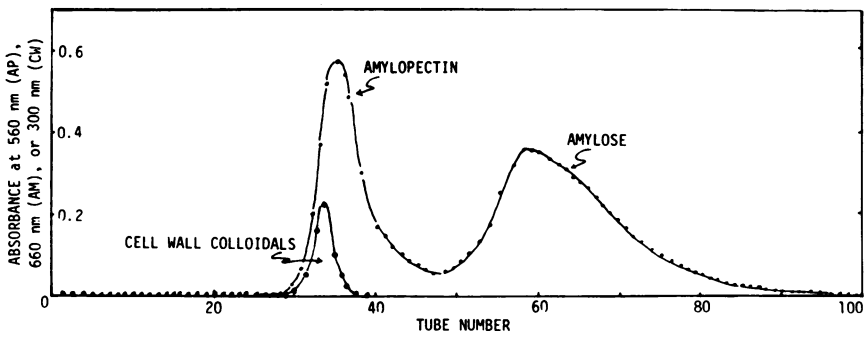

FIG. 1. Fractionation of a mixture of amylose (AM), amylopectin (AP), and cell wall contaminants (CW) from a column (3 × 30 cm) of Sepharose 2B. Eight ml of the dispersed crude starch, containing about 50 A_{660} units with the iodine-potassium iodide test, was loaded after adjustment to 0.1 M with NaCl. The column was eluted with a warm (about 32 C) solution containing 0.1 M NaCl and 0.02 M NaN_3. Flow rate was 0.9 ml/min; each fraction contained 1.8 ml. Amylose or amylopectin in each tube was identified by the procedure of Kovacs and Hill (17). Cell wall contaminants were identified by the *A* at 300 nm for turbidity.

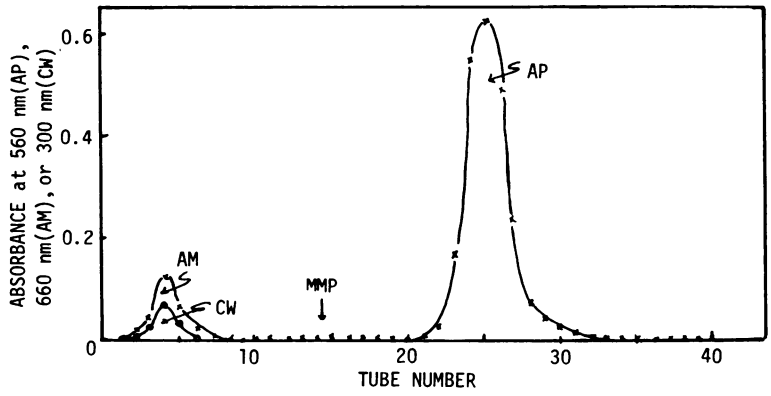

FIG. 2. Separation of a mixture containing amylose (AM), amylopectin (AP), and cell wall colloids (CW) by use of a column of Con A-Sepharose. Two (or 20) ml of the sample, with about 35 A_{560} units by the iodine-potassium iodide test, was loaded on a column of 1.2 × 12 (or 2.2 × 22) cm. The column was eluted to tube 15 with 0.9% NaCl in 0.02 M Na-phosphate buffer and was then eluted with MMP. The column was eluted at room temperature, the flow rate was 0.32 ml/min, and each fraction contained 2.5 ml. Glucans and cell wall colloids were identified as described in Figure 1.

lyophilized and dissolved in a small volume of water by immersion of a tube containing the sample in boiling water under a N_2 atmosphere (amylopectin purified up to this step was readily soluble by this process). The sample (20 ml) containing the polymer (22 A_{560} units) and MMP was rechromatographed on a column (4 × 55 cm) of Sephadex G-25. The column was eluted with water and the amylopectin fractions (40–60) free from MMP, were combined and lyophilized. MMP fractions (75–115) were identified by the periodate-formaldehyde assay (16).

Purity Tests. The absence of cell wall contaminants was established by the specific color reactions of dyes with various cell wall constitutents as follows: dichromic color with Congo red for cellulose, red color with phloroglucinol in HCl for lignin, and orange-red color with Sudan III for cuticular substances (7). Noncellulosic polysaccharides were detected as mannose after hydrolysis in hot dilute HCl (25) and hemicellulose by the procedure of Robinson (27). Protein was determined by the procedure of Ma and Zuazaga (20), and total lipid by the procedure of Bligh and Dyer (6). Absence of amylopectin in the amylose fraction was verified by enzymic assays (5, 12). Lack of amylose in the amylopectin fraction was confirmed by a constant minimum A_{660} in the reaction with iodine-potassium iodide solution (23) after treating the sample with stearic acid (24).

RESULTS AND DISCUSSION

The dispersed crude cotton leaf starch was resolved into amylose and amylopectin by chromatography on a column of Sepharose 2B. Resolution was incomplete, however, since the polymers were intercontaminated in the vicinity of fraction 47 (Fig. 1). Cell wall particles were eluted with the early fractions of amylopectin but were completely separated from amylose.

Salts associated with the amylose fractions (Fig. 1) were completely removed from this polymer by chromatography on a column of Sephadex G-50. Therefore, the sample was composed mainly of amylose and assumed to be contaminated with only a trace amount of amylopectin (see the incomplete resolution of the polymers in Fig. 1). As expected, the purity of amylose reached a constant maximum after the second purification with thymol and butanol. The iodine affinity of this glucan was 19.1 ± 0.1% (Fig. 3 and Table I). Since iodine affinity can range from 18.5 to 20.0% depending upon the source (29), enzymic assays were also performed to verify the absence of amylopectin contamination. The β-amylolysis limit of the sample was incomplete (about 84%), whereas the concurrent action of β-amylase and Z-enzyme hydrolyzed amylose completely to maltose (Table I). Since amylopectin has been reported to be unaffected by Z-enzyme (11), the data demonstrate that the purified amylose originally contained anomalous branched linkages, but was free from the branched molecules of amylopectin (see the purity of amylose in Table I).

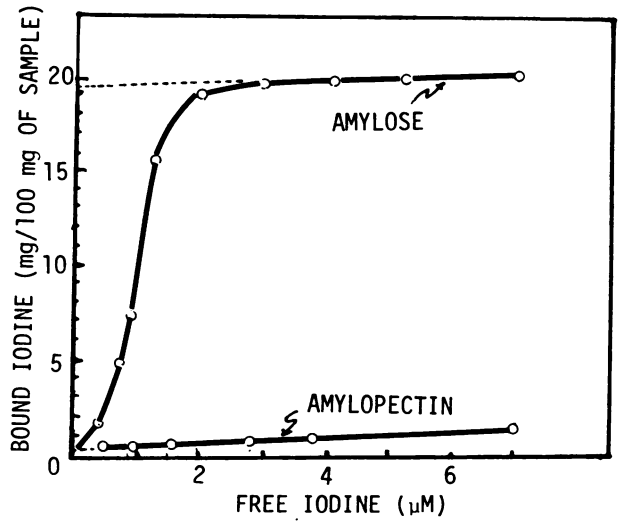

FIG. 3. Typical amperometric iodine titration curves for cotton leaf amylose and amylopectin. A sample containing amylose (6.5 mg) or amylopectin (50 mg) was titrated with a standard 0.005 N potassium iodate solution (22 ± 2 C) according to the procedure of Larson (18). The titration apparatus consisted of a DC Microvolt ammeter (HP 424A), a synchronous rotor, a Hg-filled glass electrode (platinum leads), and a calomel reference electrode. Polarization voltage was furnished by a mercury cell (1.35 v) in series with a fixed resistor (3000 ohms) and a potentiometer (100 ohms). Bound iodine was estimated at the inflection point where the extrapolated minimum slope and zero free iodine met.

Table I. *Properties of Purified Cotton Leaf Amylose and Amylopectin*

	Amylose	Amylopectin
Iodine affinity (%)	19.1 ± 0.1	<0.21
β-Amylolysis limit[a]	84.0 ± 2.0	56.0 ± 2.0
β-Amylolysis limit[b]	100.0 ± 0.5	
Purity (%)	100.0 ± 0.5	>99.0[c]

[a] β-Amylase purified from sweet potato (Calbiochem).

[b] A sample of β-amylase contaminated with Z-enzyme was prepared according to the procedure of Banks *et al.* (5) by eliminating the heat denaturation step.

[c] The purity of amylopectin was based upon the value of 19.1 iodine affinity (amylose) as 100%, which was found from this experiment.

Con A is known to form insoluble complexes specifically with branched chain polysaccharides (16). This property was tested independently with each purified cotton leaf starch component and with a mixture of glucans containing cell wall colloid. The linear polymer amylose did not form an insoluble complex with Con A; rather it was quantitatively eluted without being adsorbed. Con A interacted specifically with amylopectin, however, and retained it. The maximum adsorption capacity was about 192 μg/mg Con A. This was determined by desorption of the amylopectin from the column after it was overloaded. As expected, both cell wall colloids and amylose in the crude sample were eluted in 0.02 M phosphate buffer at pH 7.2 while amylopectin was specifically adsorbed by Con A (Fig. 2). Amylopectin was then eluted and separated from other contaminants with a second gradient of MMP solution. MMP associated with this branched glucan was then completely removed by use of Sephadex G-25. Lack of contamination of this polymer with amylose was further verified by treatment with stearic acid (24). This experiment demonstrated that the sample absorbance at 660 nm in reaction with iodine-potassium iodide solution did not change (a constant minimum) after three successive purifications. The purity of amylopectin was greater than 99%, because the iodine affinity of the polymer was less than 0.21% (Fig. 3 and Table I).

Tests were made on the purified starch components for other impurities such as proteins, lipids, and cell wall constituents (cellulose, lignin, cuticular substances, noncellulosic polysaccharides, and hemicellulose). Tests for these contaminants were qualitatively negative in both the amylose and amylopectin fractions.

The present procedure successfully isolated starch components with maximum purities, which were verified by specific biochemical and enzymic tests in addition to iodine-binding capacity. The basis of this method was the use of Con A, which allowed the complete separation of amylopectin from amylose and cell wall colloids. The responses to Con A of cotton leaf amylose and amylopectin containing contaminants were similar to those of other pure linear and branched polymers such as dextrans (B-512, B-1355) and glycogen (19). Cell wall colloids did not form insoluble complexes with the lectin. The findings in this study demonstrate that this procedure can be used for purification of starch glucans from other sources, since the basic chemical structures of starches in nature are known to be similar.

Acknowledgments—The author thanks Dr. G. Guinn for his critical review of the manuscript and Mr. S. Bortman for his technical assistance.

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