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EXTRACTION OF POLYSACCHARIDES FROM WHEAT SEEDLINGS WITH SODIUM DODECYL SULFATE*

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Abstract—A soluble polysaccharide concentrate was prepared from wheat seedling roots and coleoptiles by extraction with buffered 0·01 M sodium dodecyl sulfate. The extract separated into two peaks on Sepharose 6B, Peak 1 (P_1) at the void volume, and Peak 2 (P_2) at $V_e/V_o = 1{\cdot}55$ when eluted with Tris buffer, and $V_e/V_o = 2{\cdot}0$ when eluted with SDS buffer. The major monosaccharides released by trifluoroacetic acid and Pectinol R-10 from both peaks were galactose and arabinose in a 1:1 molar ratio, together with lesser amounts of glucose, xylose, mannose and rhamnose. Galacturonic acid and glucuronic acid were also detected in the enzyme hydrolyzates. In tracer experiments the major portion of the activity of a membranous fraction co-chromatographed with P_2.

INTRODUCTION

Plant cytoplasmic polysaccharides have been extracted from several sources with cold trichloroacetic acid (TCA) solutions [1–5] or with salt solutions [1]. Such extracts contain glycoproteins in which arabinose is linked glycosidically to hydroxyproline and they are believed to be precursors of cell-wall extensin. The advantage of TCA is that protein is not extracted and that enzymes are rapidly inactivated. However, in at least one instance, cell-wall hydroxyproline was also extracted [1].

In working with wheat root subcellular fractions, we found that the polysaccharides could be solubilized completely in neutral sodium dodecyl sulfate (SDS). In SDS solutions enzymes are also inactivated and the neutral conditions preclude hydrolyzing the acid-sensitive arabinofuranosyl links [6, 7] found in these polysaccharides. Dilute basic solutions [8] also solubilize subcellular fractions, but can induce β-elimination of galacturonans [9] and of galactosyl-serine links [7]. Consequently we compared the type of polysaccharides extracted by SDS from whole wheat root tissue with that extracted from subcellular fractions.

A neutral detergent extraction procedure introduced by Van Soest [10] has found wide use in obtaining plant cell wall preparations of low nitrogen content. The method is used to extract pectins and cytoplasmic components leaving cellulose, hemicellulose and lignin [11, 12] intact within cell walls. The procedure differs from that described here mainly in that our extractions are performed with samples chilled in an ice bath, instead of including EDTA and refluxing the samples.

RESULTS AND DISCUSSION

Gel filtration

Chromatographic mobilities of wheat seedling polysaccharides on Sepharose 2B, 4B and 6B were examined and 6B gave optimum resolution. Some carbohydrate, Peak 1 (P_1), was eluted at the void volume in each sample examined. Only in coleoptile extracts was there non-dialyzable carbohydrate, Peak 3 (P_3), with a MW too low to be resolved by Sepharose 6B. The Sepharose gels of higher exclusion limit, 2B and 4B, failed to resolve P_1, although the elution volume of Peak 2 (P_2) increased so that in the case of 2B it was not resolved from glucose.

UV absorbing substances were associated with all peaks, but primarily with P_1 and P_3. The UV absorbances of P_1 and P_3 had pronounced peaks

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at 207 nm, with minor peaks at 240 and 280 nm. In P_2 the primary peaks were at 240 and 280 nm, while the peak at 207 nm was absent. Hydrolysis of P_1 with trifluoroacetic acid (TFA) at 100° for 15 min liberated an as yet unidentified compound, which was soluble in toluene and which accounted for most of the UV absorbance. It had an R_f of 0·82 on TLC with C_6H_6–MeOH HOAc (45:8:4). It gave a blue color with 5% $FeCl_3$ in 0·5 N HCl, similar to phenols, but unlike phenols it had no absorbance at 280 nm. Only one peak was observed at 195–200 nm, somewhat lower than for the unhydrolyzed fractions. Ferulic acid and other phenolic compounds linked to polysaccharides have been reported [13, 14] in cereals but the compound found in our hydrolyzates did not correspond in R_f to ferulic acid.

The neutral monosaccharides released by TFA (1 hr at 121°) and by Pectinol R-10 were similar. A polysaccharide concentrate, containing 36% carbohydrate as galactose was hydrolyzed for 68 hr with Pectinol R-10. About 24% of the sample was recovered as four sugars in a molar ratio of arabinose (1·0), galactose (0·96), glucose (0·12) and xylose (0·05). In addition, mannose, rhamnose, galacturonic acid and glucuronolactone were identified. Similar results were obtained with TFA except that the uronic acids were not released. Since more acidic conditions are known to degrade pentose and uronic acids, a comparison was not made with mineral acids.

Pectinol R-10 is a preparation derived from *Aspergillus niger* which is known to produce α-D-galactosidase, β-D-galactosidase, α-D-glucosidase, β-D-glucosidase, 1,2-α-D-mannosidase, 1,4-β-D-mannosidase, 1,2-α-L-fucosidase, β-*N*-acetylglucosaminidase, β-*N*-acetylgalactosaminidase, xylosidase, arabofuranosidase and 1,2-α-L-rhamnosidase [15]. Arabinose in wheat seedling extracts, therefore is probably in the furanose form because it was released by this enzyme preparation, and also by dilute TFA.

The similar monosaccharide composition of P_1 and P_2, and the apparent MW of 20 million or more for P_1, prompted an investigation to ascertain if it was a colloidal form of P_2. Freshly prepared concentrates on standing for 24 hr or more at low temperatures became opaque and had to be clarified by centrifugation before being applied to columns. However, the precipitates in these instances invariably could be hydrolyzed to the phenolic compound discussed above, and to glucose. Rechromatography of a redissolved precipitate on Sepharose demonstrated that it originated from P_1. However P_2 when concentrated remained in solution and when rechromatographed did not yield P_1. Also its elution volume was not changed. P_1 is therefore a mixture of at least two components and distinct from P_2. The extremely high MW of P_1 reflects absence of degradation during isolation. A dialyzed and freeze-dried sample of P_1 gave 92% carbohydrate as galactose. Pentoses give a higher response than galactose to phenol–sulfuric acid, and so this value may be somewhat high. Also, a sample of P_1 from the labeled membranous tissue was not degraded to any great extent by pronase. It is quite likely, therefore, that P_1 is mainly polysaccharide.

The composition of our wheat seedling concentrated polysaccharide fraction was similar to that reported for Golgi fractions from maize roots [16] except for a lower glucose content. Perhaps the difference can be explained by the tendency of glucose polymers to precipitate, and thus to be lost in preparation of the concentrate.

It should be noted that P_2 ($V_e/V_o = 1{\cdot}55$ in Tris buffer and $V_e/V_o = 2{\cdot}0$ in SDS buffer) was present in roots and coleoptiles and in the membranous fraction. In some membranous fractions up to 80% of their total activity was associated with P_2. After treating P_2 from a labeled membranous fraction with pronase, about 40% of its activity was in low MW products. A similar experiment conducted with P_2 from the whole tissue showed that essentially all of the carbohydrate could be recovered without altering the elution volume. The treatment caused considerable loss of protein as shown by the disappearance of the absorbance at 280 nm, and the increased absorbance at 280 nm in the peptide peak. Extensively digested samples, however, still retained a significant peak at 280 nm, which suggests a mucopolysaccharide component. Pronase resistance could be due to many factors including the arabinose–hydroxyproline link [1]. Mild acid hydrolysis of that link made the protein susceptible to degradation by pronase.

The positive identification of rhamnose and galacturonic acid indicates that the wheat extracts are rhamnogalacturonans or pectins. These compounds isolated from sycamore cell cultures

[17, 18] and many plant sources [19] contain easily hydrolyzable side chains of arabinan, galactan, or arabinogalactan. Results from sycamore cell cultures [20] also suggest that these molecules are covalently linked to extensin. Extremely large polymers such as P_1 may represent such completed structures, while P_2 may represent an earlier state of biosynthesis of rhamnogalacturonan. The fact that both were present in the membrane structure appears to suggest that complete synthesis of cell wall polysaccharides occurs before they are deposited in the cell wall.

Fractionation of P_2

Chrispeels [2] reported the separation on CM-Sephadex of an extensin precursor derived from carrots. The compound was retained on CM-Sephadex at pH 8 at low ionic strength. Their precursor contained arabinose linked to hydroxyproline, but no galactose. Its MW, 200000, agrees reasonably well with the elution volume found for P_2. In our experience however, no retention or resolution of carbohydrate could be detected when P_2 was eluted through CM-Sephadex at pH 4–8.

Some retention of P_2, however, was obtained on DEAE-Sephadex. In this experiment a sample of P_2 obtained from a labeled membranous fraction which had prior treatment with pronase was mixed with P_2 carbohydrates from the whole root extract and applied to the column. Three carbohydrate peaks were resolved, two of which were radioactive. While the whole root extract had not been treated with pronase, it was established in separate experiments that the heights of the peaks and elution volumes of the three components were not significantly altered with pronase. Presumably pronase has no effect on the major polysaccharide components of the membranous tissue. Its effect mainly is to digest extraneous proteins. From the above experiments it appears that the membranous fraction either did not contain the third component, or this component did not become labeled. Since a 2 hr incubation period was used, the latter is unlikely. Indeed this period is long enough to label proteins and lipids [8] and the third component should have become labeled if present. It is more likely that this component is soluble and was lost during the preparation of the membranous fraction.

The monosaccharide composition of components 1 and 2 were similar and did not differ significantly from that of the whole sample. Separation on the column may possibly indicate resolution of methylated and unmethylated species.

EXPERIMENTAL

Wheat seedlings. Wheat seeds (cv Shawnee) were germinated on covered trays between 256-mesh screens sandwiched between 2 layers of filter paper. Mold growth was prevented by sterilizing screens and paper before using them, and by treating the seed with 0·1% HCHO for 30–45 min, immediately after a 4 hr soaking period in H_2O.

Preparation of polysaccharide concentrate. Roots (< 1 cm) were washed in dist. H_2O, then homogenized, while cooled in an ice bath, in 0·01 M SDS–0·1 M Na phosphate, pH 7·6 (SDS buffer) in a Sorvall Omnimixer for 10 min. The homogenate was filtered through cheese cloth and centrifuged at 5000 rpm for 10 min. The supernatant was dialyzed at 20° with $CHCl_3$ preservative for 6–10 hr in 0·01 M phosphate buffer, pH 7·0–7·6, and 36–48 hr against frequent changes of dist H_2O. Prior dialysis with phosphate buffer prevents precipitation. After dialysis the supernatant was lyophilized and stored at 4°. In a typical expt, 0·57 g of lyophilate, which contained 39–42% protein by micro Kjeldahl [21] and 15–17% carbohydrate as galactose by pHOH–H_2SO_4 [22], was obtained from 55 g of fr root tissue. A polysaccharide concentrate was prepared from the lyophilized powder as follows: 300 mg was dissolved in 6 ml DMSO, diluted to 60 ml with dist H_2O and the cloudy dispersion that developed was clarified by centrifugation at 5000 rpm for 10 min. The ppt was re-extracted with 20 ml of H_2O and re-centrifuged. The combined supernatant was applied directly to a Sepharose column or dialyzed to remove DMSO and again lyophilized. It typically constituted 85–90% of the polysaccharide extracted by SDS with carbohydrate content of 35–40% as galactose on a dry wt basis.

Preparation of a labeled membranous fraction. Seedling roots were incubated in 100 μCi D-glucose[U-^{14}C] for 2 hr and a membranous fraction (crude Golgi) was prepared that sedimented between 0·5 and 1·8 M sucrose [8]. It was washed twice by re-centrifugation through a 0·1 M phosphate buffer to the 0·5 M interphase. The sample was collected and solubilized by adding SDS buffer and occasionally shaking for 1 hr at 20°. The sample was centrifuged at 5000 rpm for 5 min and the supernatant applied to a Sepharose column pre-equilibrated with the same SDS buffer. SDS eluting agent in membranous fractions prevented precipitation or selective absorption of part of the sample on the column.

Gel filtration and ion exchange chromatography. Sepharose 2B, 4B, 6B, DEAE-Sephadex A-25 and CM-Sephadex C-25 columns (1·5 cm × 90 cm) were prepared in either 0·05 M Tris buffer, pH 8, with 0·001% sodium azide preservative or SDS buffer. Ion exchange columns were prepared as specified by manufacturers. Eluates were monitored at 254 nm and assayed for carbohydrate by the pHOH–H_2SO_4 method [22] at 480 or 490 nm.

Digestion with pronase. A sample of P_1 or P_2 was treated with 0·5 mg pronase at 37° in a 1 mM KPi buffer, pH 8, with 0·02% sodium azide preservative [8]. After 56 hr the hydrolyzate was heated gently to inactivate enzymes, then applied to a Sepharose 6B column and eluted with SDS buffer. Percentage of carbohydrate recovered, or activity of eluted samples, was compared with that of the low-MW peak formed by hydrolysis.

Fractionation and assay of sugars. Polysaccharide fractions were hydrolyzed with 2 N TFA in a sealed tube at 121° for 1 hr [23] and the acid removed by evaporation, or with N HCl for 1 hr at 100°, with acid removed by lyophilization. Polysaccharides were hydrolyzed also with 0·1% Pectinol R-10 concentrate (Rohm and Haas) in 0·01 M citrate–phosphate buffer, pH 4, at 40°. Under these conditions 1 mg of enzyme produced 0·05 mg arabinose/min from 1% beet arabinan [24]; pH 4 is optimum for arabinofuranosidase activity [24] and close to optimum for α- and β-galactosidase activity [15]. Sugars were chromatographed on Si gel plates in BuOH–Me_2CO–H_2O (4:5:1) and on Whatman No. 1 paper in BuOH–C_5H_5N–H_2O (10:3:3). For PC spots were revealed by $AgNO_3$ and for TLC, with triphenyltetrazolium chloride (TTC). Plates were dipped in a soln containing 1 g TTC dissolved in 50 ml dioxane with sufficient MeOH to dissolve the TTC (9 ml). After drying, plates were saturated with NH_3 vapor for 6 min. Development was by heating in an oven (10 min, 75–80°) containing an atmosphere of NH_3. Plates were scanned using a densitometer. With NH_3, developed plates had a more uniformly white background than when NaOH was added to the TTC soln [25]. As with the NaOH dip, response was linear up to 10 μg. It was applicable to 30–40 μg when 4–5 standards of different concn were used for each plate. Rhamnose was identified by its characteristic color reaction with aniline–diphenylamine–phosphoric acid spray [26].

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