Phytochemistry, 1980, Vol. 19, pp. 1425–1428. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0701-1425 $02.00/0

LABELING AND CHEMISTRY OF GRAPEFRUIT PECTIC SUBSTANCES*

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(*Received* 4 *October* 1979)

Key Word Index—*Citrus paradisi*; Rutaceae; grapefruit; myo-inositol metabolism; labeling of pectic polysaccharides.

Abstract—The labeling of a number of polysaccharides found in grapefruit (*Citrus paradisi*) was achieved by feeding labeled myo-inositol to ripening grapefruit through their cut fruit stem, and allowing 4 days for the metabolism of label. The pectic polysaccharides were isolated by successive extraction of the labeled grapefruit with 80% ethanol, chloroform–methanol (1:1) and finally with 0.2 M Na_2 EDTA to solubilize pectic polysaccharides. The incorporation of label from myo-inositol into galacturonosyl, arabinosyl, xylosyl and galactosyl residues of pectic polysaccharides via myo-inositol oxidation pathway was demonstrated. Ion exchange chromatography of these labeled pectic polysaccharides using DE-52 cellulose resulted in the elution of eight totally or partially resolved polysaccharides with increasing salt concentration. The results suggest that, like other plant tissues, the myo-inositol oxidation pathway is also operative in ripening grapefruit and this metabolic pathway could be successfully utilized to achieve labeling of a number of pectic polysaccharides.

INTRODUCTION

Our understanding of the elusive biochemical basis of the proposed biological role of dietary pectin in lowering of serum and/or liver cholesterol levels [1–4], would be greatly enhanced by the availability of labeled pectic polysaccharides of defined physicochemical characteristics. The present studies describe the successful labeling of pectic polysaccharides found in grapefruit (*Citrus paradisi*) by utilizing the myo-inositol oxidation pathway, the chemistry of these labeled pectic polysaccharides and their fractionation using DE-52 diethylaminoethyl cellulose column chromatography.

RESULTS

Experiments where myo-inositol-[U-^{14}C] was used as a precursor demonstrated incorporation of radioactivity in the uronosyl, pentosyl and hexosyl units of pectic polysaccharides. The specific radioactivity was found to be 270 cpm/mg of ^{14}C-labeled pectic polysaccharides. However, upon replacing myo-inositol-[U-^{14}C] by the less expensive myo-inositol-[2-^{3}H] and in large radioactive amounts (1 mCi), we were able to achieve isolation of labeled pectic polysaccharides having a specific radioactivity of 7250 cpm/mg. The results described in the present report are confined to studies with myo-inositol-[2-^{3}H]. Label was readily absorbed by the ripening grapefruit through its cut fruit stem. Within the first 2 hr following this introduction of label, more than 99% of the label was absorbed by the grapefruit. A considerable loss in the fr. wt of grapefruit was observed which dropped from 121 to 115 g at the end of the fourth day.

Table 1 summarizes the distribution of radioactivity in various labeled fractions extracted from grapefruit supplied with myo-inositol-[2-^{3}H]. Extraction of grapefruit with 80% ethanol solubilized 85.5% of the total radioactivity supplied as myo-inositol-[2-^{3}H]. Another 9.4% of radioactivity supplied was extracted with chloroform–methanol (1:1). The remaining 5% was accounted for in the insoluble residue weighing 16.2 g. Extraction of this insoluble residue with 0.2 M EDTA resulted in the solubilization of highly viscous pectic polysaccharides. These pectic polysaccharides contained 53.4% of the total radioactivity present in the insoluble residue, and accounted for 15% of the total weight of insoluble residue. The remaining 46% of the radioactivity remained bound to the EDTA-insoluble residue.

Results obtained from chemical analysis of the pectic polysaccharides are summarized in Table 2. Galacturonic acid was the only uronic acid found and composed 76% by weight of pectic polysaccharides. The remaining 24% was accounted for by neutral sugars, rhamnose, arabinose, xylose, mannose, galactose and glucose. Mannose and glucose were present in low amounts and in equal proportions and together constituted *ca* 5% of the total neutral sugar components. Rhamnose, arabinose, xylose and galactose constituted 16.3, 33.3, 13.4 and 31.5%, respectively, of the total neutral sugar components of the labeled pectic polysaccharides. It is interesting to note that rhamnose

* Supported by a grant from the Florida Department of Citrus.

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Table 1. Percentage distribution of radioactivity in various labeled fractions isolated from ripening grapefruit supplied with myo-inositol-[2-^{3}H]

Radioactivity supplied (mCi)	1
Ethanol-soluble fraction (%)	85.5
Chloroform–methanol soluble (%)	9.4
Cell wall residue*	5.1 (16.2 g)
Radioactivity in EDTA-soluble fraction (%)	53.4 (2.49 g)
Radioactivity in EDTA-insoluble fraction (%)†	46.6

* By difference.
† By difference.

Table 2. Percentage sugar composition of labeled EDTA-soluble pectic polysaccharides from ripening grapefruit

Uronic acid component (%)		76
Galacturonic acid (%)	100	
Neutral sugar components (%)		24
Rhamnose	16.3	
Arabinose	33.3	
Xylose	13.4	
Mannose	2.8	
Galactose	31.5	
Glucose	2.6	

and xylose, and arabinose and galactose were present in *ca* equal proportions.

Percentage distribution of label in the acidic and neutral sugar components of labeled pectic polysaccharides is summarized in Table 3. About 50% of the total radioactivity present in the pectic polysaccharides was recovered in the galacturonic acid component. No radioactivity was found in rhamnose, mannose and glucose. Arabinose xylose and galactose accounted for 21, 8 and 21%, respectively, of the total radioactivity found in the pectic plysaccharides.

Table 3. Percentage distribution of radioactivity in acidic and neutral sugar components of labeled pectic polysaccharides from ripening grapefruit

Component	% Radioactivity
Galacturonic acid	49.5
Rhamnose	—
Arabinose	21.4
Xylose	7.4
Mannose	—
Galactose	21.3
Glucose	—

Analysis of labeled pectic polysaccharides by ion exchange chromatography resulted in the radioactive elution profile given in Fig. 1. These results suggest the presence and incorporation of label in several pectic polysaccharides bearing different physicochemical characteristics. More than 95% of the total radioactivity loaded on to the column was recovered in various fractions.

DISCUSSION

The availability of labeled pectic polysaccharides is a prerequisite in our understanding the molecular basis of interaction between these pectic polysaccharides and cholesterol. We have achieved this labeling of pectic polysaccharides by using myo-inositol-[2-^{3}H] which has been shown to be the precursor of

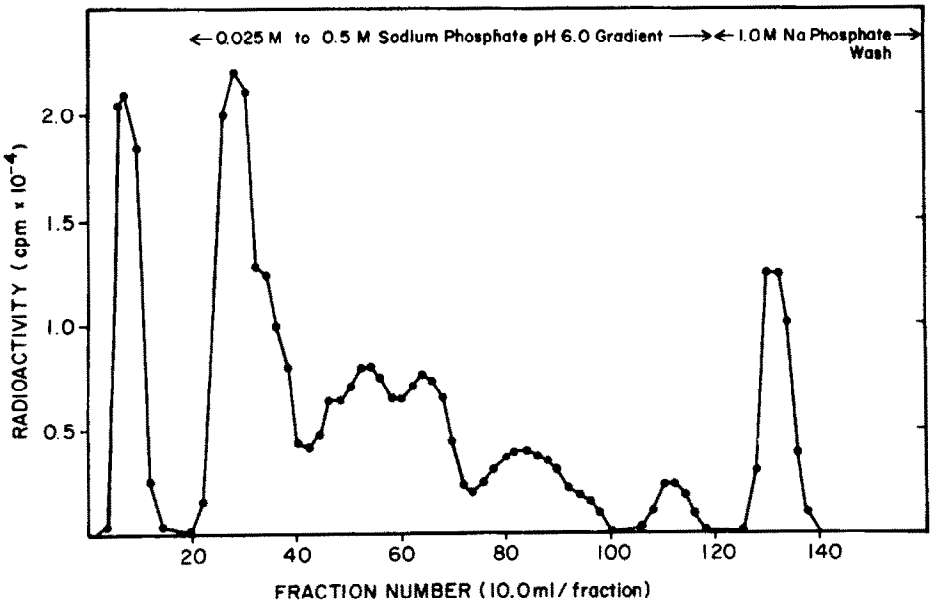

Fig. 1. Radioactive elution profile of labeled pectic polysaccharides from ripening grapefruit. Labeled pectic polysaccharides were chromatographed on a DE-52 cellulose column (1.5 × 24 cm), and eluted with a linear gradient ranging from 0.05 M Na phosphate buffer, pH 6, to 0.5 M Na phosphate buffer, pH 6. For details, see text.

pectic polysaccharides in a number of intact plant tissues as well as plant tissues grown in defined culture medium [5–7]. The results presented lend additional support to studies originally done by Loewus [5–7] that the myo-inositol oxidation pathway is also operative in grapefruit.

The chemical nature of labeled pectic polysaccharides isolated was found to be similar to other pectic polysaccharides previously obtained from citrus fruits [8]. The heterogeneous nature of these polysaccharides was suggested by results obtained from DE-52 cellulose column chromatography which partially and/or totally resolved 8 polysaccharides found in various proportions.

EXPERIMENTAL

Ripening grapefruit. Citrus paradisi (cv Thompson Pink), from plants grown under greenhouse conditions, was used. Grapefruit were cut under H_2O at the fruit stem and transported to the laboratory, submerged in H_2O.

Labeled compounds. Myo-inositol-[2-^{3}H] (5 Ci/mM) and myo-inositol-[U-^{14}C] (255 mCi/mM) were purchased from Amersham/Searle Corp.

Labeling procedure. The labels were introduced by placing the cut surface of grapefruit stem in a small glass vial containing 1 mCi of myo-inositol-[2-^{3}H] or 20 μCi of myo-inositol-[U-^{14}C]. After the labeled soln had been taken up, H_2O was added to keep the cut fruit stem submerged. Grapefruit were allowed to metabolize the labeled myo-inositol for 4 days.

Extraction of pectic polysaccharides. Labeled grapefruit was cut into small pieces and homogenized using a Virtis 45 homogenizer in hot 80% EtOH. The EtOH-soluble supernatant was removed from the insoluble residue by centrifugation of the homogenate at 2000 g for 30 min. This extraction was repeated several times until no radioactivity was detectable in the EtOH fraction. EtOH-insoluble residue was further extracted by continuous shaking in 10 vol. of $CHCl_3$–MeOH (1:1) for 3 days at room temp. At every 24 hr interval, $CHCl_3$–MeOH soluble material was removed by centrifugation at 2000 g for 30 min and fresh solvent added to continue extraction. The delipidated, depigmented and dehydrated tissue was then air-dried and weighed.

Pectic polysaccharides were extracted from this dried residue by a method described in ref. [9]. To the dried residue was added 150 ml of 0.2 M Na_2 EDTA, and pectic polysaccharides were extracted with continuous shaking for 24 hr at room temp. This extraction procedure was repeated twice and EDTA-soluble fractions thus obtained were pooled and dialysed extensively against deionized H_2O. To the dialysed EDTA-soluble fraction representing pectic polysaccharides was added enough 95% EtOH to bring the final concn of EtOH to 80%, in order to ppt. pectic polysaccharides. The precipitation was continued for 2 days at room temp. Pptd pectic polysaccharides were recovered by centrifugation at 15 000 rpm for 1 hr, dissolved in H_2O and lyophilized.

Chemical analysis of pectic polysaccharides. These were hydrolysed with 2 M CF_3COOH (TFA) at 121° for 2 hr in sealed ampules [10]. Following hydrolysis, TFA was removed by vacuum evapn and the hydrolysed residue dissolved in deionized H_2O. The pectic polysaccharide hydrolysate was passed through a Dowex 50 H^+ resin (AG50W×8, 100–200 mesh) column (1×5 cm) and the column was washed with deionized H_2O until the effluent was free of detectable radioactivity. The effluent was then loaded on to a Dowex 1 formate resin (AG 1×2, 200–400 mesh) column (1×5 cm) and the column washed with H_2O until its neutral effluent was free of radioactivity. The neutral sugar effluent thus recovered was evapd to dryness and redissolved in a small vol. (*ca* 0.2–0.5 ml) of H_2O. The acidic components bound to Dowex 1 formate resin were washed off the resin by elution with 6 M HCOOH. HCOOH was removed by vacuum evapn and acidic components dissolved in H_2O. Neutral and acidic constituants thus separated by ion exchange chromatography were further separated into individual neutral sugars or uronic acids by descending PC using Whatmen No. 1 paper. For sepn of neutral sugars, solvent system A (EtOAc–Py–H_2O, 8:2:1) was used [11]. Acidic components were separated using solvent system B (EtOAc-Py–HOAc–H_2O, 5:5:1:3) [12], Chromatograms were developed with aq. Me_2CO containing $AgNO_3$ followed by a dip in alcoholic KOH soln [13]. Contrasts between developing sugars and paper background were preserved by dipping chromatograms in 10% aq. $Na_2S_2O_3$.

Distribution of label in sugar components of pectic polysaccharides. In expts aimed to measure the radioactivity of individual neutral and acidic components of labeled pectic polysaccharides, an amount of labeled pectic polysaccharides of known carbohydrate and radioactivity content was hydrolysed and resolved into acidic and neutral sugar components by methods described above. Small aliquots of these neutral and acidic components having known amounts of radioactivity and carbohydrate were streaked on paper and chromatographed in solvent A to resolve individual neutral sugars or solvent B to resolve individual acidic sugars. After separation, controls were developed, individual neutral sugars and uronic acids corresponding in mobility to the known markers were eluted from paper with H_2O. The radioactivity and concn of each component was then determined.

GLC of neutral sugars. Pectic polysaccharides were hydrolysed with TFA [10] and alditol acetate derivatives of samples for analysis of sugars were prepared according to the procedure of ref. [14]. A glass column (1.83 m×2 mm i.d.) packed with 3% OV225 on 80–100 mesh Supelcoport (Supelco) was used for analysis. Column temp. was maintained at 200°, injection port temp. at 225° and FID port at 250°. N_2 was used as carrier gas at 20 ml/min.

DE-52 diethylaminoethyl cellulose ion exchange chromatography. A column (1.5×24 cm) was equilibrated with 0.025 M Na Pi buffer pH 6 and 100 mg of labeled pectic polysaccharides dissolved in 30 ml of equilibrating buffer was loaded on to the column. The column was washed with 200 ml of equilibrating buffer and then eluted with a linear gradient ranging from 0.025 M Na Pi to 0.5 M Na Pi pH 6 buffer, 500 ml of each. At the end of the gradient run, the column was washed with 200 ml of M Na Pi buffer, pH 6. Fractions were collected and each fraction was assayed for radioactivity.

Radioactivity in aq. samples was determined after addition of 10 ml of Aquasol (New England Nuclear) scintillation cocktail, with the use of a scintillation spectrometer. Counting efficiency was estimated to be 30% for ^{3}H and 81% for ^{14}C isotopes.

Other analytical techniques. Total carbohydrate was estimated with α-naphthol reagent using galactose as standard [15], reducing sugars were estimated using ferricyanide reagent [16] with galactose as standard, uronic acid was determined by carbazole reaction [17] with galacturonic acid as standard, and pentose was estimated using orcinol reagent [18] with arabinose as standard.

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