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THE PECTIC COMPONENTS OF PLUM FRUITS

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Abstract—Pectic substances were extracted from plum fruit tissue and the whole pectin, crude pectinic acid, purified pectinic acid and neutral fractions were prepared. Each of the fractions contained galacturonic acid, arabinose, galactose, xylose and rhamnose. High voltage electrophoresis indicated the presence of neutral and negatively charged material, and treatment with pectinesterase showed that the neutral component was completely esterified. The three cultivars of fruits studied showed close similarities. The results are consistent with proposed models of pectin structure and indicate the probable existence of a continuous gradation with respect to degree of esterification and molecular size.

INTRODUCTION

The pectic substances are a group of heterogeneous polysaccharides present in the tissues of higher plants and characterized by their solubility properties and by a major content of galacturonic acid and the neutral sugars arabinose and galactose. Early work indicated the existence of three separate and distinct homopolysaccharides (an arabinan, a galactan, and a galacturonan) and it was believed that the pectic substances are mixtures of these homopolymers [1]. More recently, however, it has become apparent that the alkaline conditions used in these early studies brought about some degradation of the native pectin molecules. By the use of extraction and fractionation procedures avoiding alkaline conditions, Northcote and his colleagues have prepared the pectic material from plant tissues with minimal degradation and shown the presence of neutral and negatively charged polymeric components, each of which was a heteropolysaccharide comprising mainly residues of arabinose and galactose, and arabinose, galactose and galacturonic acid respectively [2, 3]. Evidence was presented showing that the sugar residues occur in blocks in the intact molecule, thus accounting for the production of homopolymers on fragmentation of the heteropolysaccharide molecule by alkali.

The pectic polysaccharides are major components of the primary cell walls and middle lamella of young growing plant tissues, but are absent from the secondary walls of more mature tissues [4, 5]. The restriction of cell enlargement to cells lacking a secondary wall has led to theories of cell wall extension involving an important role for pectin [6, 7]. A possible function for pectin of the middle lamella region is the maintenance of tissue cohesion by its acting as an intercellular adhesive substance [8]. As a consequence of their location in primary walls pectic substances occur in greatest quantities in primary tissues and are particularly abundant in fruits, where a role in the ripening process has been suggested [8]. The present paper reports work carried out on the pectic components of plum fruits as a preliminary to studies of its relationships to fruit ripening and the formation of stone gum.

RESULTS

Extraction of pectic substances

Complete extraction of pectic substances was achieved by the use of 2% sodium hexametaphosphate solution, pH 3.7. A small sample of fruits (117.2 g fr. wt) of the cv Czar was skinned and stoned, the flesh cut into small pieces and extracted with 450 ml EtOH at 50° for 15 min (*ca* 80% final ethanol concentration). The extracted tissue was collected by filtration, washed with 80% ethanol, acetone, and air dried (yield 2.46 g, 2.1%). The dried plant material was ground to a powder and a sample (101.4 mg) extracted under reflux with 30 ml 2% sodium hexametaphosphate pH 3.7. Samples (0.5 ml) were removed at intervals of time and diluted to 5.0 ml with benzoic acid solution. The uronic acid content was determined by the carbazole [9] procedure after filtration. Pectin content of the dried fruit material and of a sample of fruits was determined by the procedure of McCready and McComb [10]. Alternative procedures for treatment prior to sodium hexametaphosphate extraction (freeze drying; separate extraction of juice and flesh) did not result in improved extraction.

Preparation and fractionation of the pectic components

Approximately 1 kg of fruits of each of the cultivars Czar, Pershore and Victoria were skinned and stoned, the flesh cut into small pieces and extracted for 15 min at 50° with 4 l. ethanol (final ethanol concentration *ca* 80%). The extracted tissue was filtered, washed with 80% ethanol, acetone, and air-dried. Yields are given in Table 1.

The pectic components were extracted from the ground dried plant material by refluxing for 4 hr with 2% sodium hexametaphosphate solution, pH 3.7 (6 g per 600 ml extractant). After extraction plant debris was removed by filtration and the pH of the extract was adjusted to 4.5 with NaOH. A 50 ml sample of the extract was stored for further study and 6 mg amyloglucosidase was added to the remaining solution which was incubated at room temperature overnight, followed by dialysis for 24 hr at 2–4° against several changes of distilled water.

Table 1. Yield of dry solid following extraction of fruit with ethanol

	Cultivar		
	Czar	Pershore	Victoria
Fresh weight of fruit (kg)	1.006	1.010	1.018
Weight of dry solid (g)	21.7	12.7	22.7
Yield (%)	2.16	1.26	2.23

The whole pectin was precipitated from a 200 ml sample of the starch-free extract by addition to 1 l. of absolute ethanol. The precipitate was collected by centrifugation, washed with 80% ethanol, acetone and air-dried. An attempt to fractionate the pectic substances by use of alcohol precipitation was not successful. Absolute ethanol was added to 200 ml of the extract to a final ethanol concentration of 48%. In contrast to apple fruit pectin [2], precipitates were not obtained from extracts of any of the varieties of plum fruits used in this study. However, addition of 1 M $CaCl_2$ immediately produced a gelatinous precipitate. The calcium chloride solution was added until no further precipitation was observed, the precipitate (crude pectinic acid) was collected by centrifugation, washed with 80% ethanol, acetone and air-dried.

The remaining pectin (neutral pectin) was precipitated by adjusting the ethanol concentration of the solution to 80%. The precipitate was collected by centrifugation and washed with 80% ethanol, acetone and air-dried. Yields are shown in Table 2.

Table 2. Yield of pectic fractions

	Cultivar		
	Czar	Pershore	Victoria
Whole pectin (mg)	619	776	588
Crude pectinic acid (mg)	577	617	522
Neutral pectin (mg)	262	308	148

Dry ethanol-extracted plant material (6 g) was refluxed with 600 ml sodium hexametaphosphate for 4 hr. After removal of starch, whole pectin was prepared by addition of a sample to 5 vol. ethanol, crude pectinic acid was prepared by addition of aq. $CaCl_2$ to a second sample, and the neutral pectin was prepared after precipitation of crude pectinic acid by addition of alcohol to give a final concentration of 80%.

The pectin content of the dried fruit material was determined by the method of McCready and McComb [10] and is given in Table 3. The uronic acid content of the sodium hexametaphosphate extract was determined by the carbazole procedure (Table 3). The results confirm that complete extraction of pectic substances is achieved by the procedures used.

Hydrolysis and paper chromatography

The major monosaccharides present in hydrolysates of the pectic fractions were galacturonic acid, galactose and arabinose, with traces of xylose and rhamnose also detected. These monosaccharides were present in all the pectic fractions from each of the varieties of fruits. Table 4 gives quantitative data relating to the major constituents. The trend throughout this data is the relative enrichment of the neutral fraction with arabinose. Consistent results were obtained using nitric or sulphuric acid for the hydrolysis, and using the aniline hydrogen phthalate procedure or determination of reducing sugar on thin layer plates. Hydrolysis with citric acid produced only arabinose.

In solvent system 3 two materials with a mobility less than galacturonic acid were frequently present. Elution of the less mobile component followed by hydrolysis and chromatography produced galactose and the more mobile component. Elution, hydrolysis and chromatography of this component produced galacturonic acid, arabinose, galactose, xylose and rhamnose. The two spots are probably caused by the presence of aldobiuronic acids and corresponding triuronic acids.

Electrophoresis

The whole pectin was separated by high voltage electrophoresis into two components, a neutral component which formed a distinct spot and a negatively charged component which spread out during electrophoresis indicating the presence of molecules possessing a

Table 3. Pectin content of dried plant material

	Cultivar		
	Czar	Pershore	Victoria
Pectin (as uronic acid, mg/100 mg dried material)	12.9	12.4	12.3
Pectin (as uronic acid in sodium hexametaphosphate extract mg/100 mg dried material)	12.3	12.7	11.9
% Pectin extracted	95.3	102.4	96.7

Pectin content was determined as uronic acid by the procedure of McCready and McComb [10]. Pectin extracted by sodium hexametaphosphate was determined as uronic acid by the carbazole procedure [9].

Table 4. Constitution of hydrolysates of the pectic fractions

	Sugar	Sugars present (μg/ml hydrolysate)			
		Whole pectin	Crude pectinic acid	Purified pectinic acid	Neutral fraction
Czar	Galacturonic acid	320 (1.4)	92 (1.3)	20 (0.6)	104 (1.0)
	Galactose	219 (1.0)	68 (1.0)	34 (1.0)	104 (1.0)
	Arabinose	101 (0.4)	19 (0.3)	10 (0.3)	80 (0.8)
Pershore	Galacturonic acid	162 (1.0)	20 (1.4)	65 (0.8)	118 (1.7)
	Galactose	163 (1.0)	15 (1.0)	82 (1.0)	68 (1.0)
	Arabinose	57 (0.3)	8 (0.5)	30 (0.4)	44 (0.7)
Victoria	Galacturonic acid	98 (1.04)	85 (1.0)	73 (1.1)	0 (0)
	Galactose	94 (1.0)	85 (1.0)	65 (1.0)	18 (1.0)
	Arabinose	74 (0.8)	55 (0.65)	47 (0.72)	24 (1.33)

Figures in parentheses are the ratios with respect to galactose.

range of negative charges. These negatively charged components were never separated into distinct spots. The neutral pectic fraction also separated into two components giving a pattern similar to that of the whole fraction. The crude pectinic acid fraction was separated into two distinct negatively charged spots. Neutral material was absent from this preparation. Purified pectinic acid gave rise to one distinct spot corresponding to the more mobile portion of the whole pectin and to the more mobile of the two crude pectinic acid components.

Treatment of the whole pectin with pectin methyl esterase completely removed the neutral components and produced a new component with much greater mobility than any of the naturally-occurring constituents. In some experiments traces of a negatively charged material with low mobility and corresponding to the whole pectin were present, but much of this material was also converted to the highly mobile form. Polygalacturonase treatment of the whole pectin led to an increase in size of the neutral spot and a corresponding decrease in the negatively charged components.

DISCUSSION

Sodium hexametaphosphate solution has been used for the extraction of pectic substances and produced minimal degradation [3], although some de-esterification and β-elimination have been reported to occur at certain pH values [11, 12]. Extraction of pectic substances from ethanol-extracted dried plum fruit material by sodium hexametaphosphate solution was initially very rapid; 90% of the pectic material was extracted within 15 min. This was followed by a slow extraction of remaining pectin over a 4 hr period. By this time complete dissolution of pectin had occurred.

Ethanol fractionation of plum fruit pectic substances was not successful, although it separated apple fruit pectic substances into two fractions [2]. However the use of a calcium chloride solution led to the precipitation of the major crude pectinic acid fraction, and the addition of ethanol to the remaining solution to a final concentration of 80% led to a further small precipitate, designated the neutral fraction. Pectinic acid was purified from the crude pectinic acid preparation by precipitation with cetylpyridinium chloride.

The three cultivars of plum fruits used (Czar, Pershore and Victoria) gave qualitatively identical results. Small quantitative differences were evident, probably because of factors such as tree age, the precise stage of ripening, or environmental influences. The results indicate that the pectic components of the three cultivars are of closely similar constitution.

Electrophoresis of the whole pectin resolved this material into a distinct spot of a neutral component and a zone of negatively charged material. Treatment of whole pectin with pectinesterase brought about complete disappearance of the neutral component and led to the appearance of a fast moving negatively charged component. The neutral material is therefore a completely esterified pectic component and not a polymer of neutral sugars such as the arabinan–galactan described by Barrett and Northcote as a constituent of apple fruit pectin [2]. The negatively-charged polysaccharide of the whole pectin was never resolved into separate spots. The spreading of this material indicates heterogeneity with respect to charge. This may arise from differences in the degree of esterification of homogeneous molecules, or heterogeneity with respect to the galacturonic acid content. The latter could be a characteristic of the intact pectin molecule or it could arise from random enzymic hydrolysis by endogenous polygalacturonase. Pectinesterase treatment did not completely remove the heterogeneous charged material in most experiments, but it did cause a considerable reduction in the amount of this material, some of which is therefore partially esterified. It is not clear whether the remaining negatively charged material is residual pectin which has not been de-esterified by the enzyme, or whether it represents partially degraded native pectin molecules consisting largely of neutral polysaccharides (arabinan or galactan) to which residual galacturonic acid molecules remain attached.

Treatment of the whole pectin with endopolygalacturonase produced ethanol-insoluble material which was resolved by electrophoresis into two components, one of which was neutral, the other negatively charged. Compared with the whole pectin, there was an increase in the quantity of neutral component and a corresponding decrease in the amount of the negatively charged components. The neutral material is probably arabinan and galactan released as a result of complete removal of galacturonic acid residues by the enzyme. The negatively charged material is probably undegraded pectin molecules.

The crude pectinic acid fraction was resolved by electrophoresis into two distinct negatively charged components. Purification of the pectinic acid with cetylpyridinium chloride eliminated the less mobile component and produced one spot corresponding to the faster moving component of the crude pectinic acid. This purified pectinic acid is not as mobile as the product of pectinesterase treatment of whole pectin and is probably extensively esterified.

The fraction of the whole pectin remaining in solution after precipitation of the crude pectinic acid with calcium chloride was precipitated by adjusting the ethanol concentration to 80% and designated the neutral fraction. It contained the neutral completely esterified component and also some negatively charged material. This is more heterogeneous than the crude pectinic acid fraction and may comprise the partial degradation products of endogenous endopolygalacturonase activity. The preponderance of smaller molecular weight components in this fraction would support this. It would thus consist of arabinan and galactan to which terminal residues of galacturonic acid remain attached.

These results support the conclusion of Aspinall that the pectic substances consist of a continuous spectrum of closely related species [11]. Differences in degree of esterification, molecular size, and relative proportions of uronic acid and neutral sugars have been reported in this and other papers [12, 13].

The monosaccharide residues detected in acid hydrolysates of the pectic fractions are those commonly found in preparations of pectic substances [2, 11, 12]. Models of pectin structure are based on a backbone of galacturonic acid interspersed with occasional molecules of rhamnose with arabinan and galactan side chains attached [14]. Results are consistent with this model. Arabinose was the only free sugar produced by citric acid hydrolysis, and arabinose levels were enhanced in the neutral pectic fraction. Treatment of whole pectin with endopolygalacturonase enhanced the neutral component at the expense of the negatively charged component. This is probably caused by release of blocks of neutral sugars following enzymic digestion of the galacturonan portion of the pectin molecules, and the insolubility in 80% ethanol indicates a high degree of polymerization.

Firm identification of the presumed aldobiuronic acids was not possible owing to the small quantity of material available. Hydrolysis and chromatography indicated the presence of galacturonic acid, rhamnose, galactose, arabinose and xylose. The content of rhamnose was enhanced compared with hydrolysates of the whole pectin fraction which is consistent with its location in the galacturonan backbone. Aldobiuronic acids involving covalent linkage of galacturonic acid to residues of rhamnose, galactose, arabinose and xylose have been reported from a number of plant species [15, 16].

EXPERIMENTAL

Ripe plum fruits of cvs Czar, Pershore and Victoria were provided by C. J. Jefferies of the Long Ashton Research Station, Bristol, U.K.

Hydrolysis of the pectic fractions. Acidic hydrolysis of the polysaccharides was carried out under the following conditions. (1) 0.5 M H_2SO_4 at 100° for 6 hr, (2) M H_2SO_4 at 100° for 6 or 12 hr, (3) 3% HNO_3 containing 0.05% urea at 80° for 3 hr, (4) as (3) but at 100° for 3 hr, (5) 0.05 M citric acid [17] pH 2.18 at 100° for 6 hr. Hydrolysates were neutralized, filtered and concd by evapn below 40°.

Chromatography of hydrolysates. PC was carried out on Whatman No. 1 paper by descent using: (1) EtOAc–Py–H_2O (8:2:1), (2) *n*-BuOH–EtOH–H_2O (5:4:1), (3) EtOAc–HOAc–Py–H_2O (5:1:5:3), (4) EtOAc–HOAc–HCO_2H–H_2O (18:3:1:4). The sugars were detected using aniline hydrogen phthalate [18] or $AgNO_3$ [19]. Quantitative determinations were carried out by the aniline hydrogen phthalate procedure [18]. Quantitative TLC was carried out on Si gel plates using *n*-BuOH–Me_2CO–H_2O (4:5:1). The position of the sugars was determined by exposing part of the plate to alk. $AgNO_3$ [19]. Sugars were eluted by scraping Si gel into 5 ml H_2O and were determined by the Nelson procedure [20].

High voltage electrophoresis. Separations were carried out on glass fibre paper (Whatman GF/A) in the Shandon high voltage electrophoresis apparatus. The buffer system was PV–acetate, pH 6.5 (10% PY and 0.3% HOAc by vol.). The paper was wet with buffer containing EDTA before application of samples (50 μg polysaccharide in 0.5% EDTA). Glycogen was used as a neutral marker spot. Electrophoresis was carried out at 1.5–2.0 kV for periods of 20–60 min. Papers were dried and the polysaccharides located by use of the sulphonated α-naphthol reagent.

Depolymerization. Ca 50 mg whole pectin was dissolved in 1 ml dist. H_2O and incubated overnight at room temp. with 1 mg purified endopolygalacturonase in 4 ml acetic buffer, pH 4.8 [21]. (From *Sclerotinia fructigena*, kindly provided by Dr. R. J. W. Byrde.) Following dialysis against dist. H_2O at 2–4° for 24 hr polysaccharide was prepared by addition to EtOH to a final concn of 80%.

De-esterification. Ca 50 mg whole pectin was dissolved in 5 ml dist. H_2O, the pH adjusted to 7.5 and incubated at 30° for 1 hr with 10 units of orange peel pectinesterase (Sigma). Polysaccharide was prepared by precipitation with 25 ml EtOH.

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