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HYDROLYSIS OF PLANT POLYSACCHARIDES AND GLC ANALYSIS OF THEIR CONSTITUENT NEUTRAL SUGARS

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Abstract—The release and degradation of sugars from onion cell walls and potato cell wall polysaccharides were followed during hydrolysis with trifluoroacetic acid so that the optimum hydrolysis conditions could be determined. After 6 hr hydrolysis in 2 M acid at 100°, calculated recovery factors of different monosaccharides from potato pectic fractions ranged from 61 to 96%. Lower yields of monosaccharides were obtained from intact onion cell walls, while the yield from cellulose was less than 0.2%. A new GLC column for the separation of alditol acetates derived from cell wall sugars is described.

INTRODUCTION

The sugar composition of plant cell wall polysaccharides is conventionally determined by acid hydrolysis and chromatographic analysis of the monosaccharides released. Since glycosidic bonds vary greatly in their stability to acid hydrolysis and different monosaccharides are destroyed by acid at different rates, the conditions chosen for the hydrolysis of complex polysaccharides are inevitably a compromise between destruction of the less stable sugars and incomplete hydrolysis of the more resistant glycosidic bonds. The acid hydrolysis of intact cell walls presents particularly severe problems, which have led to the development of combined enzymic–acidic hydrolysis procedures [1] and methods using concentrated mineral acids at low temperatures [2, 3]. These efficient procedures either are time-consuming or require the use of reagents that are difficult to handle. In routine work there is also a place for straightforward hydrolysis methods using dilute acid, provided that the conditions can be standardised and recovery factors estimated for each monosaccharide present. In this paper some of the parameters that affect the choice of hydrolysis conditions and the recovery of different neutral monosaccharides are examined.

A wide variety of chromatographic techniques are available for the analysis of monosaccharides, the most important being currently GLC, LC and PC [4–7]. The method which, though time-consuming, is most widely used in plant cell wall studies is GLC after conversion to the alditol acetates [4, 8–11]. Separation conditions that reduce the time required to complete an analysis by this method are described in the latter part of the paper.

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RESULTS AND DISCUSSION

Hydrolysis of soluble polysaccharides from potato cell walls

The problem of finding a compromise between the release of different monosaccharides and their degradation is illustrated by the hydrolysis time-course of two pectic fractions isolated from potato cell walls. The proportion of galacturonic acid residues in these two preparations was 33.5 and 70.4% respectively, and the main neutral components were arabinogalactans in which the galactan chains were β-(1,4)-linked. Rhamnose, xylose, mannose, fucose and apiose residues were also present [12], but the amounts of the last three were too small to permit the construction of reliable time-course curves. Starch was present in both preparations and was the main source of glucose in the hydrolysates.

Before hydrolysis, the methyl ester groups were removed from the rhamnogalacturonans under mildly alkaline conditions [12]. De-esterification increases the yield of L-rhamnose by reducing the acid stability of the α-D-galacturonosyl(1,2′)-L-rhamnose glycosidic bond. Although there have been various mechanistic interpretations of the stability to acid of glycosidic bonds involving uronic acids [13–16], it is known that the rate constant for hydrolysis of the glycosides increases in the order of carboxylate anion < unionised acid < methyl ester [14, 15], and the stability of glycuronans is similarly affected by ionisation [16].

It might be expected that at a relatively high pH (above 2) acid hydrolysis would be favoured over degradation because the carboxylate anion concentration would be higher, although it has been suggested [16] that due to the absence of intramolecular catalysis, this effect is less pronounced with aldobiuronic acids, of which 2-*O*-α-D-galacturonosyl-L-rhamnose is an example. Trials with formate buffers in the range pH 2–2.5 indicated (data not shown) that degradation was considerable during the long hydrolysis periods required. Oxidation may have

Table 1. Calculated recovery percentages for neutral monosaccharides released from two potato pectic fractions by 6 hr hydrolysis with 2 M TFA at 100°, corrected for losses due to degradation

Neutral monosaccharides	Pectic fraction I	Pectic fraction II	Average
Galactose	95.3	97.6	96
Arabinose	92.5	96.0	94
Xylose	83.3	82.5	83
Rhamnose	62.0	59.6	61
Glucose	96.0	95.5	96

contributed to the degradative losses [5]. The maximum yield of L-rhamnose after hydrolysis in formate buffers was less than could be obtained with trifluoroacetic acid (TFA), to which all the following data relate.

Galactose, glucose and mannose were released in the sigmoidal manner characteristic of linear chains depolymerised by random scission [17]. Arabinose was released more quickly and rhamnose more slowly than the other neutral monosaccharides, while arabinose and particularly xylose were more liable to breakdown following their release. Similar behaviour is found with other plant polysaccharides, although the pattern is more complex when β(1,4)-linked chains of D-glucosyl or D-mannosyl residues are present [5, 9, 18–20].

Calculated recovery percentages for the major neutral monosaccharides in the hydrolysates of the two potato pectic fractions after 6 hr hydrolysis with 2 M TFA at 100°, are shown in Table 1. These conditions represent a compromise between hydrolysis and degradation that is suitable for heterogeneous polysaccharide preparations of this type, although the optimum conditions for individual monosaccharides are obviously different. For the purpose of calculation, the quantity of each monosaccharide in the original polymer was estimated by linear extrapolation to zero time, with the exception of rhamnose for which this method would introduce substantial errors: the total rhamnose content was determined by a method devised for aldobiuronic acids [19], with the additional approximation that the stability of glycosidic bonds linking α-D-galacturonosyl oligomers to rhamnose is considered to be independent of the number of α-D-galacturonosyl residues.

The optimum conditions for polysaccharides containing β(1,4)-linked D-glucosyl or D-mannosyl residues are considerably more severe. The Saeman hydrolysis procedure [21], and related methods using H_2SO_4, depolymerise hemicellulosic polysaccharides of this type more completely than TFA hydrolysis and with less selective destruction of xylose [10, 12, 18].

Hydrolysis of intact onion cell walls

It is not possible to obtain complete hydrolysis of the polysaccharides in intact plant cell walls using dilute acids alone. Cellulose and Ca^{2+}-complexed galacturonans are particularly resistant. However, provided that appropriate recovery factors are available, acid hydrolysis can be useful for certain purposes; for example, it has been used in the authors' laboratory to determine if pectic polysaccharides and hemicelluloses had been completely removed from potato cell walls by a variety of extractive treatments [12].

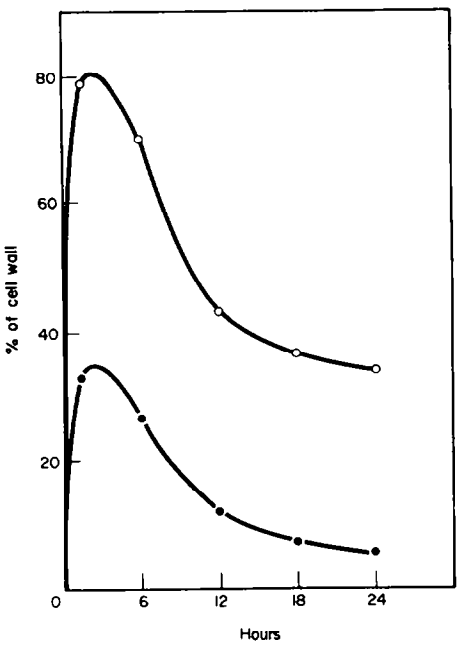

Fig. 1. Total carbohydrate (○——○) and uronide (●——●) released from onion cell walls by treatment with 1 M TFA at 120° for varying lengths of time up to 24 hr. Total carbohydrate is the sum of uronide as galacturonic acid equivalents and neutral carbohydrate as galactose equivalents, estimated by the PhOH–H_2SO_4 and carbazole–H_2SO_4 methods (see Experimental).

The optimum hydrolysis conditions depend on whether the cell wall polysaccharides must be fully depolymerised for GLC analysis, or whether it is only necessary to release the polymeric wall constituents in a soluble form for colorimetric estimation. Fig. 1 shows the release and degradation of neutral carbohydrate and uronides (both polymeric and monomeric, estimated colorimetrically) from onion cell walls by 1M TFA at 120°. For this purpose the optimum duration of hydrolysis is 1.5 hr.

Table 2. Comparison of hydrolysis methods for intact onion cell walls: percentage monosaccharide composition (estimated by GLC) of total neutral carbohydrate released under different hydrolysis conditions

	1 M TFA 2 hr at 120°	2 M TFA 2 hr at 120°	Enzymic–acidic hydrolysis[1]
Neutral carbohydrate released (mg per 100 mg cell walls)	43.2	45.4	46.0
Neutral monosaccharide (%):			
Galactose	71.8	64.3	62.4
Arabinose	6.9	7.0	6.5
Xylose	6.0	7.3	6.1
Rhamnose	6.3	6.4	5.4
Fucose	1.4	2.6	2.6
Glucose	6.0	9.0	13.5
Mannose	1.6	3.1	3.5

Slightly longer hydrolysis is required for the maximum yield of monosaccharides estimated by GLC, although there is much variation between individual sugars in this respect. In Table 2 the recoveries of monosaccharides after 2 hr hydrolysis with TFA are compared with the yields obtained by the acid–enzymic hydrolysis procedure of Jones and Albersheim [1], which gives a greater, though still incomplete [22] recovery of glucose and mannose from the onion cell wall. Both TFA hydrolysis and the Jones and Albersheim procedure are generally considered to degrade only the non-cellulosic polysaccharides in the wall, or at most, a small proportion of the cellulose. Hydrolysis with 2 M TFA solubilised only 0.14% of the carbohydrate from chromatographic grade fibrous cellulose and 0.19% from cotton wool, although it may be assumed that a much larger proportion of the less crystalline cellulose in primary cell walls [18] would be brought into solution under the same conditions.

Analysis of monosaccharides

The published methods for preparation and GLC analysis of alditol acetate derivatives from monosaccharides [e.g. 8–10, 23] are effective but somewhat time-consuming. We have found that the time requirement can be substantially reduced by a 2- to 5-fold reduction in sample size and solvent volumes, and by derivatisation of sugar samples in large batches with the help of devices such as a multi-port air manifold for evaporation of solvents from 2 ml flat-bottomed vials standing on a thermostatic hotplate.

On most of the GLC columns that have been used for the analysis of alditol acetates, temperature programming has been necessary to resolve the rhamnitol penta-acetate and fucitol penta-acetate peaks. The analysis time may then exceed 1 hr [e.g. 9, 24]. It was found that complete resolution of these two methyl hexose derivatives was possible on the polyester stationary phase Reoplex-400, although galactitol hexa-acetate and mannitol hexa-acetate were not resolved as well as on the silicone phases OV-225 and OV-275. From the R_is on these two pure phases the optimum phase composition was calculated by graphical interpolation. The proportions chosen were 0.3% Reoplex-400 and 0.6% OV-275 on acid-washed Chromosorb W. The time required to isothermally separate the major cell wall sugars on a 280 × 0.2 cm column of this packing *ca* 15 min or less; resolution was not increased by temperature programming.

EXPERIMENTAL

Potato and onion cell walls and polysaccharide fractions derived from them were prepared as described elswehere [12, 22]. Polygalacturonic acid (Sunkist), chromatographic grade cellulose (Whatman fibrous Chromedia CF12) and surgical grade cotton wool were used in some of the hydrolysis trials. Solvents were generally of analytical reagent grade. CH_2Cl_2 and, on some occasions, toluene were further purified by passing through an Al_2O_3 column (Woelm basic grade I) and stored in glass containers.

Hydrolysis. Pectic polysaccharides carrying Me ester groups were de-esterified with 0.01 M NH_4OH or 0.05 M Na_2CO_3 at 4° for 18 hr before hydrolysis. Under these conditions β-elimination is minimal [12]. Hydrolyses at 120° were carried out in sealed borosilicate glass tubes or in screw-capped test-tubes with teflon-lined caps. Hydrolyses at 100° were mainly carried out in 2 ml flat-bottomed screw-capped vials with liners cut from teflon sheet. The monosaccharides remained in these vials throughout the subsequent derivatisation.

Preparation of alditol acetates. After the hydrolysis of uronic acid-rich polysaccharides, the sugars were dissolved in either 0.25 M or 1 M NH_4OH at 4° prior to the addition of 2–4 mg $NaBH_4$. This mildly alkaline treatment prior to reduction was intended to hydrolyse any uronolactones or Me esters and prevent them from being converted to interfering products [10, 11]. Preliminary trials showed that epimerisation of neutral monosaccharides by the Lobry de Bruyn-Albreda van Eckenstein reaction was not significant under these conditions. After removal of borate by repeated evaporation with MeOH, the alditols were acetylated in Ac_2O (0.3–0.6 ml) for 2.5 hr at 100°. The Ac_2O was removed either as an azeotrope with toluene [23] or by extracting the alditol acetates with CH_2Cl_2 [25]. The alditol acetates were dissolved in 0.2–0.5 ml of Me_2CO before injection. Inositol was added after hydrolysis as an internal standard.

Colorimetric analysis. The PhOH–H_2SO_4 [26] and carbazole–H_2SO_4 methods were used in conjunction for the determination of total (free and combined) neutral sugars and uronic acids. The carbazole reagents of ref. [27] were used in a simplified procedure making use of the heat of dilution of the H_2SO_4. The sample, containing 0–70 μg of galacturonic acid or equivalent in 1 ml of H_2O, was mixed with 0.2 ml of 1.25 mg/ml carbazole in 80% (v/v) EtOH. Reagent grade H_2SO_4 (5 ml) containing Na tetraborate (0.025 M) was added very rapidly to ensure mixing, observing appropriate safety precautions and using matched tubes of at least 14 mm bore to reduce spattering. The tubes were allowed to cool to room temp. away from draughts and the *A* measured after 30 min at 525 nm or in an EEL colorimeter using filter No. 623. Provided that the neutral sugar composition was known the amounts of total neutral sugar and uronic acid could then be calculated by solving simultaneous equations based on the response factors in the PhOH–H_2SO_4 and carbazole–H_2SO_4 methods.

GLC. A FID instrument and all-glass columns (but with metal connectors from end of column to jet) were employed. The column and separation conditions used after preliminary trials were as follows: 2.8 m × 2 mm column packed with 0.3% Reoplex-400 and 0.6% OV-275 coated on acid-washed Chromosorb W, 80–100 mesh, by the fluidiser method [28]. Carrier gas was N_2 at 10 ml/min. Separations were normally under isothermal conditions at 200°. Even after thorough conditioning, the R_is on this column did not become stable until about 6 injections had been made. The packing was protected from air as far as possible when the column was not in use and the first 2–5 cm was repacked occasionally to minimise loss of resolution. Peak areas were taken as proportional to mass of monosaccharide (c.f. [1]) and were usually determined by electronic integration R_is of the alditol acetates relative to inositol hexa-acetate were rhamnose 0.19, fucose 0.2, arabinose 0.26, xylose 0.33, mannose 0.71, galactose 0.80 and glucose 0.86.

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