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A GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC ASSAY FOR GLYCOSYLASES*

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Key Word Index—Carbohydrate-cleaving enzymes; glycosyl hydrolase; GS/MS; xyloglucan; oligosaccharide; auxin; 2,4-D.

Abstract—A GC-MS method was developed to assay enzymes that cleave glycosidic bonds. The method determines the quantity and identity of each reducing sugar released by enzymes from microgram quantities of oligo- and polysaccharides. The method was developed by studying the ability of enzymes in pea stem extracts to hydrolyse xyloglucan and xyloglucan oligosaccharides. Enzymically treated substrates were reduced with sodium borohydride. The glycosidic bonds remaining in the substrates were hydrolyzed with acid and the newly formed glycoses reduced with sodium borodeuteride. The hydrogen- and deuterium-reduced alditols were then acetylated, GC-MS was used to separate the resulting alditol acetates and to determine the amount of each species that was hydrogen- and deuterium-reduced. The ratio of the amount of each alditol that was hydrogen-reduced to that which was deuterium-reduced determined the percent of the glycosidic bonds of each sugar that was enzymically hydrolysed. Enzyme extracts of 2,4-dichlorophenoxyacetic acid-treated pea stems cleaved a greater proportion of the glycosidic linkages of the xyloglucan substrates than did extracts of tissue not treated with the auxin.

INTRODUCTION

Enzymes that hydrolyse glycosidic bonds of complex carbohydrates have been assayed by a variety of techniques. These include viscometric, colorimetric, and reducing sugar assays [1, 2] as well as chromatography of cleavage products of unlabelled and radiolabelled substrates [3]. The existing assays are limited; some require relatively large amounts of viscous substrates or substrates that stain with iodine while the others require the preparation of radiolabelled or chromogenic substrates. Further, the available assays generally do not distinguish between the cleaved glycosidic linkages of the different sugars in complex substrates.

This paper describes a new assay for enzymes that cleave glycosidic bonds. The efficacy of the assay is illustrated by the ability of enzymes present in pea stem extracts to hydrolyse glycosidic linkages of xyloglucan and xyloglucan oligosaccharides.

Xyloglucans are polysaccharides that comprise ~20% (w/w) of dicot and ~5% of monocot primary cell walls [1, 4, 5]. Xyloglucans have a β-4-linked D-glucosyl backbone and sidechains composed of D-xylosyl, D-xylosyl and D-galactosyl, and D-xylosyl, D-galactosyl and L-fucosyl residues [1, 6, 7]. Sidechains composed of D-xylosyl and L-arabinosyl residues have also been reported [8, 9].

Xyloglucan, which binds strongly to cellulose, is thought to function as a structural component of primary cell walls [4, 6]. Evidence has also been obtained that a xyloglucan oligosaccharide can inhibit auxin-induced growth of pea epicotyls [10]. Therefore, it is important to be able to analyse enzymes that release and process xyloglucan oligosaccharides for such enzymes are likely to function in regulation of plant growth and development.

RESULTS AND DISCUSSION

Glycoses can be converted into volatile alditol acetates by reduction with sodium borohydride (or borodeuteride) and peracetylation with acetic anhydride. The volatile alditol acetates can be readily separated and quantitated by capillary GC (FID). The alditol acetates of different sugars can be identified by comparison of their GC retention times to those of standards and by their CI mass spectra obtained by GC-MS. The alditol acetate derivatives of hexoses, pentoses, and deoxyhexoses can be easily distinguished by the masses of their characteristic fragment ions. An abundant fragment ion in the CI mass spectrum of every alditol acetate is formed by protonation of the alditol acetate followed by loss of acetic acid, that is, the $[M+1-60]^+$ ion. Selective monitoring of the $[M+1-60]^+$ ion is the basis of the assay described below.

The assay we have developed depends on sodium borohydride reduction of those glycoses liberated from complex carbohydrates by enzymes. The sodium borohydride reduction is followed by acid hydrolysis of the remaining glycosidic linkages; the glycoses thus released

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are reduced with sodium borodeuteride. The resulting mixture of hydrogen- and deuterium-reduced alditols are acetylated and analysed by GC-MS. All of these reactions are carried out in a single test tube. The degree of hydrolysis of the glycosidic linkages of each sugar is calculated from the relative abundance of the hydrogen- and deuterium-reduced alditols.

The assay for cleavage of glycosyl linkages depends on quantitatively comparing the abundance of the $[M+1-60]^+$ ions for glycoses reduced with sodium borohydride with the abundance of the $[M+1-60]^+$ ions of glycoses reduced with sodium borodeuteride. However, the $[M+1-60]^+$ ion of an aldose reduced with a single deuterium atom has the same mass as the hydrogen-reduced species containing one ^{13}C atom, i.e. the $[M+1-60+1]^+$ ion.

In order to determine the percentage of $[M+1-60+1]^+$ ions of aldoses reduced with hydrogen, seven sugars (two deoxyhexoses, two pentoses, and three hexoses) were mixed, reduced with sodium borohydride, acetylated and analysed by GC-MS (CI). The computer controlling the mass spectrometer was used to integrate the total ion current of each alditol acetate and the ion currents of the $[M+1-60]^+$ and $[M+1-60+1]^+$ ions expected from the alditol acetates of deoxyhexoses (*m/z* of 317 and 318), of pentoses (*m/z* of 303 and 304), and of hexoses (*m/z* of 375 and 376). The ratio of the abundance of the $[M+1-60]^+$ to the abundance of the $[M+1-60+1]^+$ should be the same for any compound containing the same number of carbon atoms, that is, the ratio is the same for the alditol acetates of all deoxyhexoses, for all pentoses and for all hexoses.

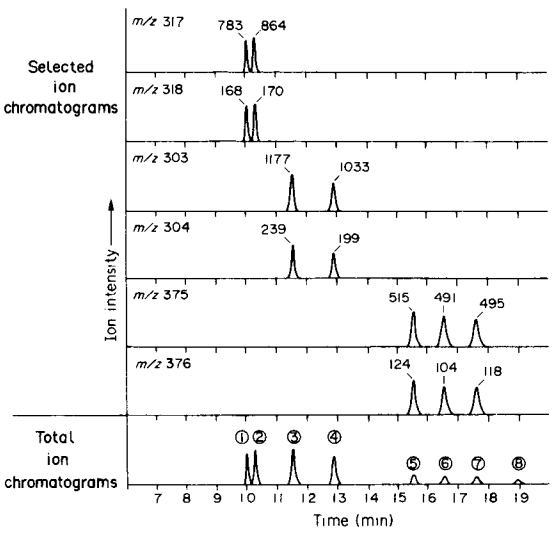

Fig. 1. Results obtained from GC-MS (CI mode), using selected ion monitoring, of hydrogen-reduced alditol acetates of sugar standards. The masses of the $[M+1-60]^+$ fragment ions of deoxyhexoses, pentoses, and hexoses are 317, 303, and 375 amu, respectively, and they are 318, 304, and 376 amu when they contain one ^{13}C atom per alditol acetate ($[M+1-60+1]^+$ ions). The peaks (labelled 1 and 2) in the total ion chromatogram (and selected ion peaks directly above each of those peaks) are the alditol acetate derivatives of the deoxyhexoses rhamnose and fucose, respectively; peaks 3 and 4 are those of the pentoses arabinose and xylose, and peaks 5, 6 and 7 are those of the hexoses mannose, galactose, and glucose, respectively; peak 8 is that of myoinositol, the internal standard. The number associated with each peak in the selected ion chromatograms indicates relative ion abundance.

The relative abundance of the $[M+1-60+1]^+$ ion of hydrogen-reduced alditol acetate (17.0% for deoxyhexoses, 16.5% for pentoses and 18.9% for hexoses, calculated from data) must be subtracted from the abundance of the $[M+1-60]^+$ ions of deuterium-reduced alditol acetates in order to accurately determine the ratio of hydrogen- and deuterium-reduced alditols. (In practice, we have subtracted the relative abundance of the $[M+1-60+1]^+$ ion actually detected by our mass spectrometer, which is close to but not exactly the abundance predicted by calculation from natural isotopic abundance.) An example of the results produced by the mass spectrometric analysis of a single sample, in this case of hydrogen-reduced alditol acetate standards, is shown in Fig. 1.

The utility of the assay was illustrated by treating two xyloglucan substrates (Figs 2a and b) with the enzymes present in extracts of pea stems. The progress of the enzymic cleavage of glycosidic linkages could be followed over time. A portion of each glycosidic linkage in the substrates was hydrolysed (Fig. 3). The results presented were obtained with extracts isolated from a single group of plants, which was divided into two lots, one of which was pretreated with 2,4-D five days before harvest. Indistinguishable results were obtained with extracts isolated from another similar group of plants.

The data presented were corrected for the presence of sugars in pea stem extracts (Table 1) and for the percentage of these sugars which was reducible prior to hydrolysis (Table 2). The amounts of these reducible sugars in the stem extracts did not change with incubation of the extracts without xyloglucan substrates. The data was also corrected for the presence in the oligosaccharide substrate of one reducing glucose residue (Fig. 2b and Table 2). It is impressive that it was possible to determine the proportion of the galactosidic bonds that were cleaved in the xyloglucan substrates in the presence of an amount of galactose in pea stem extracts that was as much as six times the amount of galactose present in the xyloglucan substrates.

It is interesting that only a portion of the glycosidic bonds of the substrates could be cleaved by the enzymes in the extracts. Preliminary data (not presented) indicated that the limited hydrolysis could not be explained by lability of the enzymes or by product inhibition in either the +2,4-D or −2,4-D tissue extracts. It is likely that the oligosaccharide products of the partial enzymic hydrolysis were resistant to further enzymic hydrolysis which, if true, could relate to the biological activity of xyloglucan oligosaccharides [10].

It has been previously reported that enzyme extracts from soybean hypocotyls, when used to treat xyloglucan similar to that used in our study eventually converted all of the polysaccharide to monosaccharide [3, 11]. If all of the glycosidic bonds of the substrates were cleaved in those experiments, the soybean extracts must have contained enzymes not present in our pea stem extracts.

Treatment of the pea seedlings with 2,4-D five days before preparing the enzyme extracts resulted in the extracts having an ability to hydrolyse a greater portion of galactosidic, glucosidic, and xylosidic bonds (Fig. 3). Our data suggest that the extracts of the 2,4-D pretreated tissues had a different array (mixture) of enzymes.

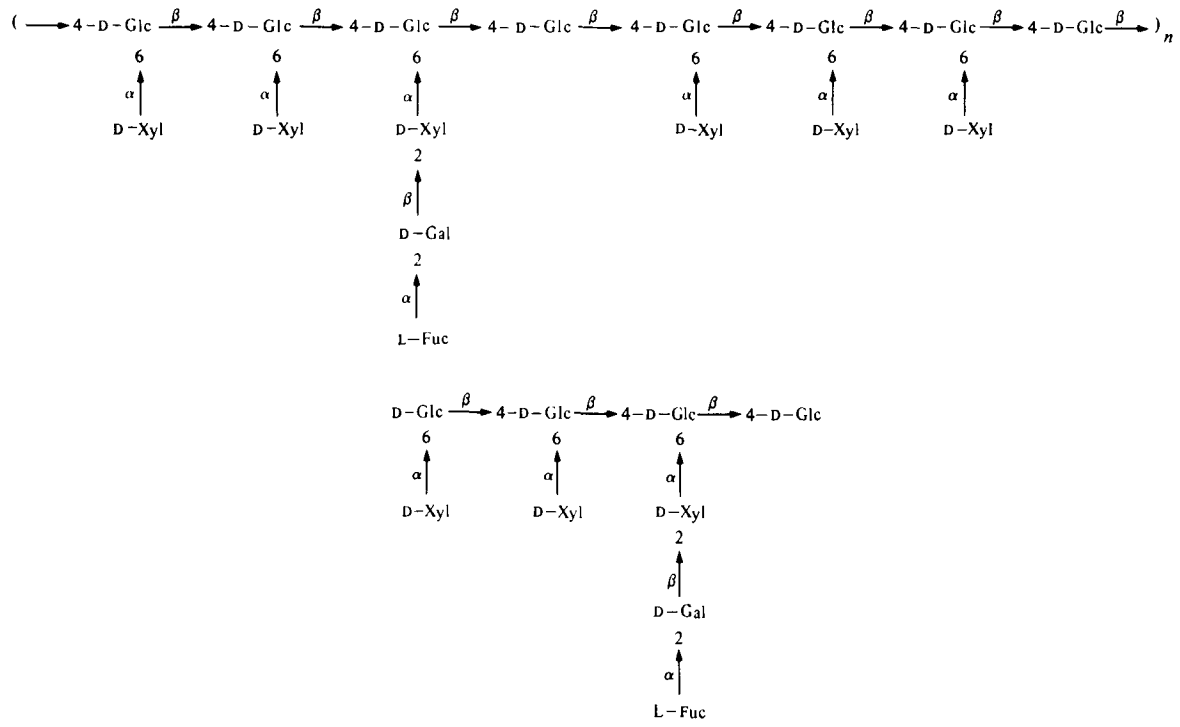

Fig. 2. Structures of the two xyloglucan substrates. The structure of the xyloglucan polymer is idealized. It appears that the distribution of the nonasaccharide and heptasaccharide constituents of xyloglucan is primarily in alternating sequence [1]. Some variation in the glycosyl residues attached to the xylosyl residues is known to exist. The oligosaccharide substrate is a mixture of closely related oligosaccharides enriched (~90%) in the nonasaccharide shown.

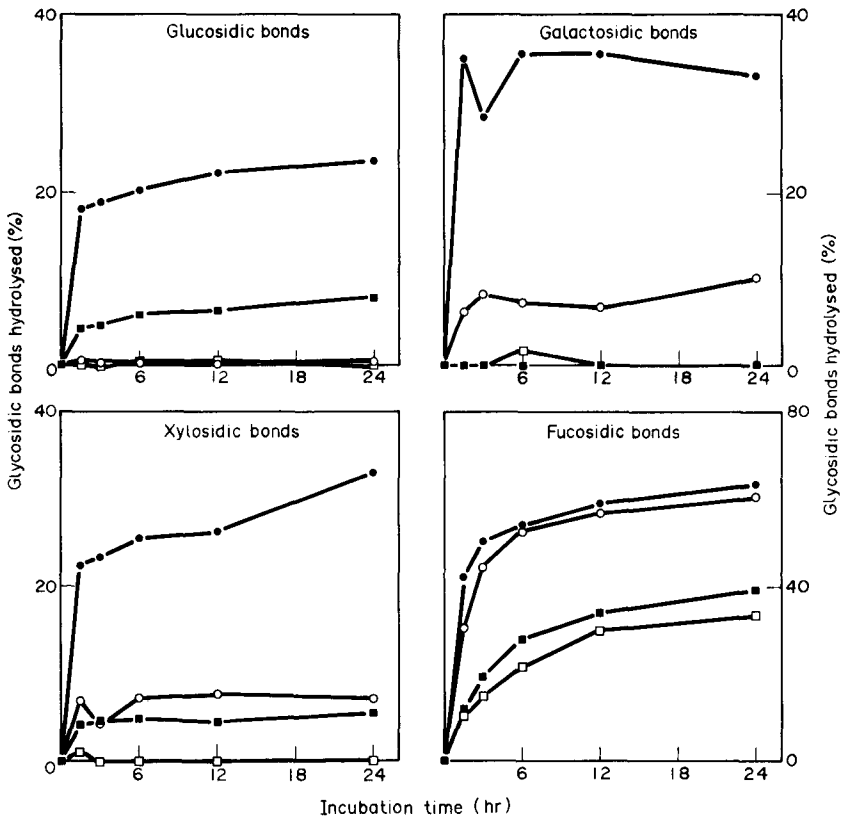

Fig. 3. Effect of time on the percent of the glucosidic, galactosidic, xylosidic and fucosidic bonds of the xyloglucan substrates that are cleaved by the pea stem extracts. Xyloglucan polymer (squares), and oligosaccharide (circles) were treated with extracts of stem tissue from 2,4-D-treated (closed symbols) and non-treated (open symbols) pea plants.

Table 1. Amount (μg) of each glycose per 100 μg of substrate and per 400 μl of an extract of pea stems that were either pretreated or not pretreated with 2,4-D [The glycoses were quantitated by GC (FID)]

Glycosyl residue	Substrate		Pea stem extracts	
	Xyloglucan	Oligosaccharide	Pretreated with +2,4-D	Not pretreated with −2,4-D
Fucosyl	6.8	9.6	3.9	Trace
Galactosyl	9.1	12.4	59.5	19.2
Glucosyl	51.8	48.7	6.8	Trace
Xylosyl	32.7	29.3	5.0	2.9

Table 2. Percent of glycoses in substrates and in pea stem extracts that were reducible prior to hydrolysis

Glycosyl residue	Substrates		Pea stem extracts	
	Xyloglucan	Oligosaccharide	Pretreated with +2,4-D	Not pretreated with −2,4-D
Fucosyl	0	0	26	—
Galactosyl	0	0	8	7
Glucosyl	0	25	50	—
Xylosyl	0	0	0	0

EXPERIMENTAL

Plant tissue. Pea seedlings were grown as described [12]. Twelve-day-old etiolated stem tissue was harvested 5 days after spraying with a soln containing 4.5×10^{-3} M 2,4-D, 0.1% Tween 80 and 0.1 M NaCl, pH 7.0, or with an otherwise identical soln not containing 2,4-D [12].

Enzyme extract. All procedures were carried out, where possible, in an ice bath and otherwise at 2–4°. Harvested pea tissue (100–200 g) was homogenized in a blender in a vol. of buffer equal to twice the weight of tissue. The extraction buffer was 1 M NaOAc, pH 5.2, containing 0.05% sodium thimerosal (Sigma). The homogenate was passed through 200 mesh nylon cloth and the filtrate centrifuged at 13000 *g* for 30 min. A portion (5 ml) of the resulting supernatant was subjected to gel-permeation chromatography to remove low M_r components (Bio-Gel P-6, 100–200 mesh, 1.5 × 48 cm bed, pre-equilibrated with 50 mM K-Pi, pH 6.0, containing 0.05% sodium thimerosal). Protein-containing material was separated from low M_r carbohydrates by this chromatography, and was pooled as enzyme extract. Protein content was determined by the method of Bradford [13] using bovine liver catalase (Sigma) as the standard. Sugar in the P-6 column eluant was determined by the anthrone method [14].

Substrates. Xyloglucan was purified from suspension-cultured sycamore cell extracellular polysaccharide as described [6]. A monasaccharide-rich fraction was derived from the xyloglucan by β-1,4-endoglucanase hydrolysis and purified by gel-permeation chromatography as described [15]. Structures of the substrates are shown in Fig. 2.

Enzyme assays. Enzyme reaction mixtures contained 400 μl of extract obtained from ~ 170 mg of fresh pea stem tissue. The mixtures also contained 100 μg substrate, 50 mM K-Pi, pH 6.0, and 0.05% sodium thimerosal. The amount of protein present in the reaction mixtures was 0.26 mg when using extracts of seedlings not treated with 2,4-D and 2 mg with extracts of 2,4-D treated seedlings. Reactions, in 13 × 100 mm tubes fitted with teflon-lined caps, were incubated at 35° with agitation. The reactions were terminated by freezing at −20°. Samples containing extracts in the absence of substrate were incubated and analysed in order to determine the amount of carbohydrate in the enzyme extracts and the amounts that were hydrogen- and deuterium-reduced.

Formation of alditol acetates. Frozen reaction mixtures were thawed upon addition of 250 μl 1 M NH_4OH containing 2.5 mg $NaBH_4$ to convert reducing sugars to hydrogen-reduced alditols [14]. The reduction reaction was allowed to proceed for 2 hr before quenching with glacial HOAc [14]. The mixture was warmed to 40° and dried by evapn using a stream of filtered air. Further drying was achieved by twice adding and evapg 1 ml of *iso*-PrOH. Borate was removed by four additions and evapns of 1 ml of 10% HOAc in MeOH followed by four additions and evapns of 1 ml of dry MeOH.

The remaining glycosidic bonds in each sample were hydrolysed by adding to each tube 0.5 ml 2 N TFA containing 50 μg myo-inositol (as an int. standard). The tubes were sealed and heated for 2.5 hr at 121°. The reaction mixtures were then dried as above and reduced with 5 mg $NaBD_4$ in 0.5 ml 1 M NH_4OH. The reduction was allowed to proceed for 1 hr at room temp. The samples were then dried and borate removed as described above.

The resulting mixture of hydrogen- and deuterium-reduced alditols was per-*O*-acetylated by adding 5 drops pyridine and 5 drops Ac_2O and heating the mixture in a tightly capped tube for 20 min at 121°. The resulting alditol acetates were evapd to dryness at room temp. in a stream of filtered air. Further drying was achieved by two additions and evapns of 1 ml of toluene.

Water (1 ml) and CH_2Cl_2 (1 ml) was added to each sample and the tubes shaken vigorously. After the solvents separated, the alditol acetate-containing CH_2Cl_2 fraction was removed and evapd to dryness. The alditol acetates were then dissolved in 40 μl Me_2CO in preparation for analysis by GC-FID and GC-MS.

GC-FID analysis. Representative samples were analysed by GC. Inositol was used as an int. standard (see 'Formation of alditol acetates'), and samples containing known amounts of standards were used to calculate response factors for each alditol acetate. For this work a 15 m fused silica (Supelco) column was used isothermally at 220° in the splitless mode, 25 ml/min H_2, and 250° injector and detector.

GC-MS analysis. Approximately 1 part in 40 of each sample was separated and analysed by GC-MS using a 30 m Supelco SP-2330 fused-silica column in the splitless mode, 25 ml/min H_2, 250° injector, and a temp. program of 80° for 2 min, increasing 30°/min to 240°, and holding at 240° until the end of the 20 min program. The mass spectrometer was operated in the CI mode using isobutane as the ionization gas.

The ratio of hydrogen- to deuterium-reduced alditol acetates arising from the L-fucosyl, D-galactosyl, D-glucosyl and D-xylosyl residues present in samples was determined using SIM of the appropriate masses for the $[M+1-60]^+$ ions (see Results and Discussion). The observed ratio was corrected for the contribution of the ^{13}C isotope, for the presence of a reducing glucosyl residue on the oligosaccharide substrate, and for the presence of sugars in the enzyme extracts.

While determination of the ratios of hydrogen- to deuterium-reduced alditols in samples was reliably achieved by mass spectrometry, this technique does not give precise quantitation of the *amounts* of alditol acetates present. The amounts of alditol acetates and the ratio of hydrogen- to deuterium-reduced species in samples can be accurately obtained, respectively, from GC (FID) and MS. Thus, the amounts of each hydrogen-and deuterium-reduced species can be accurately calculated. The following equation was used to determine the extent of enzymic cleavage of each type of glycosidic linkage:

$$\frac{a\,(b+c)-(dc)}{b} = \%\ \text{enzymically hydrolysed}$$

where a = percent hydrogen reduced alditol acetate (determined by MS), b = μg of each sugar in the substrate (determined by FID), c = μg sugar in the pea stem extract (determined by FID), and d = percent hydrogen reduced alditol acetate in pea stem extract (determined by MS). Corrections for the reducing glucose (25%) in the oligosaccharide substrate and for the natural isotopic abundance of ^{13}C were made prior to substituting the data into the formula.

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