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SEPARATION OF MACROMOLECULAR COMPONENTS OF PLANT CELL WALLS: ELECTROPHORETIC METHODS

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Abstract—Qualitative, quantitative and preparative electrophoretic methods of separating polymeric substances derived from plant cell walls are described. Analytical electrophoresis is carried out on glass-fibre paper trimethylsilylated to alleviate the problem of endosmotic flow, and wetted with the help of a surfactant. Carbohydrate-, polypeptide- and hydroxyproline-containing substances can be located on this material using specific spray reagents and phenylpropanoid derivatives by their UV fluorescence. Preparative column electrophoresis is carried out on a urea density gradient.

INTRODUCTION

In the past few years a clearer picture of the macromolecular construction of plant cell walls has been emerging, hastened by numerous advances in analytical technique. New methods of isolating [1,2] and fragmenting [3–7] cell walls have been introduced and once the polymeric fragments have been brought into solution and separated from one another they can be examined by a much wider range of degradative procedures (e.g. [8–10]) than was formerly available. At the same time the older technique of methylation analysis of polysaccharides has been made more efficient and much more convenient [3,11], while there are signs that the direct examination of wall polymers by physical methods may become more important in the future [2,12–14].

By comparison, the separation and purification of polymers derived from plant cell walls have received little attention recently, although some new methods have appeared and are of undoubted value (e.g. [15–19]). There is now a great deal of interest in covalent linkages between components of the wall that were once thought to be unconnected, e.g. carbohydrates and proteins. Before it is assumed that two such components which cannot be separated are covalently linked to one another, it must be ensured that the resolution of the separation techniques used is adequate. In such cases the strictest criteria of purity and homogeneity should clearly be applied. With the methods currently available this is difficult, although not impossible, (e.g. [3,18,20–22] and other examples) to ensure.

We have therefore attempted to develop a set of techniques for separating plant cell wall polymers easily at the required level of resolution, on the analytical as well as the preparative scale. Quantitative as well as qualitative analysis was considered desirable to allow changes within the wall to be studied by direct examination of its constituent polymers. Further requirements were that the polymers were to be separated without prior modification (such as methylation [9] or dyeing [15,16] in the case of polysaccharides); and that the apparatus required was to be as simple as possible without sacrifice of performance, as in some parts of the world sophisticated instrumentation is neither readily available nor easy to maintain. Fractionation methods involving more than one molecular property, including chromatography on most polysaccharide-based ion exchangers, were considered less suitable in view of the continuous variation in both the size and the composition of at least some cell wall polymers [4,9,17,21]. Instead, molecular-sieve chromatography (MSC) and electrophoresis were used separately. In this paper the electrophoretic methods are described. Existing MSC procedures are satisfactory for most purposes, but it is hoped that some improvements will be described in a later publication.

RESULTS

Qualitative analysis

In preliminary trials with a number of support materials, the best results were achieved with glass-fibre paper. Its large pore size eliminated molecular sieving effects such as occur in polyacrylamide gels and cellulose acetate membranes, and because it was inert there were no restrictions on the detection methods that could be used. However, the high rate of endosmotic flow in unmodified glass-fibre paper [23] was a serious disadvantage.

It was found that electroendosmosis could be almost eliminated by treating the glass-fibre paper with a trimethylsilylating agent. The trimethysilylated glass fibre paper (TGFP) would float indefinitely on water, but could be wetted in buffer to which a surfactant had been added. Endosmotic flow through this support material was negligible at pH 5, and too slight to cause any problems at pH 9 under the conditions used.

A variety of polysaccharides, proteins and lignin–carbohydrate complexes have been separated on this material, in a standard tank designed for cellulose acetate membrane electrophoresis (Shandon Ltd). Hemicellulosic xylans and lignin–carbohydrate complexes tended to aggregate in solution and remain at the origin, but this could be prevented by heating for 10 min at 100° immediately beforehand [25] and pre-equilibrating the TGFP strips at the running voltage for the same length of time: the elevated running temperature (*ca* 40–60° depending on the conditions) prevented re-aggregation. Otherwise no adsorption problems were encountered, but if the separation of bacterial lipopolysaccharides or similar polymers is contemplated the possibility of hydrophobic interactions with the TGFP should be kept in mind.

A modification of the sulphonated α-naphthol reagent of Barrett and Northcote [23] located polysaccharides with good sensitivity. Proteins could be detected by staining with Amido black 10B, but the stain was not readily removed from pectic acids if these were present: a more satisfactory procedure was to hydrolyse with H_2SO_4 on the TGFP and spray with ninhydrin. Hydroxyproline-containing polymers could be detected, with lower sensitivity, by substituting isatin for ninhydrin and overspraying with acid Ehrlich's reagent.. Carbohydrates were hydrolysed with H_2SO_4 in the same way, and the resulting monosaccharides were eluted with MeOH for chromatographic characterisation. Lignin-carbohydrate complexes were located by their UV fluorescence, most of which coincided with the α-naphthol positive band although there was slight tailing towards the anode (cf. [4] and [20]).

Quantitative analysis

Polymer bands separated on TGFP were located, prior to quantitative assay, by running separate narrow marker strips which were dried and sprayed as described above. This allowed the main strips, run alongside them, to be kept wet until the bands had been located and cut out (re-wetting partially dried TGFP is difficult). Diffusion during this time was slight, and migration distances differed by ±4% or less between strips run at the same time.

In principle any appropriate assay method can be used after elution of the polymers from the TGFP wth water, the main limitation being the sensitivity available. Up to 50 μg of polymeric material could be separated on strips 10 cm wide, but the limit for strongly charged polysaccharides was lower. Quantitative analysis of carbohydrate-containing substances was possible without elution from the support: standard colorimetric procedures were adapted for use in the presence of the shredded TGFP, which was filtered off before the absorption was measured. The PhOH–H_2SO_4 method was not sensitive enough to be used in this way, but the sulphonated α-naphthol method of Devor [26] and the carbazole method for uronic acids [27] gave satisfactory results with 10–50 μg of carbohydrate. The TGFP did not increase the blanks but tended to reduce the sensitivity.

Preparative electrophoresis

Preparative density-gradient electrophoresis on a mg scale was carried out in a standard water-jacketed column of 110 ml capacity, designed for isoelectric focusing (LKB Produkter AB). Urea gradients were used: their maximum density was less than could be obtained with sucrose and their zone stabilising capacity was therefore reduced, but the urea did not interfere when the fractions collected on draining the column were assayed for carbohydrate by the PhOH–H_2SO_4 method or examined by electrophoresis on TGFP.

The buffers used were more dilute than in electrophoresis on TGFP, the potential gradient was smaller and running times were longer. Nevertheless, the results were comparable, as can be seen from Figs. 1 and 2. This helps to avoid confusion when a preparative separation is monitored on the analytical scale. The maximum loading advisable with this procedure was *ca* 5–20 mg (the lower figure applies to pectic acids.)

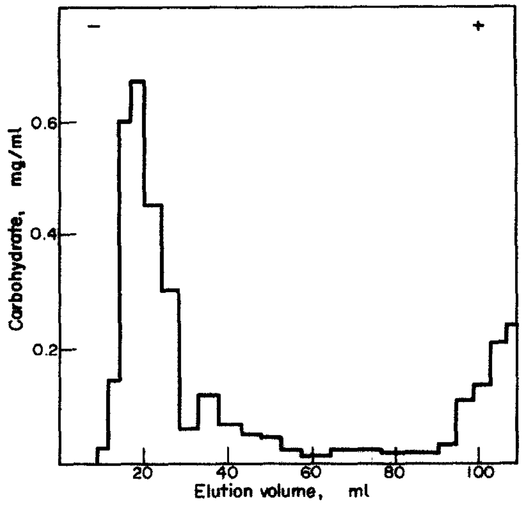

Figure 1. Preparative separation of de-esterified potato tuber pectin by density-gradient column electrophoresis. Conditions: Acetate–EDTA buffer, 0.02:0.004 M, pH 5, Voltage, 100V. Temperature, 15°. Running time, 13 hr. Fractions assayed for carbohydrate by PhOH–H_2SO_4.

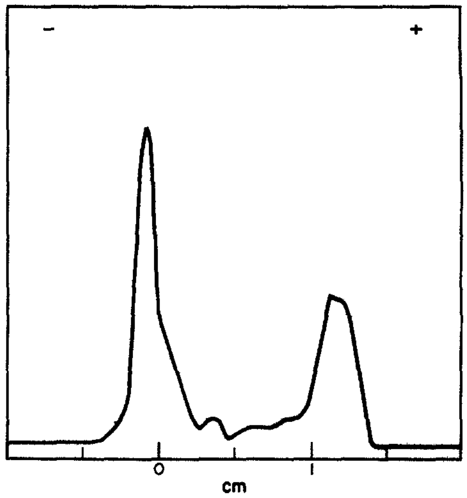

Figure 2. Rapid qualitative separation of de-esterified potato tuber pectin on TGFP. Conditions: Acetate–EDTA buffer, 0.1:0.02 M, pH 5. Potential gradient, 13V cm^{-1}. Running time, 10 min. Location by sulphonated α-naphthol spray and reflectance scan.

DISCUSSION

When the mechanism of electroendosmosis was considered it was obvious how the problem of endosmotic flow in unmodified glass-fibre paper could be overcome. Briefly, the surface of the glass fibres carries fixed negative charges. These induce a net positive charge in the shallow overlying layer of buffer, which is drawn towards the cathode when a voltage is applied. This endosmotic flow can be reduced by using a buffer in which the cation, e.g. $C_5H_6N^+$ [23], interacts with the surface charges; or it can be nearly eliminated by chemically modifying the glass surface so that its fixed charges are blocked. The latter approach was adopted here.

In density-gradient electrophoresis, the urea may help to prevent aggregation in addition to forming the gradient. However, while urea and other chaotropic solutes in water are highly efficient in disaggregating proteins, their effect on at least some other cell wall polymers seems relatively slight (see [4] for example. This is supported by our own experience with charged polysaccharides [28]).

Preparative electrophoresis of macromolecules other than polysaccharides has not been attempted here: it would not be expected to cause problems, but superior resolution of proteins (which have defined isoelectric points) can of course be obtained by existing isoelectric focusing techniques.

The analytical and preparative methods described above are now being used in the study of cell wall structure. Analytical-scale electrophoresis has made it very simple, for instance, to follow the removal of particular components from the wall during sequential extraction with different solvents, and the progressive de-esterification of pectins by mild alkaline treatment (see Table 1) [28]. The same technique promises to be useful when fractions from different wall samples are compared. However, some caution is necessary here. The macromolecules to be separated are not always as clear-cut and definable as proteins, for example, or monosaccharides on a PC. Wide and continuous variation in the structure and properties of plant polysaccharides is an established fact, even if in many cases it results from partial degradation during the extraction process. Some polymeric 'components' may need to be described not only by their electrophoretic mobility but by the dispersion their mobility shows; and the borderline between pairs of such 'components' may be indistinct. In general it would seem inadvisable to rely on electrophoretic methods in isolation, without some further characterisation of the individual polymers being separated.

Table 1. De-esterification of potato tuber pectin with Na_2CO_3, monitored by electrophoresis on TGFP. Acetate–EDTA buffer, pH 5; potential gradient 9V cm^{-1}

Duration of Na_2CO_3 treatment (min)	Electrophoretic mobility* (cm/min) Acidic component	Neutral component
0	0.024	0
1	0.034	0
4	0.046	0
16	0.054	0
60	0.064	0
120	0.067	0

* Towards the anode. Mobilities of standard polysaccharides under these conditions: blue dextran 0.008 cm/min, citrus galacturonan (citrus polygalacturonic acid) 0.067 cm/min.

EXPERIMENTAL

Materials for trial separations. Lupin seed pectin was prepared as described in ref. [29]. Potato pectin was extracted with cold H_2O from cell walls of potato tubers (var. King Edward), and contained a galactan and a highly esterified galacturonan [28], which was de-esterified by treating a 5 mg/ml soln with Na_2CO_3 (0.05M); de-esterification was complete after 2 hr at room temp. [28]. Aquilinan was prepared earlier [30]. Other polysaccharides used were of various commercial origins. BSA and citric acid-soluble collagen were obtained from Sigma, London. Lignin-carbohydrate and lignin-hemicelluose complexes were extracted from timothy (*Phleum pratense* L.) using methods given in ref. [4].

Buffers. (a) 0.1 M acetate pH 5.0 containing 0.02 M EDTA. (b) 0.1 M Tris–acetate pH 6.5, containing 0.02 M EDTA. (c) 0.05 M Na tetraborate, pH 9. (d) 0.02 M molybdate, pH 4.8 (cf. [31]). Buffer (a) was used for general work, particularly with charged polysaccharides; (b) for lignin–carbohydrate complexes; (c) and (d) for neutral polysaccharides. The same buffers, diluted by a factor of *ca* 3, were used for preparative separations.

Preparation of TGFP. Glass-fibre paper (Whatman GF/C) was cut into strips usually 15 cm long, and heated at 400° for 2 hr to burn out organic material [23], then immersed for 18 hr in a glass dish of CCl_4 containing 2% dimethyldichlorosilane 'Repelcote' Hopkin & Williams Ltd.), rinsed in toluene and dried. The TGFP strips were wetted by floating for 18 hr in trays of the appropriate buffer, to which Tween-20 (0.2% by vol.) had been added.

Analytical electrophoresis. TGFP strips were blotted only lightly before being placed in the tank. Before application of the sample it was often found advantageous to pre-equilibrate for 10–20 min at the running voltage. The sample, usually 1–5 μg in *ca* 2 μl of buffer for qualitative work, was applied at or near the mid-point of the strip as a band 1–2 cm long. Separation conditions were; potential gradient 3–10 V cm^{-1}, current 1–2.5 mA cm^{-1} width of strip, running time 10 min–6 hr. To limit evaporation the power input was not allowed to exceed 8 mW cm^{-2} unless the running time was very short.

Evaporation drew the buffer from both ends towards the centre: this effect was utilised in checking for adsorption onto the support, by applying an aliquot of the sample 3 cm from each end of the strip and separating for a short time at 20 V cm^{-1}. Obvious differences between the two separation patterns, or material remaining at both application points, indicated some interaction with the support.

Location and quantitation. When the separation was complete the strips were immersed in EtOH to immobilize the polymers and dried in hot air. When it was necessary to remove the buffer this was done by repeated soaking in EtOH (MeOH for molybdate) and blotting with filter paper. Carbohydrate-containing substances were located by spraying with α-naphthol (200 mg α-naphthol and 1 ml H_2SO_4 in 50 ml MeOH) (cf.[23]), and heating for 10 min at 110° (Sensitivity, 200 ng). Residual molybdate was reduced to give a blue background; this could be prevented by thorough washing with MeOH. It was also possible to hydrolyse polysaccharides with H_2SO_4 (0.35 M in 95% MeOH) on the TGFP. The strips were sprayed after removal of the buffer and heated for 1 hr at 100°. Monosaccharides were extracted from appropriate areas of the strip by shaking with 80% MeOH (2 ml) and $CaCO_3$ (*ca* 1 mg). The paper and salts were filtered off under suction and the filtrate evaporated for chromatography of the sugars. Both of these reagents could also be applied by dipping.

Proteins and glycoproteins were stained with 0.1% amido black 10B, MeOH (5 vol.) being added to both staining and washing solns. Better results were achieved by hydrolysing with H_2SO_4, as described above, and then spraying with ninhydrin

(0.2% w/v in Me_2CO, with 15% v/v C_5H_5N added immediately beforehand (cf. [32]). Excess C_5H_5N was removed with hot air in a fume cupboard. Heating for 3 min at 100° gave a red colour with polypeptides. For hydroxyproline, isatin was used instead of ninhydrin: the procedure was the same except that it was preferable to continue the acid hydrolysis for 18 hr. The TGFP was then oversprayed with acid Ehrlich's reagent [32] and a red colour developed after 5 min at room temp. Phenylpropanoid-derived material was located by its UV fluorescence after removing the buffer and fuming with NH_3. Except in this case permanent records of electrophoretic separations were made using a TLC scanner (Joyce-Loebl Ltd.) in the reflectance mode. For quantitation, bands were cut out as necessary and the polymers eluted with H_2O using a wick arrangement. Alternatively, quantitative assay of polysaccharides was carried out by cutting the wet bands, carrying 10–50 μg of carbohydrate, into small pieces with a razor and shaking vigorously with 1 ml of H_2O. The sulphonated α-naphthol [26] or carbazole [27] reagents were than added and the assay carried through in the normal way. Dark streaks on the TGFP indicated when wetting or mixing was not adequate. The TGFP was filtered off on sintered glass or glass-fibre paper before measurement of A. For calibration, known amounts (0–50 μg) of starch or polygalacturonic acid in 0.25% solns were applied to TGFP with a syringe and the above procedure followed.

Preparative methods. Electrophoresis was carried out in a 110 ml vertical column with a 0–50% (w/v) linear gradient of urea; the cathode was at the lower end. Separate anode and cathode reservoir electrolytes were not used, but might be required for separations lasting more than 16 hr [33]. The sample was applied in a vol. of 5 ml, 8 cm above the cathode. The density of the sample soln was adjusted as follows: 10 ml of the gradient was collected, mixed, and weighed in a volumetric flask. The sample was dissolved in 3.7 ml of buffer 1.35 × normal strength and 1.8 g of urea added. H_2O and urea were then added or removed as necessary in a 5 ml volumetric flask. The temp. was allowed to return to ambient after each addition of urea. The sample soln was then pumped onto the column and the gradient resumed on top of it. Separation conditions; potential gradient 2–4 cm^{-1}, total power input not more than 1 W, running time 10–16 hr. When the column was drained fractions (normally 3 ml) were collected and assayed for carbohydrate by $PhOH–H_2SO_4$. Polysaccharides were recovered by pptn with EtOH (3 vols.).

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