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DISC ELECTROPHORESIS OF SOLUBLE PROTEINS OF CONIFER FOLIAGE

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Abstract—A method is described for separation of protein bands from low-protein content conifer foliage containing high levels of interfering phenolic substances. Phenolic–protein complexes are dissociated by use of 8 M urea and this is followed by removal of proteins from the solids, and their separation, by discontinuous electrophoresis on polyacrylamide gels containing 3 M urea and run at low pH, so that the phenolics do not migrate. The simplicity of sample preparation makes the method practical for large numbers of samples.

INTRODUCTION

REPORTED separations of protein from conifer tissues appear to be confined to pollen,[1] seeds[2] and buds.[3] These have low levels of interfering substances and their high-protein content favors ease of extraction. In contrast, conifer foliage is low in protein and high in phenolic substances which form insoluble H-bonded complexes with protein.[4] These complexes resist extraction and prevent electrophoretic separation of proteins. This report describes techniques which have partially overcome these problems and permit separation of a dozen or more protein bands from foliage of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco.) and other conifers. We are presently utilizing these techniques for semi-quantitative comparisons of protein changes in Douglas fir, following cone-inducing and non-cone-inducing nitrogen fertilizer treatments.

RESULTS

Our attempts at extraction of Douglas fir foliage protein by published techniques, shown to be useful for a variety of other plant materials, were all unsuccessful. These included: (1) Use of insoluble polyvinylpyrrolidane (PVP) in a 0·1 M phosphate extracting buffer, pH 7·3, with or without use of 0·2 M sodium ascorbate or 0·1 μM dithiothreitol as reducing agents.[4,5] (2) Defatting before extraction with cold acetone; with cold acetone–petroleum ether (4:1, v/v)[2] or with cold acetone–water (5:1, v/v).[6] (3) Attempts at phenolic removal preceding protein extraction by use of cold dimethylsulphoxide,[4] or by homogenizing with basic alumina or with anionic resins (Dowex 1-X8, 2-X8, and 3) in 0·1 M phosphate buffer, pH 7·3.[7] In all three cases, extracts from 10 g fr. wt. of foliage had been concentrated to 2 ml

[1] W. E. BINGHAM, S. L. KRUGMAN and E. F. ESTERMANN, *Nature* **202**, 923 (1964).
[2] D. J. DURZAN, *Can. J. Botany* **44**, 359 (1966).
[3] T. O. PERRY, *Am. Inst. Biol. Sci. Bull.* **13**, 73 (1963).
[4] W. D. LOOMIS and J. BATTAILE, *Phytochem.* **5**, 423 (1966).
[5] B. H. MCCOWN, G. E. BECK and T. C. HALL, *Plant Physiol.* **43**, 578 (1968).
[6] D. S. BENDALL and R. P. F. GREGORY, in *Enzyme Chemistry of Phenolic Compounds* (edited by J. B. PRIDHAM), p. 7, Pergamon Press, Oxford (1963).
[7] M. A. STAHMAN, *Ann. Rev. Plant Physiol.* **14**, 137 (1963).

by use of dry Sephadex G-25 or by freeze-drying, and extracts were run on basic discontinuous polyacrylamide gels.[8]

These methods produced seed extracts which heavily overloaded the gels, and extractions with PVP plus dithiothreitol produced bud extracts with detectable protein. The lack of success with Douglas fir foliage seemed attributable to a high phenolic/protein ratio, as compared to that of seeds and buds. Interference by phenolics was confirmed by running PVP–dithiothreitol–0·1 M phosphate buffer, pH 7·3, extracts of defatted, newly flushed foliage on cellulose acetate electrophoresis strips at pH 8·0. Ferric chloride staining revealed a heavy band of phenolics at the origin and another half-way along the strip. Amido black staining showed that practically all the protein had stayed at the origin; a faint protein streak just behind the phenolics had migrated.

Perry[3] placed acetone powders of *Pinus thunbergii* L. buds directly on basic discontinuous polyacrylamide gels and separated their proteins. The proteins of Douglas fir foliage did not migrate under the conditions of this method. However, when fresh or freeze-dried foliage was homogenized with small amounts of 8 M urea and the slurry applied directly, much unseparated protein ran into the gel. This means of dissociating the phenol–protein bonds was suggested by Anderson and Sowers'[9] demonstration of the efficiency of 8 M urea for breaking hydrogen bonding between PVP and phenolics.

Increasing the concentration of the running gel to 16%, from the initial 7·5%, produced faint, streaky bands close to the electrophoresis front. Along with the proteins ran brownish compounds which darkened as the run proceeded, indicating that under basic gel buffer conditions some phenolics were migrating and being oxidized, with possible re-bonding with proteins causing the inadequate protein separation. By employing acid pH conditions, set up according to the rules presented by Lindh and Brantmark,[10] ionization and migration of the phenolics was suppressed and proteins were separated as cations.

Virtually no separation was achieved when acid pH conditions were tested with non-discontinuous gels. Separation was reduced when urea was omitted from either the spacer gel or the running gel, or when 8 M urea was used in place of 3 M urea in the gels. Defatting the tissue, by homogenizing with cold acetone before making up the urea slurry, slightly decreased the protein yield from freeze-dried foliage and caused minor changes in the relative intensities of bands from fresh foliage. Freeze-dried foliage gave protein patterns identical to those from fresh foliage except that bands were slightly more diffuse.

Migration of protein from the sample slurries was increased with increasing urea concentrations up to 8 M, but was not further increased by use of a saturated solution for slurry preparation. In contrast, protein extraction from inner bark required saturated urea solution containing 15 mg sodium ascorbate/ml. Undissolved urea crystals introduced with the extracting solution further prevented oxidation of bark phenols during slurry preparation, and increased protein yield. It was essential to immediately cover freshly collected disks with the extracting solution.

Four extractions of freeze-dried foliage with 8 M urea were sufficient to remove all protein. Further extracts did not contain protein in quantities detectable by electrophoresis. However, samples applied as extracts did not produce better resolved bands than those applied as slurries, and protein was diluted. Concentration of extracts would have involved

[8] L. ORNSTEIN, *Ann. N. Y. Acad. Sci.* **121**, 321 (1964).
[9] R. A. ANDERSEN and J. A. SOWERS, *Phytochem.* **7**, 293 (1968).
[10] N. O. LINDH and B. L. BRANTMARK, in *Methods of Biochemical Analysis*, Vol. 14, p. 79, John Wiley, New York (1966).

time-consuming extra steps with potential losses. Slurries were thus preferred for rapid semi-quantitative work.

Satisfactory resolution of proteins was dependent upon use of a small pore running gel (16%) which, in acid-buffered gels, reduced the rate of migration of the fastest moving band to about R_f 0·26. Thus, extended running times (2–3 hr) were necessary with the result that the front disappeared from the gel; with shorter runs, a frontal band was always present. This band had a remarkably consistent intensity for samples of a given species. It gradually diffused, and disappeared on long standing after staining, suggesting lower molecular weight materials, possibly peptides. Basic amino acids stained strongly with amido black at R_f 0·65, but were rapidly lost by diffusion during normal destaining. Therefore, they were undetected

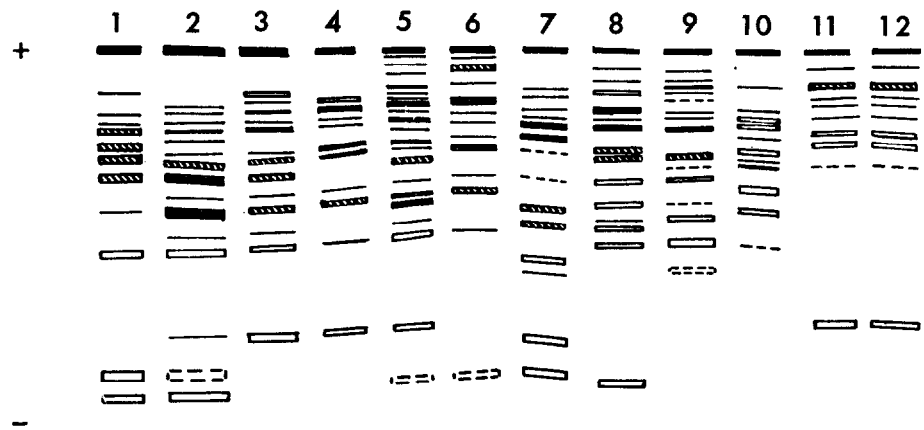

FIG. 1. POLYACRYLAMIDE DISC ELECTROPHORESIS PATTERNS OF SOLUBLE PROTEIN FROM CONIFER FOLIAGE, APPLIED AS 1:5 SLURRIES IN 8 M UREA.

Spacer gel 4% polyacrylamide, pH 4·35; running gel 16%, pH 3·35; 120 min run. The numbers refer to the following materials: Freeze-dried Douglas fir foliage, of various ages from the same tree: (1) new flush; (2) 2 weeks old; (3) 6 weeks old; (4) 12 months old. Fresh, 8-month-old (December) conifer spp. foliage: (5) Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco); (6) White spruce (*Picea glauca* (Moench) Voss); (7) Grand fir (*Abies grandis* (Dougl.) Lindl.) (8) Western white pine (*Pinus monticola* Dougl.); (9) Western red cedar (*Thuja plicata* Donn); (10) Western hemlock (*Tsuga heterophylla* (Raf.) Sarg.); (11) and (12) duplicate December samples of Douglas fir inner stem bark, prepared as in text, for comparison with Douglas fir foliage (sample 5).

unless electrophoretic destaining times were very short and foliar amino acid levels abnormally high, as occurs following heavy nitrogen fertilization.

Examples of the type of protein separations possible are shown in Fig. 1. Changes in protein patterns as foliage matured are shown by samples 1–4, and protein differences between conifer species by samples 5–10. Variation in protein composition between tissues is illustrated by comparison of samples 11 and 12, inner stem bark of Douglas fir, with sample 5, foliage collected at the same time from the same tree.

DISCUSSION

The final procedures adopted, based on dissociation of phenolic–protein complexes with minimal quantities of 8 M urea, direct sample application as a slurry, and use of low pH, small pore, discontinuous polyacrylamide gels containing 3 M urea, minimized interference from

phenolic substances and permitted useful separations of soluble proteins from conifer foliage. Urea will disrupt protein–protein hydrogen bonds, as well as protein–phenolic bonds. Therefore as a result of these procedures many proteins will have an unfolded random configuration, and multichain proteins may be dissociated into sub-units. Protein dissociation can be necessary, as when dispersing agents are used to dissociate artifact aggregates of basic proteins[11] or to dissociate multichain proteins for identification and characterization by immuno-electrophoresis and molecular size estimation.[12] It could be undesirable if multichain enzymes are dissociated to inactive sub-units or if they assume inactive unfolded configurations. Brewer[13] found that yeast enolase in gels containing 8 M urea had a specific activity of about half that of the original enzyme, which he states is "the normal recovery of activity from urea-treated enzyme". Bartels[14] dissociated proteins from tannins in beech leaves with phosphate buffer, pH 7·8, containing 4% caffeine and 3% Na ascorbate and obtained active aldolase, malate dehydrogenase and glutamate-aspartate transaminase. Substitution of 4% or 6% caffeine for 8 M urea in slurries of Douglas fir foliage decreased electrophoretic protein removal (about equal to that obtained with 3 M urea slurries). However, caffeine or other dissociating agents might prove useful if urea were found to inactivate an enzyme of interest.

Use of 8 M urea in the gels caused trailing bands, but this effect was absent when 3 M urea was used. This suggested that dissociation of protein sub-units occurred in 8 M urea gels but was not serious in 3 M urea gels.

The exact conditions of pH for discontinuous electrophoresis are set by the properties of the ions used.[10] Since dilute buffers are used to prevent the trailing ion overrunning the proteins initially, the pH of the gel buffers can be affected by impurities, such as cyanates, in the relatively large amounts of urea in the gels. Poulik[12] suggests removal of impurities with mixed bed resins but states that good-quality urea may be used without purification. Since phenolic substances do not migrate under the acid gel conditions, the system may be useful for initial purification of proteins on a preparative scale, and for isolation of specific substances separable by electrophoresis. By this means, the substances concentrated at R_f 0·65 were recovered, permitting their identification as amino acids. Sample preparation is rapid, a convenience for comparisons involving large numbers of samples, with as little as 20 mg tissue/sample slot yielding good results.

EXPERIMENTAL

Sample Preparation

Slurries from fresh foliage were prepared by homogenizing of 1 part (fr. wt.) with 5 parts (v/v) of cold 8 M urea, in a 8 ml capacity, sealed, stainless-steel capsule with steel ball pulverizer, powered by a "Wig-L-Bug" dental amalgamator. The capsule was chilled to −5° before homogenizing for 20 sec. The 1:5 dilution contained the minimum amount of urea solution that could be used to obtain pipettable slurries.

Freeze-dried foliage, previously ground to 40-mesh, required dilutions of 1:5–1:10, depending upon method of slurry preparation. Protein yield, resolution of bands, and clarity of background, decreased slightly with method of slurry preparation (1:10 dilution), in the following order: (1) ultrasonic disintegration in extracting solution, (2) dry powdering in "Wig-L-Bug" before mixing with extracting solution, (3) wet homogenizing in "Wig-L-Bug" with extracting solution, (4) wet grinding in glass–glass tissue grinder with extracting solution, (5) direct mixing of 40-mesh sample with extracting solution. In methods 1, 3, and 4, an ice-salt water bath was utilized for cooling. Measured quantities of the slurries (50–200 μl) were transferred

[11] C. I. KNIGHT, in *Techniques in Experimental Virology* (edited by R. J. C. HARRIS), Academic Press, London (1964).
[12] M. D. POULIK, in *Methods of Biochemical Analysis*, Vol. 14, p. 455, John Wiley, New York (1966).
[13] J. M. BREWER, *Science* **156**, 256 (1967).
[14] H. BARTELS, *Proc. XIVth Congress Int. Union of Forest Res. Org.* **3**, 551 (1967).

immediately after preparation to sample slots of the water-cooled gels, by means of disposable capillary tube pipets.

Electrophoresis

The gel quantities and current conditions stated below apply to the slab-type vertical gel electrophoresis cell (EC Corp., Philadelphia, P.A., U.S.A., Model 470) used in this study, but are adaptable to the tube-type or other discontinuous electrophoresis apparatus. A 3-mm thick gel was utilized, providing superior separations to the 6-mm preparative scale gels. The slabs were poured to depths of 13 cm of running gel and 2 cm of spacer gel. The 12 cm slab width allowed simultaneous runs of up to ten samples. Teflon slot formers were fabricated to provide various sizes and numbers of 2·7 mm wide (narrow dimension) × 10 mm deep sample slots. The original perspex slot formers tended to stick when used with acid gels.[15]

Catalyst for acid gels.[15] Amounts of the following were added to each gel solution immediately before pouring to give stated percentages based on vol. of gel solution: 0·1% ascorbic acid, 0·0025% $FeSO_4$, 0·03% H_2O_2.

Gel plug. 40 ml of catalysed 16%, w/v "Cyanogum 41" in buffer (0·06 N KOH made to pH 3·35 with citric acid) were poured at the bottom of the gel space with the cell assembly inclined at a 50° angle until the plug set.

Running gel. 70 ml of catalysed 16%, w/v "Cyanogum 41" in buffer plus urea solution (0·06 N KOH made to pH 3·35 with citric acid, then made 3 M with respect to urea just prior to use).

Spacer gel. 20 ml catalysed 4%, w/v "Cyanogum 41" in buffer plus urea solution (0·06 N KOH made to pH 4·35 with citric acid, then made 3 M with respect to urea just prior to use). The spacer gel was poured over the solidified running gel and the teflon slot former set in place.

Electrode buffer. When the spacer gel had set, the electrode chambers were filled with 2 l. buffer (0·037 M glycine made to pH 3·85 with citric acid), the slot former removed, and samples pipetted into the slots.

An initial potential of 250 V (about 45 mA) was applied for 5 min and the run completed at 450 V (about 80 mA). The electrophoresis front migrated about 8 cm in hr 1. After 2 hr the front had migrated into the gel plug, but sacrifice of this reference was necessary for complete separation of all resolvable proteins which migrate slowly under the small pore, acid gel conditions. Decreasing the concentration of the running gel from 16% to 12% increased R_f values by about 1/3, but resolution of some bands was lost.

Gels were stained for 1 hr in amido black 10B, C.I. No. 20470, 0·7% in 10% acetic acid, and destained in the EC Corp. model 489 electrophoretic destainer for 30–90 min, as required, using H_2O–MeOH–HOAc (5:5:1). The residual background color may be further decreased by overnight storage in 0·1% $FeSO_4$, 3% HOAc, prior to photography or densitometry.

[15] E. M. JORDAN and S. RAYMOND, *New Catalyst System for Acid Polyacrylamide Gel*, Technical Bull. No. 134, Supplement, E-C Apparatus Corp., Philadelphia, Pa. (1966).