HOW TO EXTRACT AND CHARACTERIZE DEHYDROGENASES FROM WOODY PLANTS

Bruce E. Haissig, *Principal Plant Physiologist,*
Rhinelander, Wisconsin,
and **Arthur L. Schipper, Jr.,** *Principal Plant Physiologist,*
St. Paul, Minnesota

Forest biology research increasingly explores physiological mechanisms that control tree growth in order to discover physiological bases for selecting strains that yield more or better wood (Gordon 1971, Wolter and Gordon 1975). Such studies may require knowledge of metabolic changes that cause or accompany variations in growth of the tree and differentiation of tissues. Metabolic changes can be estimated by various means, which include quantifying variations in the activities and types of enzymes (Firenzuoli *et al.* 1968, Barnett and Naylor 1969). Interest has also recently increased in genetic analysis of trees through study of isoenzymes (Conkle 1971a, 1971b; Feret 1971, 1978; Feret and Stairs 1971a, 1971b; Hamaker and Snyder 1973, Juo and Stotzky 1973).

Enzyme analysis is possible only if enzyme extraction can be exactly reproduced and the enzymes stablized for hours or days, as governed by analytical methods. Extraction and stabilization of enzymes from animals, lower plants, and herbaceous plants have been extensively investigated because these organisms are long time subjects of biological research. Only more recently has interest arisen in the enzymes of woody plants. Therefore, least is known of extracting and stabilizing their enzymes.

Methods developed for securing and stabilizing enzymes from other organisms have been tried on trees with limited success. Thus, most studies with woody plants have only involved enzymes that are relatively stable in crude extracts. Less stable enzymes may prove more indicative of physiological status or genetic constitution.

Best results have been obtained when enzymes were extracted during or shortly after tree seed germination, or when leaves were used (see earlier references). Woody tissues are difficult to disrupt without physically destroying enzymes. Disrupted woody tissues release tannins and phenols that promptly denature enzymes, either directly or after enzymatic oxidation. Thorough extraction and stabilization of enzymes from woody tissues assume importance because difficulties in disruption often make it impossible to use large amounts of tissue to offset deficiencies in technique.

Thus, we have extensively examined methods for the extraction, stabilization, and characterization of dehydrogenases from woody plants. Results and previously unpublished data are summarized here as an aid to other investigators, many of whom have contacted us seeking further, more detailed information concerning application of our findings.

Our studies have yielded methods for extraction and stabilization of dehydrogenases through prolonged periods of characterization. These methods can be applied, with suitable modifications, to other enzymes.

The first section deals with methods, for those readers who are interested only in technique; the second section discusses the rationale for the methods, and cites supporting evidence. Previously unpublished supportive technique data have been included, as have typical results to be expected from various techniques.

METHODS

Collection

Collect samples by clipping pieces or punching out discs with, for example, a leather punch. Immediately place tissue in standard lyophil containers or in 4-dram screw cap vials. The latter fit 1/2-inch diameter Virtis port fittings. Freeze tissue in a mixture of dry ice and acetone (-86C), acetone-filled mechanically refrigerated bath (-90C), or, preferably, in liquid nitrogen (-196C). Do not cover the samples with water before freezing, or allow acetone to leak into the containers. Collect samples in the field by freezing in powdered dry ice, or (preferably) in liquid nitrogen. Keep samples cool during transport.

Lyophilize samples for 15 to 24 hours. Resinous tissue may require longer drying times unless containers are less than half full. Check the lyophil for proper operation by lyophilizing a water sample, which should remain frozen until the ice has sublimed. Melting indicates an improperly constructed or functioning unit.

Storage

Store samples, if necessary, at -20C or less in plastic bags or jars that contain anhydrous calcium sulfate or a molecular sieve. Samples store best in a cabinet cooled with dry ice, or in a mechanically refrigerated ultra-low temperature freezer. Always store over desiccant.

Milling

Mill samples to pass a 20-mesh screen of a Wiley mill. Very small samples can be collected by aspirating the milling chamber (Haissig 1972). Samples should be milled just before homogenization, if possible. Milled tissue should be stored at the lowest possible temperature, over desiccant, in evacuated or inert gas-filled containers, such as vacuum desiccators.

Homogenization

Technique

All operations are performed at about 5C in a cold room. Place from 10 to 50 mg of milled tissue in a V-bottom glass homogenizer (Duall, Kontes Glass Co., Vineland, New Jersey 08360)[1]. Add 2 ml of buffer (see below) and suspend the tissue with a small spatula. Homogenize the sample for 30 to 45 seconds with a motorized (1100 rpm) glass pestle. A suitable apparatus is available commercially (Kontes Glass Co.) or can be easily constructed. In either instance, the user must supply a rheostat (Powerstat, Superior Electric Co., Bristol, Connecticut 06010) and timer (Gralab Model 300, Dimco-Gray Co., Dayton, Ohio 45402) to control speed and duration of homogenization. Rinse tissue adhering to the pestle into the homogenizer with 1 ml of buffer. Mix contents of homogenizer and decant into centrifuge tube. Centrifuge at 40,000 to 100,000 xg for 30 minutes at 5C. Transfer samples to 1 dram screw cap vials. Certain homogenates contain lipid-like substances that float on the supernatant, even after prolonged centrifugation at 100,000 xg. Supernatants can be freed of these substances by withdrawing supernatant with a syringe from below the floating layer.

Buffers

Use 100 mM Tris-HCl,[2] pH 7.5, containing 5 percent (w/v) glycerol, 100 mM EtSH, and 1 mM NAD (or NADP). Substitute 1 mM DTT for EtSH, and increase nucleotide coenzyme concentration to 10 to 50 mM if electrophoresis will be conducted without prior removal of the higher concentration

---

[1] *Mention of trade names does not constitute endorsement of the products by the USDA Forest Service.*

[2] *Abbreviations: EtSH—mercaptoethanol, DIECA—diethyldithiocarbamate, DTT—dithiothreitol, NAD—nicotinamideadenine dinucleotide, NADP—nicotinamideadenine dinucleotide phosphate, AP—ammonium persulfate, TEMED—N,N,N',N', tetramethylethylene-diamine, PVP—insoluble polyvinylpyrollidone (Polyclar AT), G-3-PD—glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12), G-6-PD—glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49), 6-PGD—6-phosphogluconate dehydrogenase (E.C. 1.1.1.44), MD—malate dehydrogenase (E.C. 1.1.1.37), PPO—phenolase (E.C. 1.10.3.1), PR—peroxidase (E.C. 1.11.1.7), MES—(2[N-Morpholino] ethane sulfonic acid, HEPES—N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Tris—Tris (Hydroxymethyl) aminomethane, BSA—bovine serum albumin.*

of EtSH. Affinity chromatography requires buffers of low ionic strength. Use 10 to 25 mM HEPES - NaOH, pH 7.5, containing 20 percent (w/v) glycerol and 10 mM DTT.

Purification and Characterization

Numerous methods exist for the partial purification and characterization of enzymes. The following methods afford a starting point from which more detailed analyses begin.

Acrylamide Gel Electrophoresis

Equipment consists of a Bio-Rad Model 150 (Bio-Rad Laboratories, Richmond, California 94804) electrophoresis cell, or equivalent, and a compatible power supply.

Allow gel solutions to warm to room temperature. Add 25 mg of AP to 25 ml of running gel solution while the latter is being stirred. Quickly add 1.2 ml of the solution to each 5 mm (I.D.) x 125 mm acid washed gel tube and layer distilled water over the acrylamide solution. Use a 5 ml plastic syringe that has been fitted with a 6 cm piece of 1/32-inch I.D. teflon tubing. After polymerization, add 0.2 ml of spacer gel solution to each tube, and then withdraw the solution with the syringe. Flush 25 ml of spacer gel solution with nitrogen gas for 15 seconds. Stir the solution and add 25 mg of AP. Add 0.2 ml of this solution to the top of each running gel tube and layer with water. Allow polymerization. Install the tubes in the cell, add electrode buffer, and apply sample.

From 100 to 400 µl of sample are added to each tube with a micro syringe (Drummond Scientific Co., Broomall, Pennsylvania 19008). Apply about 150v until samples have entered the running gel, after which voltage can gradually be increased to about 250v (about 2.5 ma per tube). Colored impurities usually run at the front, which obviates need for a tracking dye. If necessary, add a small amount of bromphenol blue to each sample or to the upper electrode buffer.

Remove gels from tubes and stain by standard methods (Brewer 1970). Trials are necessary to balance amount of sample applied and duration of staining. Always include controls (eliminate substrate or coenzyme) to check for non-enzymic reduction of tetrazolium dye.

Blue background and diffuse banding in gels can be reduced or eliminated by exposing gels to stain for 15 to 30 minutes at 5C in darkness, then decanting staining solution. A good scanning-further reduction of dye for up to 15 hours at 5C in darkness. Do not add water or buffer after decanting staining solustion. A good scanning-recording device markedly simplifies and improves data collection (e.g., attachment to Pye-Unicam SP 1800 spectrophotometer).

Extracts sometimes do not contain sufficient enzyme for a satisfactory staining reaction. In such instances, increase the volume of extract added to the gel, or concentrate the sample by $(NH_4)_2SO_4$ precipitation (see later), Minicon B-15 or A-25 membrane concentrators (Amicon Corp., Lexington, Massachusetts) (Hicks and Haissig 1974), or Molecular Separators (Millipore Corp., Bedford, Massachusetts 01730).

Salting-out

Assay the crude extract for enzyme activity and measure the volume (v_1). Add crystalline $(NH_4)_2so_4$ to the cold extract. The initial percentage (w/v) of $(NH_4)_2SO_4$ should not precipitate the enzyme of interest but should precipitate many of the other macromolecules in solution. Usually from 50 to 80 percent $(NH_4)_2SO_4$ effects initial precipitation.

Allow the mixture to stand for several hours at 5C to insure complete precipitation. Centrifuge (40,000 xg, 30 minutes), decant the supernatant, and save the pellet. Assay and measure the volume of the supernatant (V_2). Dissolve the pellet in a measured amount of extraction buffer (V_3) and assay.

Make the supernatant 100 percent (at 5C) in $(NH_4)_2SO_4$ again by adding crystalline salt and allow the mixture to stand for several hours. Centrifuge, decant, and save the supernatant. Save the pellet. Measure the volume of supernatant (V_4) and assay. Dissolve the pellet in 1 ml of extraction buffer (V_5) and assay.

Calculate the total units of enzyme activity in V_1 through V_5 in order to determine changes in activity at each step. If activity is found in V_3 or V_4, adjusting $(NH_4)_2SO_4$ concentrations will rectify the situation. A suitable schedule places most of the initial activity in V_2, then V_5.

V_5 can be freed of salts by dialysis against extraction buffer, but this step is often unnecessary.

Gel Filtration

Columns of 75 cm x 2.5 cm and longer often yield the best results. Select a packing material, such as Sephadex G-150 (Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey 08854), with a molecular weight exclusion limit greater than the estimated molecular weight(s) of the enzyme(s) of interest. Hydrate the gel according to manufacturer's instructions in extraction buffer. Decant the fines several times, and thoroughly degass the room-temperature gel suspension with an aspirator. Coenzymes can be excluded from buffer in preparation of the gel and packing of the column to reduce cost.

Stabilize the gel bed by pumping buffer through the column overnight. The peristaltic pump should be set for a flow rate that will not collapse the gel bed but will give a suitably fast elution. These conditions are determined empirically.

Apply about 1 ml of enzyme solution to the column and elute at the predetermined flow rate with freshly prepared extraction buffer. Collect fractions of about 5 to 10 ml volume.

Assay the fractions for enzyme activity, and do electrophoresis on samples from desired tubes. Concentrate samples, if necessary or desired, by $(NH_4)_2SO_4$ precipitation or with membrane concentrators.

Column Electrofocusing

We use LKB equipment, which includes 110 ml column, ampholyte solutions, power supply, fraction collector, gradient mixer, and peristaltic pump (LKB Instruments, Inc., Rockville, Maryland 20852). A Radiometer model 26 pH meter and 0-14 pH miniature combination electrode are used to measure pH of each fraction at the collection temperature (London Co., Westlake, Ohio 44145 and Sargent-Welch Scientific Co., Skokie, Illinois 60076).

Our procedures closely follow the manufacturer's recommendations, with important exceptions that are noted under Reagents.

Fractions collected after electrofocusing, like those from gel filtration, can be further analyzed by electrofocusing, acrylamide gel electrophoresis, gel filtrations, or other established methods.

Affinity Chromatography

Various proprietary media exist for affinity chromatography of dehydrogenases (P-L Biochemicals, Inc., Milwaukee, Wisconsin 53205; Bio-Rad Laboratories, Richmond, California 94804). Specificity of the media varies and may profoundly influence the quality of results, so care should be used in selection.

Purification of G-3-PD from jack pine seedlings is a typical example of affinity chromatography.

Agarose-hexane-NAD Type 1 (P-L Biochemicals) is washed free of glycerol and sodium azide with extraction buffer. The agarose is then slurried into a column constructed from a 5 ml disposable plastic syringe barrel. A porous, hydrophilic, polypropylene disc at the bottom of the syringe barrel retains the agarose (Bolab, Inc., Derry, New Hampshire 03038). Commercial columns are also available (Isolab, Inc., Akron, Ohio 44321). Settled medium is further washed with extraction buffer to stabilize the bed. Settled bed volume is about 4 ml.

Samples are homogenized and the crude extract is assayed for enzyme activity. A known volume (about 2 ml) of crude extract is applied to the column after the buffer in the column has been run almost into the bed. Crude extract is then run slowly into the bed, and the column is washed at full flow rate with about 25 ml of extraction buffer. Effluent volume is measured and enzyme activity determined. The column is then eluted at full flow rate with extraction buffer that contains 10 mM to 25 mM NAD. Fractions of 1 or 2 ml volume are collected and assayed for enzyme activity.

Enzyme activity at all stages is recorded as total Δ OD min (Δ OD/min/0.1 ml x number of 0.1 ml volumes per fraction). Thus total enzyme activity can be accounted for at each step, and gains or losses in activity will be apparent.

Fractions are subjected to further analysis or purification at once.

Columns are cleaned for reuse by washing with several bed volumes of: 50 mM MES-NaOH, pH 5.5, containing 2.0 M NaCl and 0.1 percent BSA; then, 50 mM Tris-HCl, pH 8.5, containing 2.0 M NaCl and 0.1 percent BSA; and finally, extraction buffer. Columns thus treated can be reused many times. Periodic resuspension of the medium with nitrogen gas aids removal of foreign materials and prevents marked decreases in flow rate.

REAGENTS

Extraction Buffer

Weigh 50 gm of glycerol into a 1000 ml beaker. Add 750 ml distilled water, 12.1 gm Tris, and 7.0 ml EtSH. Insert a glass electrode, stir the solution, and adjust the pH to 7.5 with concentrated HCl. Make up to 1 liter with distilled water. The resulting solution is 100 mM in Tris, 5 percent in glycerol, and 100 mM in EtSH. Store frozen at -20C.

Add NAD or NADP to a portion of thawed buffer just before use. Do not store buffer containing NAD or NADP.

Add 2.38 gm HEPES to 750 ml distilled water that contains 200 gm of glycerol. Insert a glass electrode, stir the solution, and adjust pH to 7.5 with 1.0 N NaOH. Make up to 1 liter. The resulting solution is 10 mM in HEPES and 20 percent in glycerol. Store frozen at -20C. Add 77.2 mg of DTT per 50 ml of thawed buffer just before use. The resulting buffer is 10 mM in DTT. Check pH. Do not store buffer containing DTT for more than a day.

Electrophoresis Electrode Buffer

Dissolve 12.1 gm Tris and 58.0 gm glycine in 1500 ml of distilled water, and make up to 2 l. The resulting buffer is 50 mM in Tris. Dilute each part of buffer with 9 parts of distilled water before use. Insert a glass electrode and adjust pH of the solution to 8.3 by adding crystalline Tris or glycine.

Electrophoresis Spacer Gel

Dissolve 60.6 gm Tris in 750 ml of distilled water. Add 40 gm Cyanogum-41 and 1 ml TEMED. Insert a glass electrode, stir, and adjust pH of the solution to 6.7 with concentrated HCl, then dilute to 1 liter. The resulting solution is 500 mM in Tris. Refrigerate.

Electrophoresis Running Gel

Dissolve 60.6 gm Tris in 750 ml of distilled water. Add 80 gm Cyanogum-41, and 1 ml TEMED. Insert a glass electrode, stir, and adjust pH of the solution to 8.5 with concentrated HCl, then dilute to 1 liter . The resulting solution is 500 mM in Tris. Refrigerate.

Electrofocusing Density Gradient Solutions

Heavy Fraction

Weigh 36 gm glycerol into a 100 ml beaker, and add 25 ml of distilled water. Stir in 0.4 ml EtSH and 1.8 ml of pH 3-10 ampholyte (LKB) solution. Make up to 50 ml with distilled water.

Light Fraction

Add 0.6 ml of pH 3-10 ampholyte solution and 0.4 ml EtSH to 20 ml of distilled water. Add a measured amount of enzyme extract and make up to 50 ml with distilled water.

DISCUSSION

Collection

Under many circumstances, samples are too numerous, or are collected too far from the laboratory to allow extraction of fresh tissues. It may be easier to transport tissue frozen in dry ice or in liquid nitrogen and lyophilize at the laboratory. Lyophilization of samples eliminates problems connected with sample milling and storage, as long as the enzyme of interest is not cold labile. Moreover, fast freezing of tissues before lyophilization increases extraction of certain enzymes or does not markedly lower extracted activity (Haissig and Schipper 1972).

Successful lyophilization depends upon very fast freezing of the sample and a perfectly functioning lyophil. Sample containers must be rapidly aspirated so that samples remain frozen

until all moisture has sublimed. The condenser must effectively collect water vapor so that samples dry thoroughly before removal. Samples are being vacuum dried, not lyophilized, unless these conditions are met.

Storage

Enzyme activities decline in stored tissues, with the highest rates of decline related to elevated temperatures, moisture levels, and oxygen levels. Assay of enzymes extracted from samples readily detects such decline. However, our observations indicate that qualitative changes apparently occur in enzyme molecules upon storage even though activity has not detectably declined. Qualitative variations may markedly influence electrophoretic characterization. Isoenzyme bands become increasingly diffuse with prolonged storage of tissue. Lyophilization magnifies the problem, apparently because of freezing. Whatever the reason, lyophilization somewhat decreases the sharpness of electrophoretic separations. Enzymes extracted from fresh tissue produce the best electrophoretic results, but practicality may necessitate devising a storage method that is applicable to the tissue and enzyme of interest.

Milling

Homogenization of whole tissues, other than leaves, root tips, and buds, has proven impractical (Haissig and Schipper 1972). Enzyme activities are lost or markedly lowered before woody tissues are fully disrupted. Enzyme activity may be obtained in such extracts, but never the highest attainable activity. Fracturing tissues frozen with liquid nitrogen or an acetone-dry ice mixture is generally less effective and more expensive than milling (Haissig and Schipper 1972).

Unfortunately, milling increases surface area of the sample, and thereby exposes enzymes to oxygen, moisture, and air-borne denaturants. Storage conditions for milled tissues must consequently be more rigorous than conditions for unmilled tissue. Inert gases, such as welding grade nitrogen, adequately shield well dried, milled tissues that are maintained at -20C or lower.

Homogenization

Duall (Kontes Glass Co.) homogenization of milled, lyophilized tissues has proven superior to homogenization of tissues that have been dehydrated or pulverized by other methods. Duall homogenization is fast, reproducible, and yields quantifiable enzyme activities where suitable extraction media are used (Haissig and Schipper 1972). In particular, quantifiable enzyme activities are obtained from very small samples (10 to 50 mg) of even difficult to disrupt tissue such as xylem. The method is only semiquantitative, but exceeds other methods in enzyme recovery because the sample is well contained, and easily transferred to centrifuge tubes. More sample is lost adhereing to the surface of a conventional mortar and pestle than is required for Duall homogenization. In addition, samples need not be strained before centrifuging. However, the method requires practice. Inexperience in operation of the Duall homogenizers invariably results in too little or too much homogenization, with concommitent low yields of enzyme activity. Practice until a peak enzyme activity can be reproduced for a given type of sample.

In addition, homogenizer size must be exactly 3 ml. Smaller pestles do not withstand the needed force, and larger homogenizers are difficult to keep cool. If needs dictate more extract, supernatants should be pooled after homogenization, or another method of homogenization should be developed.

Extraction Media

The extraction medium solubilizes and, hopefully, stabilizes the enzyme molecules. Any component of the extraction medium that does not have a demonstrable effect on solubility or stabililty should be eliminated (Haissig and Schipper 1972). Untested additives complicate buffer preparations, increase costs, and often subsequently prove deleterious or valueless. In addition, additives may interfere with subsequent steps of purification or characterization. Additives that have been tested singly may have different effects when used in combinations.

Similarly, pH, chemical composition, and ionic strength of buffers should be adjusted with

purpose, never arbitrarily. Elevation of ionic strength or use of monionic detergents may release so-called bound enzyme fractions (Haissig and Schipper 1971, 1972; Shipper 1975), which may or may not meet experimental objectives. At times an arbitrarily selected buffer and ionic strength may inadequately buffer a tissue extract, as shown by pH measurements with a glass electrode.

Extraction media for enzymes from plant tissues have usually been designed to protect enzymes from tannins (Goldstein and Swain 1965) or phenols or from polymeric brown pigments that form after the oxidation of phenols by phenolase (Haissig and Schipper 1975a and references therein). Plant tissues usually contain sufficient phenols and phenolase so that, in the presence of molecular oxygen, brown pigments form in extracts, and co-precipitate with enzymes (table 1). The pigments may not form immediately in tissue extracts of some species, but in others, such as extracts of *Salix fragilis* stem tissue, distinct browning occurs during homogenization. Consequently, enzymes may be completely denatured before an initial assay can be done unless preventive measures are used.

Various methods have been used to prevent formation of brown pigments (Haissig and Schipper 1971, 1972, 1975a). Of these, addition of insoluble PVP has been widely employed. PVP adsorbs tannins and phenols. Reasonable amounts of PVP remove all tannins from aqueous solution but not phenols (fig. 1). Moreover, PVP treatment may enhance phenolase activity (table 2) and reduce activity of desired enzymes (Haissig and Schipper 1975a). However, other methods of removing tannins and phenols, such as using anion exchange resins or celluloses, are less desirable than PVP either in removal of tannins or phenols or in maintaining desired enzyme activity (Haissig and Schipper 1971, 1975a) (fig. 2). Thus, adding PVP is a good starting point for enzyme stabilization, except for particular instances where it inhibits enzyme activity (Haissig and Schipper 1975a).

Table 1.—*Estimation of phenolics (as phenol) and phenolase (PPO) activity in woody and herbaceous plants*

Species	Type of tissue	Phenolics $\bar{x}$ µg ± S.E. per mg dry weight	PPO Units ± S.E. per gm dry weight
Woody			
Aspen	Dormant twig	32 ± 0.7	348 ± 12
Black walnut	" "	20 ± 0.4	84 ± 24
Barberry	" "	16 ± 0.5	144 ± 0
Red pine	" "	15 ± 0.1	167 ± 24
Sugar maple	" "	14 ± 0.5	36 ± 0
White spruce	" "	13 ± 0.5	312 ± 48
Silver maple	" "	10 ± 2.0	276 ± 53
Nonwoody			
Collards	Leaves	22 ± 0.7	252 ± 21
Spinach	"	17 ± 0.7	288 ± 2
Barley	"	5 ± 0.2	1,064 ± 98
Sweet potato	Tuber	4 ± 0.1	156 ± 67
Tomato	Stem & leaves	4 ± 0.1	347 ± 14
Control			
Mushroom	Cap	13 ± 0.3	2,988 ± 101

A further step in enzyme stabilization consists of blocking formation of brown pigments by continually reducing quinones that are generated from phenols by phenolase. Reduction of quinones prevents their condensation and prevents formation of brown pigments as long as sufficient reducing agent is present (fig. 3).

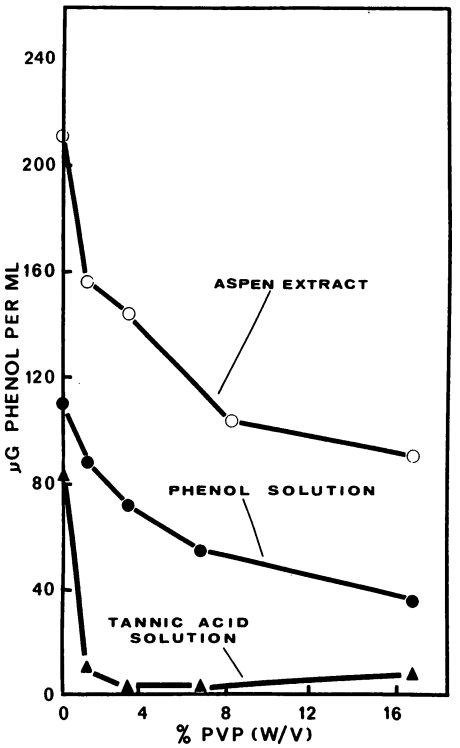

Figure 1.—*Effect of PVP during tissue homogenization on removal of phenolics from aspen extracts and on removal of phenol and tannic acid from aqueous solution. The decrease in the level of phenolics in the aspen extract between 0 and 1.7 percent (w/v) PVP probably results from the removal of tannins.*

Table 2.—*Comparison of phenolase (PPO) activity in extracts of woody and herbaceous plants prepared with and without 1.7 percent (w/v) polyvinylpyrrolidone (PVP)*

Species	Type of tissue	Ratio units per gm dry weight + PVP / - PVP
Woody		
Sugar maple	Dormant twig	6.0
Black walnut	" "	5.9
Aspen	" "	2.4
White spruce	" "	1.3
Barberry	" "	1.2
Silver maple	" "	0.9
Red pine	" "	0.8
Ginkgo	" "	0.7
Herbaceous		
Tomato	Stem & leaves	2.4
Sweet potato	Tuber	2.0
Collards	Leaves	2.0
Spinach	"	1.4
Barley	"	0.8
Control		
Mushroom	Cap	0.9

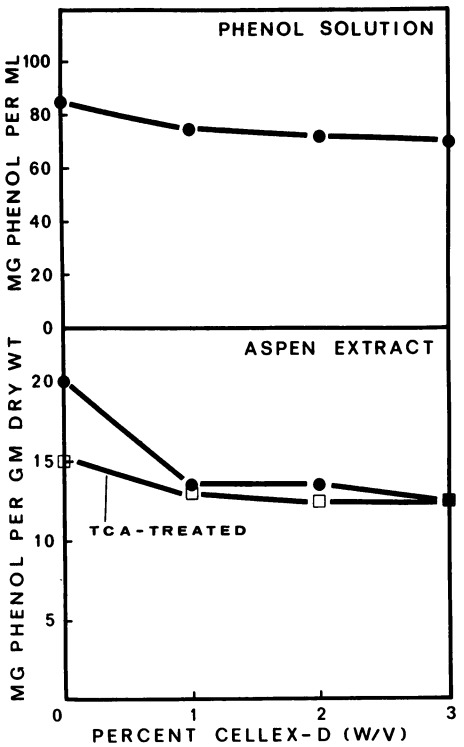

Figure 2.—*Effect of DEAE cellulose on removal of phenol from aqueous solution and aspen extracts. Extracts were prepared with buffer containing 0.1 percent (w/v) bovine albumin and DEAE cellulose and were (TCA) or were not treated with an equal volume of 10 percent trichloroacetic acid before phenolics were estimated. Note that more than 1 percent (w/v) DEAE cellulose removes differences between TCA- and non-TCA-treated aspen extracts, which indicates removal of bovine albumin from non-TCA extracts.*

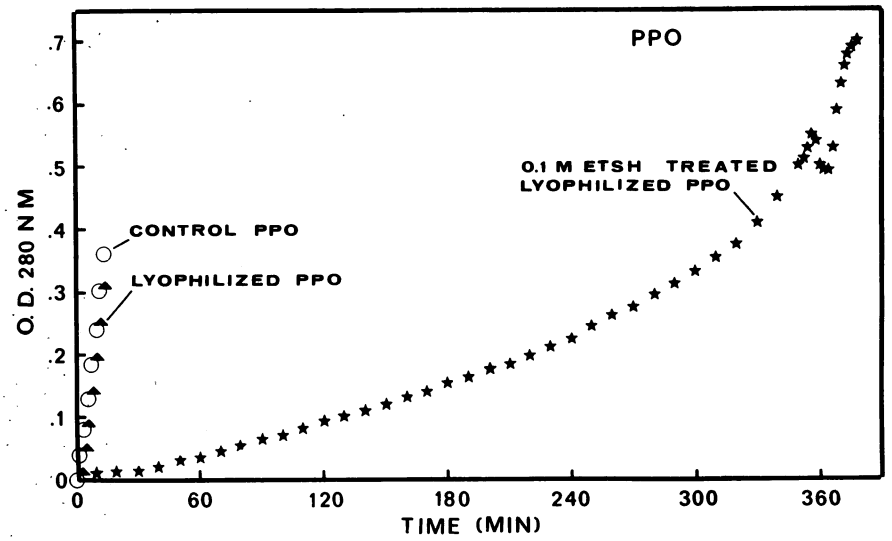

Figure 3.—*Influence of mercaptoethanol (EtSH) on mushroom phenolase (PPO) activity. Full enzyme activity is restored by lowering the concentration of EtSH by lyophilization. The effect of low levels of EtSH and other reducing agents, is overcome after quinones generated by PPO activity oxidize the reducing agent.*

Cysteine, DIECA, EtSH, and DTT reduce quinones. However, cysteine tends to autooxidize, which greatly shortens the life of buffers that contain cysteine. DIECA may reduce activity of desired enzymes in comparison with no treatment (fig. 4) or EtSH or DTT treatment (Haissig and Schipper 1975a) (fig. 5). In our experience, about 10 mM EtSH or 1 mM DTT prevent formation of brown pigments, even during prolonged experiments, but higher concentrations are often required to maximize desired enzyme activity (Haissig and Schipper 1975a, 1975b) (fig. 6). Neither EtSH or DTT inhibit desired enzyme activity in concentrations of at least 100 mM.

Combined PVP-EtSH treatments have proved effective in securing and retaining dehydrogenase activity, except G-3-PD activity (table 3). PVP treatment strongly inhibits G-3-PD activity initially or speeds loss of activity in comparison with EtSH alone. Other dehydrogenases may perform similarly.

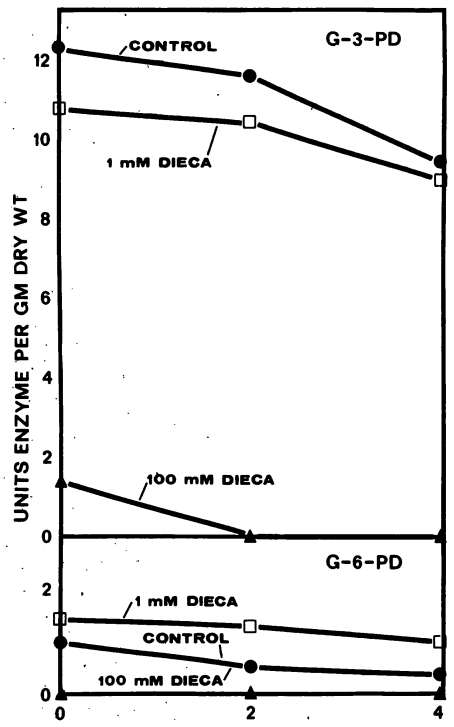

Figure 4.—*Influence of diethyldithiocarbamate (DIECA) in the extraction buffer on the activity of glyceraldehyde-3-phosphate (G-3-PD) and glucose-6-phosphate (G-6-PD) dehydrogenases in aspen extracts.*

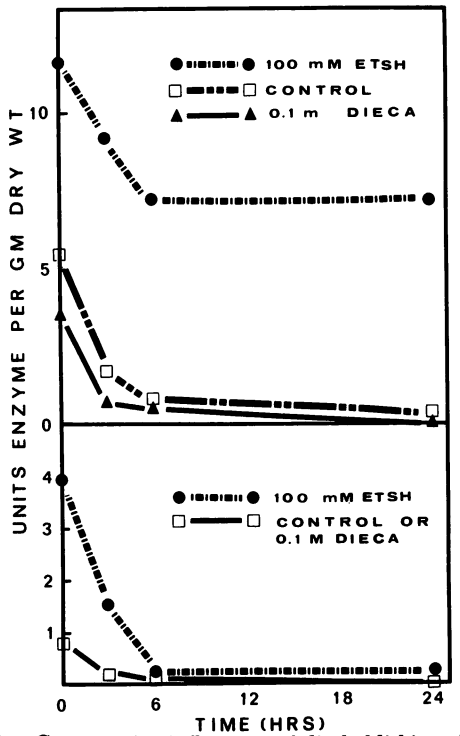

Figure 5.—*Comparative influences of diethyldithiocarbamate (DIECA) and mercaptoethanol (EtSH) in the extraction buffer on the activity of glyceraldehyde-3-phosphate (G-3-PD, upper graph) and glucose-6-phosphate (G-6-PD, lower graph) dehydrogenases in aspen extracts.*

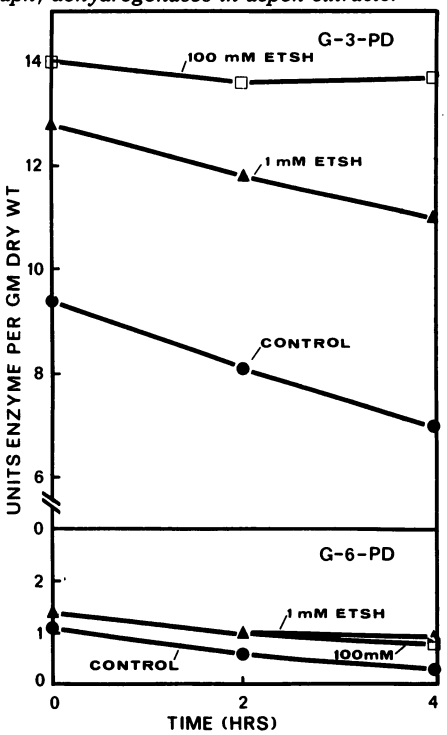

Figure 6.—*Influence of mercaptoethanol (EtSH) in the extraction buffer on the activity of glyceraldehyde-3-phosphate (G-3-PD) and glucose-6-phosphate (G-6-PD) dehydrogenases in aspen extracts.*

Table 3.—*Influence of insoluble polyvinylpyrrolidone (PVP) and mercaptoethanol (EtSH) on decay of sweet potato dehydrogenase activity*

Treatment	$\bar{x}$ units[1] per gram dry weight by enzyme and time							
	6-PGD		G-6-PD		G-3-PD		MD	
	0 hrs	72 hrs	0 hrs	72 hrs	0 hrs	72 hrs	0 hrs	72 hrs
Control	0	0	0.2	0	0	0	0.7	0
1.7 percent PVP	0.6	0	4.0	2.5	2.0	1.0	3.5	2.8
3.3 percent PVP	0.6	0	5.4	3.0	1.7	1.1	5.0	3.4
100 mM EtSH	2.3	1.9	8.7	1.2	67.7	31.4	13.0	1.6
100 mM EtSH + 1.7 percent PVP	2.4	2.2	9.8	2.9	70.4	18.1	15.4	13.6
100 mM EtSH + 3.3 percent PVP	2.2	2.1	9.4	3.5	62.6	15.5	14.0	13.2

[1]Standard errors of means did not exceed ± 7 percent.

The combined PVP-EtSH treatment frequently increases initial enzyme activities, but activity of some dehydrogenases is retained over the long term only by additional treatment. Our experience with these third-order treatments has been limited primarily to dehydrogenases, but the principles probably apply equally to any enzyme that catalyzes product formation from two or more reactants.

Addition of one of the reactants to the extraction buffer stablizies long-term activity (Haissig and Schipper 1975b). Although the actual cause remains unclear, the extraction of dehydrogenases in the presence of PVP (3 percent), a reducing agent (10 to 100 mM), and oxidized nucleotide coenzyme (1 mM or more) markedly enhances initial and long-term activity in comparison with PVP-reducing agent treatment alone (Haissig and Schipper 1975b) (figs. 7 and 8).

Including a higher concentration of oxidized nucleotide coenzyme (10 to 50 mM) allows a reduction in concentration of reducing agent (1 to 10 mM). Lower concentrations of reducing agent make possible the electrophoresis of samples, which, if prepared with 100 mM EtSH or 10 mM DTT, would cause high background in gels that are stained with tetrazolium dyes.

Purification, Concentration, Characterization

Purification invariably results in loss of enzyme and an increase in specific activity. Unfortunately, loss of enzyme cannot be afforded when initial amounts of enzyme are small, as often occurs when extracting woody tissue. A mode of purification must be sought that yields pure enough enzyme to meet experimental objectives and simultaneously yields a suitable quantity of enzyme in an appropriate volume of buffer. Purification must usually be followed by concentration of enzyme because most methods of purification dilute the sample.

Membrane concentrators will reduce almost any volume of enzyme solution. However, concentration may wholly denature enzyme, or yield

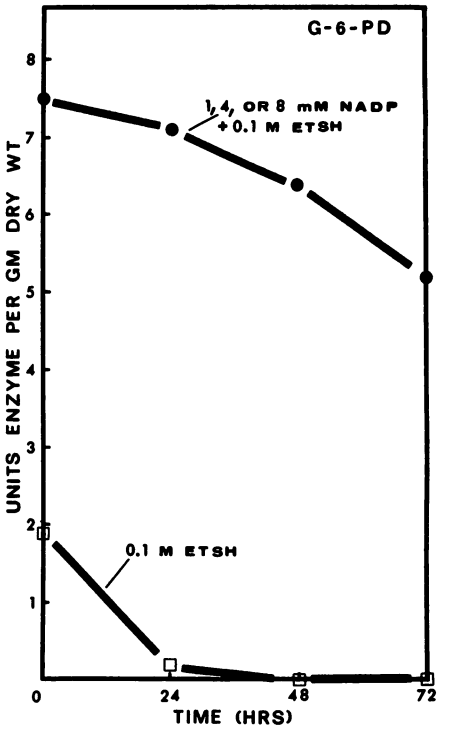

Figure 7.—*Influence of NADP in extraction buffer on G-6-PD activity with time in crude aspen extracts.*

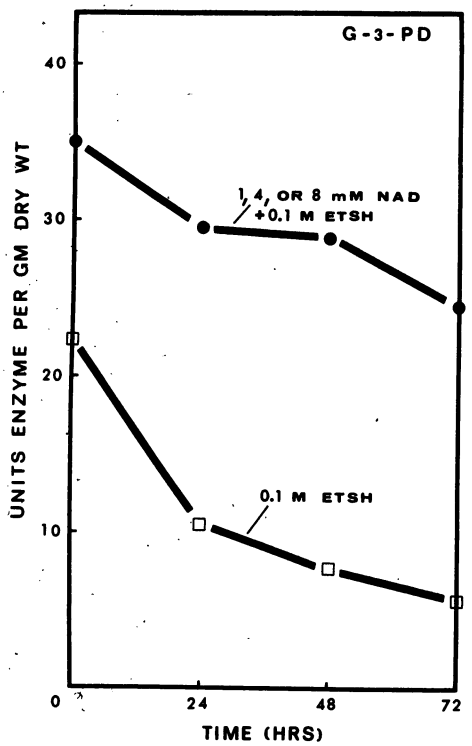

Figure 8.—*Influence of NAD in extraction buffer on G-3-PD activity with time in crude aspen extracts.*

solutions with enzyme activity that is out of proportion to the reduction in volume (table 4). Concentration may also produce syrups. Preliminary trials should test conditions of concentration so that desirable results are obtained.

Dialysis frees an enzyme of low-molecular-weight substances and may enhance specific activity upon removal of inhibitors. We have found dialysis particularly useful in cleansing extracts before further purification. In particular, dialysis seems to remove phenolics that tend to limit life of gel filtration columns. However, activity losses during prolonged dialysis often exceed half of initial activity unless the buffer is carefully selected for stabilization characteristics and flushed with nitrogen gas during dialysis. Fast dialysis equipment can also be used.

Ammonium sulfate precipitation has proven useful because it simultaneously purifies and concentrates the enzyme. Again, however, loss of enzyme activity may be great because complete precipitations at the various stages of fractionation require time during which enzyme denatures.

Table 4.—*Influence of 10-fold volume reduction by Minicon -A25 concentrators on enzyme activity per unit volume of extract*

Enzyme	Species	Specific activity[1]		Increase in specific activity per unit volume[2]
		Initial	Final	
PR	Aspen	66.5 ± 1.2	442.0 ± 31.8	6.6
	Jack pine	10.9 ± 0.1	62.8 ± 9.0	5.8
	Paper birch	60.5 ± 4.0	599.3 ± 9.6	9.9
	Black spruce	18.6 ± 1.1	148.3 ± 3.0	8.0
	White spruce	11.0 ± 0.0	103.6 ± 8.0	9.4
G-3-PD	Aspen	15.1 ± 0.2	76.9 ± 0.6	5.1
	Jack pine	0.7 ± 0.0	2.2 ± 0.2	3.1
	Paper birch	0.3 ± 0.0	0.0 ± 0.0	0.0
	Black spruce	1.6 ± 0.1	11.1 ± 1.0	6.9
	White spruce	1.0 ± 0.0	2.61± 0.4	2.6
MD	Aspen	8.6 ± 0.3	118.8 ± 6.2	13.8
	Jack pine	3.3 ± 0.2	17.7 ± 1.3	5.4
	Paper birch	2.1 ± 0.1	8.5 ± 1.7	4.0
	Black spruce	1.2 ± 0.1	22.7 ± 2.1	18.9
	White spruce	0.6 ± 0.0	10.1 ± 0.0	16.8
G-6-PD	Aspen	2.7 ± 0.2	17.6 ± 2.9	6.5
	Jack pine	0.5 ± 0.0	3.1 ± 0.2	6.2
	Paper birch	0.3 ± 0.0	1.4 ± 0.2	4.7
	Black spruce	0.8 ± 0.0	4.5 ± 1.1	5,6
	White spruce	0.6 ± 0.0	3.4 ± 0.1	5.7
6-PGD	[3]			

[1]Mean units per gram dry weight per 100 µl extract ± standard errors of three replications.

[2]Experimental conditions should have theoretically yielded a 10-fold increase in specific activity per unit volume because the initial sample volume was reduced by one-tenth, and no measurable activity losses occurred in unconcentrated portions of the extracts during the experiment.

[3]No activity detectable in concentrated extracts.

In addition, loss of enzyme occurs unless precise concentrations of ammonium sulfate are determined for each fractionation step. In spite of certain disadvantages, ammonium sulfate fractionation works for small volumes of extract, once acquaintance is gained with the method.

Gel filtration yields best results when small volumes of relatively clean extract are used—for example, enzyme purified and concentrated by ammonium sulfate fractionation. The method will both purify and characterize and has a unique capability for approximating molecular weight. Two disadvantages of the method are the length of time that is required to elute enzyme from large columns and the large effluent volume in which enzyme is obtained. However, a high degree of purification may be obtained and specific activity may increase, so that more activity is eluted from the column than was applied (fig. 9).

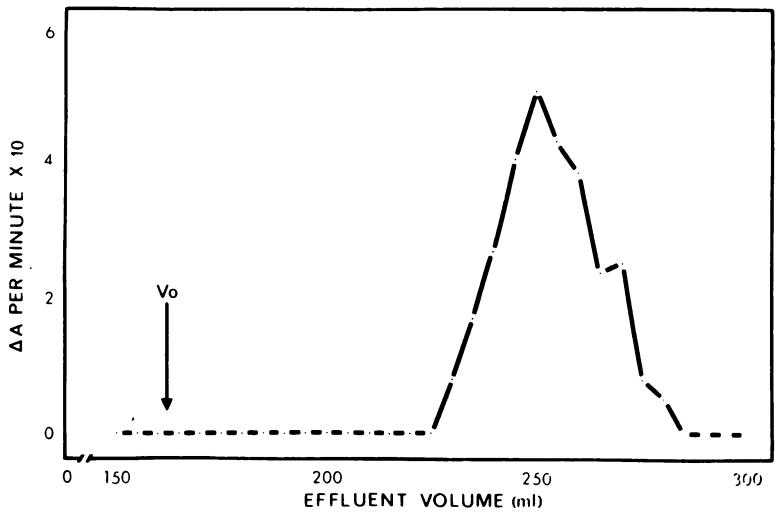

Figure 9.—*Separation of G-3-PD from jack pine seedlings (192 hours) on sephadex G-150. 1.92 U of G-3-PD were applied to column, and 2.58 U were recovered, for a 134 percent increase in activity.*

Column electrofocusing, like gel filtration, works best with clean enzyme preparations. Suitably prepared columns provide highly reproducible determinations of isoelectric point and enzyme or isoenzyme separations based thereon (fig. 10). The primary disadvantages of the method that we have encountered are prolonged times to properly focus enzyme and isoelectric precipitation of the enzyme of interest or other molecules in the extract. In addition, half or more of initial enzyme activity may be lost during the long focusing times. And, of course, power failures of even short duration destroy separations.

Affinity chromatography affords a theoretically superior method for enzyme purification because highly specific medium-enzyme combinations can be used. In addition, purified enzyme is obtained in a relatively small effluent volume, which can be less than the applied volume of enzyme solution (fig. 11).

Excellent separations can be obtained with small columns, which allow working with small volumes of extract, such as those volumes used for characterization by gel electrophoresis. However, it cannot be assumed that affinity chromatography

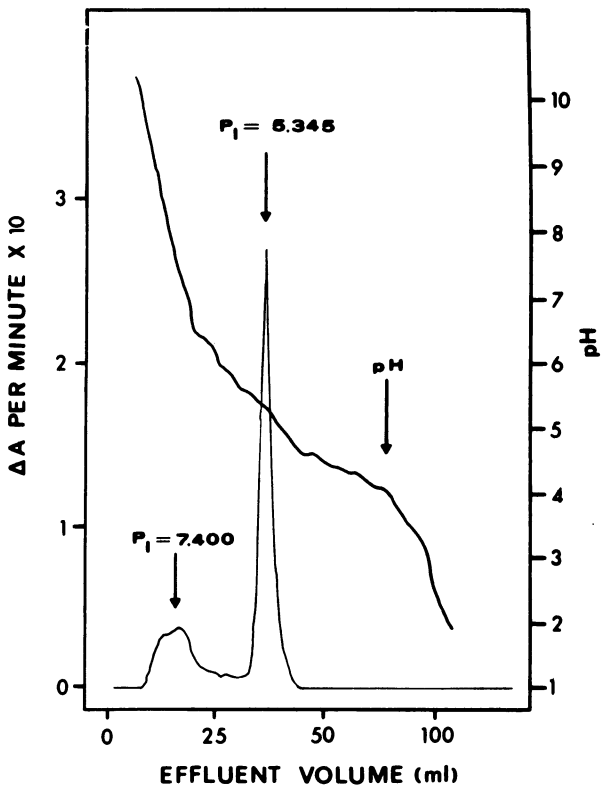

Figure 10.—*Column electrofocusing of G-3-PD (7 days, 2 percent LKB ampholyte, 300v). 15.97 U of G-3-PD were applied to column, and 8.57 U were recovered, for a 46 percent loss in activity.*

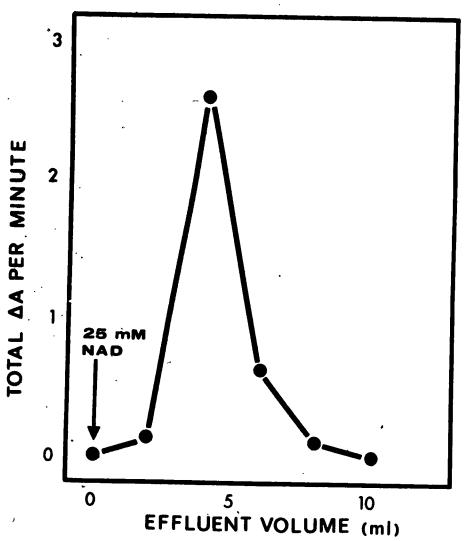

Figure 11.—*Affinity chromatography of G-3-PD from jack pine seedlings. Wash contained 29.4 percent of applied activity, effluent contained 69.5 percent and 1.1 percent was not accounted for.*

automatically results in a high or even usable level of purification, as might be predicted on a theoretical basis. First, impurities may elute at pH and ionic strength similar to those that elute enzyme. Second, we have found that under some conditions, a large fraction of the enzyme molecules may be irreversibly or nearly irreversibly bonded to impurities during extraction. This fraction of the population, though bound to impurities, may show high catalytic activity in wet assays both before and after affinity chromatography (Haissig and Schipper 1977).

Best results may be obtained only by preventing bonding of impurities to enzyme during extraction or by removing impurities before chromatography. A workable approach at these stages may, however, be vexing to obtain. As an alternative, enzyme of high purity (presumably the uncontaminated portion of the population) may be obtained by collecting the earliest elution volume with activity. All fractions with activity cannot be pooled using this approach, and much enzyme is lost.

Gel electrophoresis, and modifications such as gel electrofocusing, have become standard techniques for the simultaneous purification and characterization of enzymes in woody plants and other extracts. Discontinuous acrylamide disc gel electrophoresis provides good resolution of isoenzymes (fig. 12). Slab acrylamide gel electrophoresis has been much less useful to us,

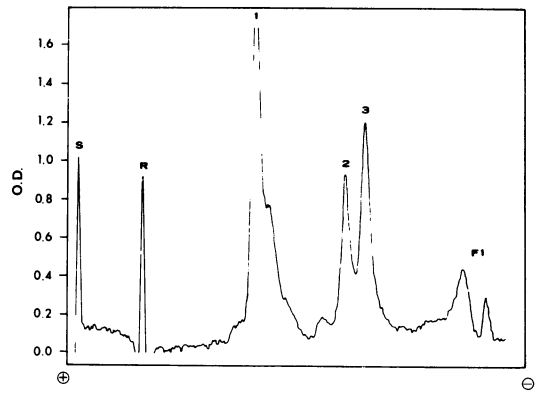

Figure 12.—*Acrylamide gel electrophoretic separation of G-3-PD from jack pine seedlings (48 hours) showing three isozymes. Peaks S, R, and F are start of spacer gel, start of running gel, and frontal bands, respectively.*

apparently because the amount of power applied denatures too much enzyme for use with extracts that are basically low in enzyme activity.

In general, crude extracts have been prepared and electrophoresed without either purification or determination of activity before assay. In our experience, this approach has serious faults, at least in the study of dehydrogenases.

Enzyme may be completely denatured during electrophoresis, but presumed absent from the extract. Moreover, the degree of purity of enzyme in crude extract may vary with developmental stage of the plant, organ, or tissue from which enzyme was extracted. Thus, the density and number of isoenzymes found may vary with developmental stage or physiological status, but not because of a true relation between the enzyme and metabolic levels or rates. Rather, the new chemical composition of the crude extract may have modified the structure or catalytic ability of the enzyme to create an artifact. Such occurrences are possible, for example, when levels of phenolics vary in crude extracts. However, other low-molecular-weight chemicals may also modify structure or catalytic activity.

The basic approach to electrophoresis must be cautious. Available methods of purification seem to preclude the need to use unassayed, crude initial extracts.

LITERATURE CITED

Barnett, James P., and Aubrey W. Naylor. 1969. Alcohol dehydrogenase activity and ethanol utilization in germinating longleaf and slash pine seeds. For. Sci. 15(4):400-403.

Brewer, G. J. 1970. An introduction to isozyme techniques. 186 p. Academic Press, New York.

Conkle, M. Thompson. 1971a. Inheritance of alcohol dehydrogenase and leucine amino-peptidase isozymes in knobcone pine. For. Sci. 17(2):190-194.

Conkle, M. Thompson. 1971b. Isozyme specificity during germination and early growth of knob-cone pine. For. Sci. 17(4):494-498.

Feret, Peter P. 1971. Isoenzyme variation in *Picea glauca* (Moench) Voss, seedlings. Silvae Genet. 20:46-50.

Feret, Peter P., and Gerald R. Stairs. 1971a. Enzyme electrophoresis—application of molecular biology to forest genetics. p. 72-80. *In* 18th Northeastern Forest Tree Improv. Conf. Proc.

Feret, Peter P., and Gerald R. Stairs. 1971b. Peroxidase inheritance in Siberian elm. For. Sci. 17(4):472-475.

Feret, Peter P. 1978. Perspectives on utility of isoenzyme analysis for improving growth and yield. *In* Fifth North American Forest Biology Workshop Proc., Univ. Florida, Gainesville, Florida. [In press.]

Firenzuoli, A.M., P. Vanni, E. Mastronuzzi, A. Zanobini, and V. Baccari. 1968. Enzymes of glyoxylate cycle in conifers. Plant Physiol. 43:1125-1128.

Goldstein, J. L., and T. Swain. 1965. The inhibition of enzymes by tannins. Phyto-chemistry 4:185-192.

Gordon, John C. 1971. Changes in total nitrogen, soluble protein, and peroxidases in the expanding leaf zone of eastern cottonwood. Plant Physiol. 47:595-599.

Haissig, Bruce E. 1972. Near total recovery of small tissue samples after milling. For. Sci. 18(3):261-262.

Haissig, Bruce E., and Arthur L. Schipper, Jr. 1971. Effect of ionic strength on recovery of plant enzymes in extracts prepared with anion exchange resin. Biochem. Biophys. Res. Commun. 45:598-605.

Haissig, Bruce E., and Arthur L. Schipper, Jr. 1972. Easy extraction of enzymes from small amounts of woody tissue. Anal. Biochem. 48:129-146.

Haissig, Bruce E., and Arthur L. Schipper, Jr. 1975a. Effect of sequestering and reducing agents on stabilization of plant dehydrogenase activity in crude extracts. Phytochemistry 14:345-349.

Haissig, Bruce E., and Arthur L. Schipper, Jr. 1975b. Effect of oxidized nucleotide coenzymes and mercaptoethanol on stabilization of plant dehydrogenase activity in crude extracts. Physiol. Plant. 35:249-255.

Haissig, Bruce E., and Arthur L. Schipper, Jr. 1977. Glyceraldehyde-3-Phosphate dehydrogenase activity and NADH/NAD levels during early development of *Pinus banksiana.* (Abstr.) Plant Physiol. 59:86.

Hamaker, J.M., and E.B. Snyder. 1973. Electrophoresis patterns of needle enzymes in longleaf and Sonderegger pines. U.S. Dep. Agric. For. Serv. Res. Note SO-151, 8 p. Southeast. For. Exp. Stn., Asheville, North Carolina.

Hicks, Ray R. Jr., and Bruce E. Haissig. 1974. Rapid concentration of woody plant enzymes before electrophoresis. p. 363. *In* Third North American Forest Biology Workshop Proc., Colorado State Univ., Fort Collins, Colorado.

Juo, Pei-Show, and G. Stotzky. 1973. Electrophoretic analysis of isozymes from seeds of *Pinus, Abies,* and *Pseudotsuga.* Can. J. Bot. 51:2201-2205.

Schipper, Arthur L., Jr. 1975. Changes in dehydrogenase and peroxidase activities of aspen infected with *Hypoxylon mammatum*. Phytopathology 65:440-445.

Wolter, Karl E., and John C. Gordon. 1975. Peroxidases as indicators of growth and differentiation in aspen callus cultures. Physiol. Plant 33:219-223.

☆ U. S. GOVERNMENT PRINTING OFFICE: 1978—768-880/69