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PREPARATION OF EXTRACTS FROM JUNIPER LEAVES FOR ELECTROPHORESIS

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Abstract—The action of phenolase and subsequent reaction; in plant tissues with a high phenol to protein ratio causes a reduction in enzyme activity during extraction. To overcome the action of phenolases in Juniper leaves, an extraction buffer was developed which contained sodium tetraborate, sodium ascorbate, sodium meta-bisulfite, sodium diethyldithiocarbamate, and germanium dioxide. This extraction buffer was used in combination with an extraction procedure using liquid nitrogen, polyvinylpyrrolidone, *n*-butanol, and diethyl ether. Modifications in the buffer or extraction procedure resulted in a reduction in both the quality and number of isoenzymes obtained. Differences obtained in isoesterase banding by changing the extraction procedures should caution the systematic botanist that extraction methods may have critical effects in isoenzyme studies.

INTRODUCTION

A high ratio of phenolics to protein in leaves is common in gymnosperms, is often found in perennial angiosperms, and appears occasionally in annual angiosperms [1, 2]. These large amounts of phenolics are known to complex with enzymes following cellular destruction [3–5]. Thus when leaf material of plants with high concentrations of phenolics (such as *Juniperus*) are ground at 20° with a minimum amount of buffer (i.e. Pi buffer, pH 7) one obtains a highly viscous fluid which browns rapidly. Electrophoresis of this extract yields some peroxidase activity; no activity for malate dehydrogenase, succinate dehydrogenase, or glutamic-oxaloacetic transaminase and smeared staining with no distinct banding for esterases [6]. Reactions catalyzed by the phenolase enzyme complex (Monophenol, dihdroxyphenylalanine: oxygen oxidoreductase. E.C. 1.14.18.1) and subsequent reactions are primarily responsible for the loss of enzyme activity in plant extracts [7–13].

Methods for eliminating these problems in protein extraction have been outlined in papers by Loomis and Battaile [5], Loomis [4], and Anderson [14]. Attempts at resolving these problems by following previously used techniques for sample preparation [3, 15, 16] and with various phenolase inhibitors [6] were unsuccessful. The purpose of this paper is to present techniques for obtaining active enzymes from plants in which phenols cause difficulty in protein extraction and to examine the effects of different extraction procedures upon isoenzyme variation.

RESULTS

The results of electrophoresis of the eleven extracts are summarised in Table 1. Changes in the chemical composition of the extraction buffer in most cases resulted in either a loss of enzymatic activity (as reflected by the absence of enzyme bands in the gels) or a reduction in band clarity. In all extracts showing enzymatic activity, changes in the extraction buffer did not alter the number or location of bands seen in the gels. Sample 1 (control), which was prepared using the normal extraction buffer and the normal extraction procedure, gave the best enzyme banding for the three enzyme systems investigated. Removal of sodium teraborate (sample 2) reduced the gel banding quality for peroxidases and α-terpineol (alcohol) dehydrogenases and resulted in a loss of esterase activity. The omission of sodium ascorbate (sample 3)

Table 1. Sample descriptions and summary of zymograms. Quality of zymogram banding evaluated at: + + +, sharp bands with clear gel background; + +, some band smearing with clear gel background; +, band smearing with discoloration in gel background; −, no banding. The number of enzyme bands for each sample is indicated in brackets next to each gel quality evaluation

Sample description	Zymogram evaluation and number of bands		
	Peroxidase	Esterase	α-Terpineol dehydrogenase
1. Control	+ + +(9)	+ + +(2)	+ + +(1)
Buffer without			
2. Na tetraborate	+(9)	—	+(1)
3. Na ascorbate	+(9)	+ +(2)	+ +(1)
4. Na meta-bisulfite	+ +(9)	—	+(1)
5. DIECA	+ +(9)	—	—
6. germanium dioxide	+ + +(9)	+ +(2)	—
7. DMSO	+ +(9)	+ + +(2)	+ + +(1)
Extraction without:			
8. *n*-BuOH	+ +(9)	+ +(7)	—
9. di Et_2O	+ + +(9)	—	+ +(1)
10. *n*-BuOH and diEt_2O	+(9)	+(7)	—
11. PVP	+(9)	+(2)	—

from the extraction buffer caused a loss of banding clarity three enzyme systems. Sample 4, obtained when the buffer did not contain sodium metabisulfite, showed a reduction in the clarity of enzymatic banding patterns for peroxidases and α-terpineol (alcohol) dehydrogenases and a loss of esterase activity. When sodium diethyldithiocarbamate (DIECA) (sample 5) was not included in the extraction buffer no activity for esterases or α-terpineol (alcohol) dehydrogenases was detected and the clarity of peroxidase banding was moderately reduced. When germanium dioxide was omitted from the extraction buffer (sample 6) esterase gel clarity was slightly reduced, peroxidase banding equal to that of the control, and α-terpineol (alcohol) dehydrogenase activity lost. An extraction buffer which did not contain dimethyl sulfoxide (DMSO) (sample 7) gave results similar to that seen in the control for esterases and α-terpineol (alcohol) dehydrogenases and a slight reduction in peroxide gel clarity.

In almost every case, modification in the extraction procedure either resulted in a reduction of enzyme banding quality or a loss of enzymatic activity. When enzymatic bands did develop the number and location of these bands were identical for all extracts except in the esterases of samples 8 and 10. These samples showed identical and distinctive isoenzyme banding patterns for esterases. The omission of the *n*-BuOH step from the extraction procedure (sample 8) gave a slight reduction in quality for peroxidase and esterase banding with a loss of α-terpineol (alcohol) dehydrogenase activity. This extract yielded an esterase isoenzyme pattern identical to that seen for sample 10 (neither *n*-BuOH nor Et_2O) with five additional isoesterases not seen in the other extracts. Sample 9, which was prepared with the omission of Et_2O, showed peroxidase activity equal to that of the control, no activity for esterases, and a slight reduction in banding quality of α-terpineol (alcohol) dehydrogenase. When both *n*-BuOH and Et_2O were omitted from the extraction procedure (sample 10), banding patterns similar to that seen in sample 8 (without *n*-BuOH) were seen. In this sample there was no α-terpineol (alcohol) dehydrogenase activity and the peroxidase and esterase gels showed an increase in discoloration of the gel background and smearing of the bands. Thus, sample 10 showed the same esterase banding pattern seen in sample 8 (i.e. five additional isoesterases). Removal of the PVP from the extraction procedure (sample 11) greatly reduced the banding quality of the peroxidase and esterase gels due to the increase in band smearing and background discoloration, and caused a loss of α-terpineol (alcohol) dehydrogenase activity.

DISCUSSION

Extracts obtained using a buffer composed of five inhibitory compounds and DMSO in combination with the original extraction procedure gave the highest clarity polyacrylamide gels for the 3 enzymes tested. With the exception of the omission of DMSO, modification in the extraction buffer or procedure resulted in either a reduction in the clarity of gel banding or a loss of enzymatic activity. When DMSO was not included in the buffer, results nearly equivalent to those found in the control were obtained. DMSO was added to the buffer because of its ability to stabilize enzyme extracts during prolonged storage [19] and its removal from the extraction buffer had little effect.

The effect of changes in the extraction protocol varied considerably, depending on the enzyme system tested. Peroxidases were least affected by alterations in the extraction buffer or procedure. Peroxidase activity was never lost, although in most cases the clarity of the gel banding patterns was reduced. α-Terpineol (alcohol) dehydrogenase was very sensitive to changes in the extraction procedure, with either a loss of activity or reduction in banding quality. The esterases showed either a loss of activity, reduction in banding quality, or changes in isoenzyme banding patterns when the extraction methods were changed. Of particular interest is the increase in the number of isoesterases detected when *n*-BuOH was removed from the extraction steps (samples 8 and 10). The staining method used for esterases allows for the detection of both α- and β-esterases [18].

β-Esterases are often inhibited by alcohols [20] and differences in zymograms of extracts having esterase activity may be explained by this fact. When esterase activity was present and *n*-BuOH used (samples 1, 3, 6, 7, 11) two isoesterases were seen; these perhaps being α-esterases. Seven isoesterases developed in active extracts (samples 8 and 10) when *n*-BuOH was omitted; the two α-esterases (?) and 5 additional isoenzymes. These five additional isoesterases may have been β-esterases which were inhibited by *n*-BuOH.

As summarized by Anderson [14], the formation of oxidation products of the phenolase complex and subsequent reactions can be inhibited in a variety of ways: removal of the phenolic substrates; inhibition of the phenolase complex activity; and removal of the quinones by reduction back to *o*-diphenols or by condensation with compounds that yield a product which will not be inhibitory to enzymes. In order to achieve these results in extracts of *Juniperus* foliage it was necessary to include 5 inhibitory compounds in the extraction buffer and PVP and two organic solvent separations in the extraction procedure.

Borate inhibits the action of the phenolase complex by binding to *o*-diphenols, forming a covalently bound phenol–borate complex [21] which cannot act as a substrate for the enzyme [22]. A competitive inhibitory condition is created with the borate and phenolase competing for the *o*-diphenols and thus reducing the amount of *o*-quinones produced [23]. Germanate is believed to function via a mechanism similar to that of borate [23]. PVP binds phenolics [4]. DIECA inhibits the phenolase which requires copper at its active center [14]. In addition, DIECA, as a thiol, reacts with quinones forming covalently bonded phenol–thiol complexes which are not inhibtory to enzymes [11]. The use of DIECA for its copper chelating ability in an extraction buffer will of course inhibit other enzyme systems having a requirement for this metal (e.g. ascorbic acid oxidase, cytochrome oxidase), thus reducing the total number of enzyme systems which may be investigated. The phenolase complex is inhibited by ascorbic acid by mechanisms not yet fully understood. Ascorbate also reduces quinones (formed by phenolase) back to phenols [8]. The presence of metabisulfite in the extraction buffer causes the formation of covalent PhOH–sulfite complexes which will not react with protein [8]. Bisulfite may have undesirable side effects in extracts due to its inhibitory action on other enzyme systems, especially some dehydrogenases [24]. BuOH and Et_2O separatory steps were included in the extraction procedure primarily

to remove phenols not removed by the PVP. The use of organic solvents appears essential in obtaining a broad spectrum of enzyme activity in Juniper but their effect on changes in isoenzymes banding patterns (i.e. the effect of BuOH on isoesterases) should be kept in mind.

Loomis [4] discusses in considerable detail the control of pH in plant extracts. We performed our extractions at pH 7 for several reasons. The optimum results in isolation of plants enzymes are usually obtained in a pH range from pH 6.5 to 7.2 [4]. The amount of phenolics bound to PVP decreases as pH increases [4]. Although more phenols could be removed at a pH less than 7 by PVP, other inhibitors present in the buffer are most effective at *ca* pH 7. DIECA loses efficiency in extractions when the pH exceeds 7.5 [14]. Borate and germanate function most efficiently at pH 7.5 [23].

Juniper tissue was ground in liquid nitrogen since it allows fracture of the tough leaf tissue, it cools the extract and provides an oxygen free atmosphere during the initial cellular destruction phase of the extraction procedure. Molecular oxygen is required for the conversion of monophenols to diphenols and quinones by the phenolase complex [9].

Loomis [4] in discussing the aim of his publication says, "Our purpose here will be to describe, in as general a way as possible, the special problems of plant enzymology, and the 'bag of tricks' that have been developed to deal with these problems." In *Juniperus*, obtaining an extract with a broad spectrum of enzyme activity involves using almost the entire 'bag of tricks.' The action of phenolase could not be significantly reduced by any single inhibitor [6] and phenolase inhibition could be achieved only by using a combination of inhibitors, each functioning by way of a different chemical mechanism.

When isoenzyme analysis first became available it was thought that crude extracts could be used and, in certain organisms, this is apparently true, but for others (i.e. *Juniperus*) this is not the case. The chemical adaptive strategy 'developed' by the organism under investigation will determine the extraction methods required for studying its isoenzymes. A general guideline which might be used is that the best results will be obtained from the 'cleanest' extracts prepared with the fewest inhibitory chemicals and simplest extraction procedure.

EXPERIMENTAL

Each sample was prepared from 10 g of foliage collected from the same *Juniperus scopulorum* Sarg. tree and extracted on the same day. Electrophoresis was performed on all samples concurrently and within 72 hr from the time of extraction.

Extraction buffer. The standard extraction buffer developed for use with Juniper was a 0.1 M Tris–maleate buffer pH 7 containing: 0.2 M Na tetraborate, 0.25 M Na ascorbate, 0.02 M Na meta-bisulfite, 0.02 M DIECA, 0.01 M germanium dioxide, and 10% DMSO. The buffer was prepared by dissolving the germanium dioxide in boiling H_2O; this soln was allowed to cool to 25°, then the other components were added and the pH adjusted.

Extraction procedure. PVP powder (10 g) was mixed with 50 ml extraction buffer and allowed to hydrate for 24 hr at 6°. Following hydration a buffer–PVP paste was obtained. 10 g of Juniper foliage were placed in a cold mortar with 5 g alumina and ground with liquid N_2 until a fine green powder was obtained. This powder was then added to the buffer–PVP paste, mixed, and allowed to warm at room temp. for 30 min. The Juniper–buffer–PVP paste was then expressed through nylon. Liquid obtained was centrifuged at 400 *g* for 10 min at 6°. Supernatant was retained and mixed with an equal vol. of *n*-BuOH in a separatory funnel. A colloidal suspension was obtained which was centrifuged at 400 *g* for 10 min at 6°. Following centrifugation a 3 phase suspension was obtained composed of an upper BuOH layer, a middle semi-solid layer, and a lower H_2O layer. The aq. layer was removed and extracted with an equal vol. of Et_2O. The aq. layer was removed and concentrated by dialysis in powdered sugar for 48 hr at 6°. Following dialysis all samples were stored at −20° until electrophoresis. The control sample (Sample 1) was prepared using the extraction buffer and the extraction procedure outlined above.

Modifications in the extraction buffer. 6 modified 0.1 M Tris–maleate buffers pH 7 were made with the omission of one of the following: Na tetraborate, Na ascorbate, Na meta-bisulfite, DIECA, germanium dioxide, or DMSO. Extracts were made from Juniper foliage using these 6 modified buffers and the normal extraction procedure (see below) to obtain samples 2–7.

Modifications in the extraction procedure. A series of modifications in the normal extraction procedure were made involving: omission of the *n*-BuOH extraction, omission of the Et_2O extraction, omission of both the *n*-BuOH and the Et_2O extractions, and omission of the PVP. When the PVP was omitted the amount of extraction buffer used was reduced from 50 to 25 ml since the PVP retains about 25 ml of extraction buffer following the expression of the Juniper powder–buffer–PVP paste in the normal extraction procedure. 4 extracts were made according to these modifications in the extraction procedure using the normal extraction buffer to obtain samples 8–11.

Electrophoresis. Starting at the bottom of the vertical cell, layers of 8% (55 mm), 6% (10 mm), and 4.5% (5 mm) acrylamide in pH 9, 0.375 M Tris–sulfate buffer were cast. The 12 sample wells (10 mm deep) were formed in 8% acrylamide, pH 9, 0.075 M Tris–sulfate gel. All samples were made up to 15% sucrose and 20 μl of each sample was pipetted into the well. The wells were capped with 8% acrylamide, pH 9, 0.075 M Tris–sulfate. The gel slab was subjected to electrophoresis in cold (10°) Tris–borate, pH 9, 0.065 M buffer with a pulsed power supply at a constant 350 V. The power settings were 5 min at 75 pulses per second (pps) (45 mA), 5 min at 150 ppsec (90 mA), 5 min at 225 ppsec (125 mA), and 60 min at 300 ppsec (150 mA). This electrophoretic procedure allows for separation of anionic isoenzymes.

Gel staining procedures. Gels were stained for peroxidases following the method of ref. [17] and for esterases using the staining procedure of ref. [18]. Alcohol dehydrogenases in *Juniperus* showed the darkest banding zymograms when terpene alcohols (α-terpineol or borneol) were substituted for EtOH. Zymograms of Juniper where EtOH, α-terpineol, or borneol is used as the substrate show identical banding patterns [6]. The α-terpineol (alcohol) dehydrogenase stain contained: 0.1 M Pi buffer pH 7, 0.02 M α-terpineol dissolved in enough Me_2CO to make the final staining soln 25% (v/v) Me_2CO, 0.2 mM PMS, 1 mM M MTT, 2 mM NaCN, 8.5 mM $MgCl_2$ and 1 mM NAD. Gels were stained for α-terpineol (alcohol) dehydrogenase 6 hr at 37° followed by 8 hr staining at 25°.

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