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EFFECT OF SEQUESTERING AND REDUCING AGENTS ON STABILIZATION OF PLANT DEHYDROGENASE ACTIVITY IN CRUDE EXTRACTS

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Abstract—Initial and long-term loss of dehydrogenase activity in crude extracts of herbaceous, and, especially, of woody tissue occur partially because of the inhibitory influence of phenolics. In addition, oxidation of phenols by phenolase results in subsequent enzyme oxidation. Preparation of crude extracts with insoluble PVP, in comparison with anion exchange resins or celluloses, best decreases phenolic concentrations and least decreases dehydrogenase activity in crude extracts. However, removal of phenolics during tissue homogenation does not maximize dehydrogenase activity. Therefore, other methods must be used to stabilize dehydrogenase activity. Sodium ethylenediaminetetracetic acid or sodium azide promoted activity of both purified mushroom and crude plant phenolases. Quinone reduction with diethyldithiocarbamate (DIECA) or mercaptoethanol eliminated apparent phenolase activity, but DIECA inhibited dehydrogenase activity. Elevated concentrations of EtSH diminished initial decay of dehydrogenase activity. Combined use of EtSH and insoluble PVP further stabilized 6-phosphogluconate, glucose 6-phosphate, and malate dehydrogenase, but not glyceraldehyde 3-phosphate dehydrogenase.

INTRODUCTION

Difficulties abound in the stabilization of enzymes in crude plant extracts, especially in the little-studied extracts of woody tissue. Previous research suggests that phenols and tannins are the major enzyme denaturants in plant extracts, although pH, osmotic strength and enzyme solubility must also be considered [1,2]. Tannins denature enzymes by cross linking with them through hydrogen, ionic, or covalent bonds [3]. Phenols denature proteins directly, through hydrogen bonds, or by covalent bonding after their enzymatic oxidation to quinones via the action of phenolase [4–6]. Quinones, besides denaturing enzymes directly, may condense in a series of nonenzymatic reactions to form brown polymerized polyphenols, after which the brown polymers coprecipitate with enzymes. Brown polymer formation, observed as browning of an extract, usually results in complete loss of enzyme activity. Phenolase may also directly denature enzymes, itself included, by oxidizing tyrosine residues [6].

Various stabilization techniques to overcome rapid loss of enzyme activity are used but none has proved generally effective. All techniques are based on removal of phenolics, removal of phenols or copper to prevent phenolase activity, or chemical reduction of quinones, but little is known about the amount of phenolics or phenolase in crude extracts, the efficiency with which various agents remove phenolics from such extracts, or the many possible side effects that removal of phenolics may have on enzyme activities [1,4,5,7]. Some highly effective reducing agents, such as dithiothreitol (DTT) and EtSH have not been tested. Finally, the effects of copper chelating agents on crude plant phenolase have not been thoroughly examined. Most attempts have been confined to stabilization of enzymes during tissue homogenization or shortly thereafter. Few investigators have measured the subsequent rate of decay. Moreover, the causes of activity losses in specific experiments have usually been deduced rather than demonstrated.

Our experience has shown that enzyme activity decays more rapidly in woody than in non-woody plant extracts which is enough to preclude or confound subsequent analysis. We therefore investigated two types of enzyme activity loss that occur in woody plant extracts. First, the loss that occurs during homogenization and centrifugation was examined; this was termed "initial decay" because it occurs before an initial enzyme activity determination. The magnitude of this decay cannot be accurately measured, but it may be estimated for a given treatment relative to the initial activity of a suitable control. Second, the readily quantified "long-term decay" that occurs later was examined. The present paper thus describes effects on initial and long-term dehydrogenase activity of treatments that either sequester phenolics or metals, or block completely or partially the reaction sequence leading to brown polymer formation.

RESULTS AND DISCUSSION

Extracts of woody tissue turn brown within 2 or 3 hr of preparation even when kept ice cold. Homogenates of some tissues (e.g. *Salix fragilis* stems) brown during preparation. Such browning, and probably at least part of the concomitant loss of enzyme activity, result from the oxidation of phenols by phenolase. Three lines of evidence support this conclusion. First, browning occurs via an enzymatic reaction; boiled extracts do not brown. Second, the rate of browning increases with oxygenation and decreases in oxygen-poor extracts in comparison with untreated controls. Phenolase requires molecular oxygen in the oxidation of phenols to *o*-diphenols and of the latter to *o*-quinones [6]. Third, the woody plant tissue contained both substrate and enzyme.

Removal of phenolics

Substrate removal is effective only if all or nearly all phenols can be removed and if substrate removal does not itself diminish the activity of other enzymes. Insoluble PVP [4] removed phenol and tannic acid in a concentration-dependent manner from unbuffered aq. soln. PVP treatment almost completely removed tannic acid [8], but only partially removed phenol and the phenolics (estimated as phenol) from buffered tissue homogenates.

In spite of the incomplete removal of phenolics, we ascertained the effect of PVP on initial and long-term decay of enzyme activity. The test employed (malate dehydrogenase (MD), phosphogluconate dehydrogenase (6-PGD), glucose 6-phosphate dehydrogenase (G-6-PD) and glyceraldehyde 3-phosphate dehydrogenase (G-3-PD) extracted from red pine, white spruce, aspen, sugar maple, barberry, black walnut, barley, tomato, spinach, and sweet potato. PVP generally enhanced (5-fold or more) initial enzyme activities and sometimes lessened rates of decay, even for tissues such as sweet potato tuber that contain low total phenolics, but its effect was pronounced in the woody species. In species other than sweet potato, PVP treatment invariably decreased initial G-3-PD activity (Fig. 1).

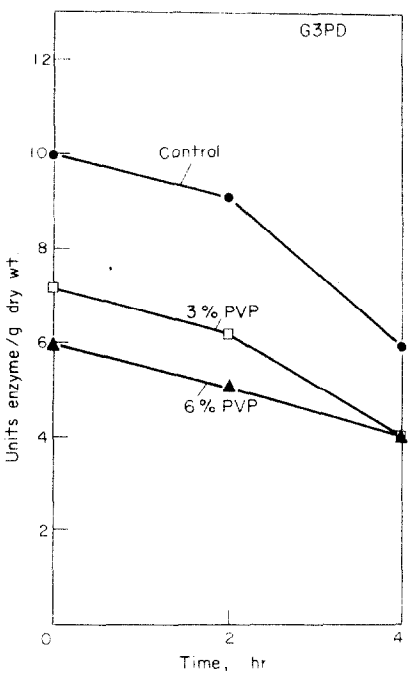

Fig. 1. Effect of PVP during tissue homogenization on activity of glyceraldehyde 3-phosphate dehydrogenase (G-3-PD) in aspen extracts.

PVP treatment did not yield dehydrogenase activity from sugar maple and black walnut; the PVP-treated extracts browned more rapidly than did untreated controls. The more rapid browning was due to an increase of phenolase activity, possibly due to the removal of phenolics that inhibit

phenolase, but not of oxidizable phenols. Activation of phenolase by PVP apparently does not lower initial activities or hasten the decay of dehydrogenases if extracts contain low initial levels of substrate phenols or the PVP adsorbs appreciable substrate phenol. Thus, a combination of factors determines whether or not PVP treatment will increase initial enzyme activity and retard activity decay. In addition, PVP may sensitize some enzymes, such as G-3-PD, to the action of phenolase by inducing conformational changes that expose tyrosine residues, which phenolase could then oxidize.

PVP is not an ideal agent for removing phenol; nor have we found an agent that both removes phenols and does not decrease initial enzyme activity. Anion exchange resins such as AG 1-X8 remove phenols from solution, but they also remove enzymes with low *iso*-electric points [2]. Anion exchange celluloses remove phenols less effectively from solution, but they do remove enzymes such as G-3-PD.

Effects of chelating and reducing agents

Several chemicals have been reported to "inhibit" phenolase activity [9–14]. These compounds competitively inhibit the enzyme with respect to substrate oxygen (cyanide) or phenol (benzoic acid), complex copper (DIECA, cyanide, sodium azide, EDTA, CO) or decrease the apparent rate of quinone formation either by complexing with quinone (DIECA) or by reducing the quinone (sulfhydryl-containing reagents). Of these, copper chelation and quinone reduction have been most used with plant extracts.

We tested the effects of DIECA, sodium azide, EDTA, and EtSH because of the varied mechanisms by which these chemicals prevent brown polymer formation. Both DIECA (1 mM) and EtSH (10 mM) eliminated apparent quinone formation by phenolase. Sodium azide stimulated or did not greatly change apparent quinone formation, while EDTA markedly stimulated apparent quinone formation (Fig. 2). Because the copper-complexing reagents we tested did not inhibit phenolase, the effect of DIECA on quinone formation was probably indirect, by complexing with or reducing quinone, rather than through direct inhibition of the enzyme by removing copper [5,11,12].

We tested the effects of DIECA on dehydrogenase activity because DIECA proved at least 10× as effective as EtSH in eliminating apparent quinone formation. DIECA at 100 mM inhibited G-3-PD and G-6-PD activity. At 1 mM DIECA G-3-PD activity was somewhat decreased and G-6-PD activity increased. In addition, 1 mM DIECA decreased the rate of decay of both enzyme activities over a 4-hr period. At 0·1 mM DIECA, mushroom phenolase was completely inhibited but 0·1 mM DIECA neither increased initial activity nor lessened rates of decay over 24 hr for either G-3-PD or G-6-PD in comparison with no treatment or 100 mM EtSH.

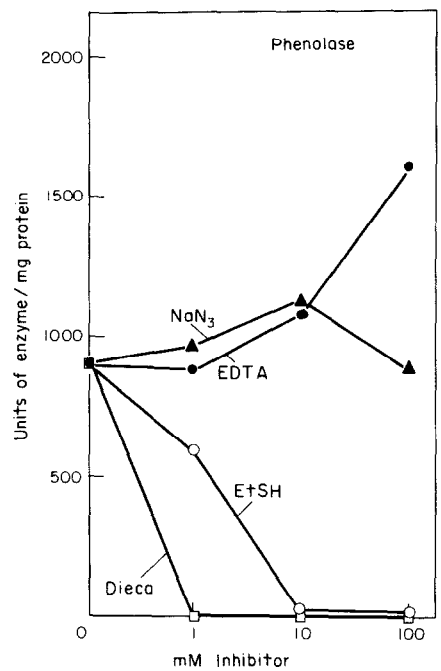

Fig. 2. Influence of EDTA, NaN_3, EtSH and DIECA on the activity of mushroom phenolase.

A similar test indicated that EtSH increased initial enzyme activity, sometimes spectacularly (G-3-PD), and lessened the rate of enzyme decay over a 4-hr period. Maximum initial dehydrogenase activity was obtained with 100 mM EtSH or 10 mM DTT, and in no instance have these concentrations inhibited dehydrogenase activity (Fig. 3).

Combined effects of phenol removal and reducing agent

Removal of phenolics by PVP and control of phenolase activity with EtSH afforded a potential means to diminish both initial and long-term decay of dehydrogenase activity. In practice, a pronounced initial and long-term (72-hr) stabilizing effect was noted for MD and 6-PGD obtained from aspen, black walnut, barberry, red pine, sugar maple, white spruce, spinach, tomato, barley, and sweet potato. The combined treatment usually also markedly increased initial G-6-PD activity, but either did not lessen or even increased rates of long-term activity decay. The use of PVP with EtSH always increased the rate of decay of G-3-PD activity.

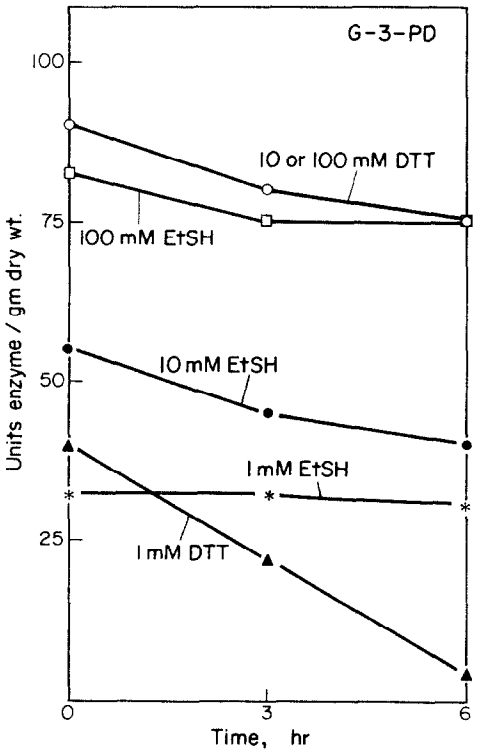

Fig. 3. Influence of EtSH and DTT in the extraction buffer on the activity of glyceraldehyde dehydrogenase (G-3-PD) in aspen extracts.

EXPERIMENTAL

Plant material. Sample collection and storage methods were reported elsewhere [2]. The following species were used: aspen (*Populus tremuloides*), black walnut (*Juglans nigra*), apple (*Malus* sp.), barberry (*Berberis* sp.), red pine (*Pinus resinoa*), sugar maple (*Acer saccharum*), white spruce (*Picea glauca*), silver maple (*Acer saccharinum*), spinach (*Spinacia oleracea*), tomato (*Lycopersicon esculentum*), pinto bean (*Phaseolus vulgaris* cv. Top Crop), collards (*Brassica oleracea* var. *virdis*), barley (*Hordeum vulgare*), sweet potato (*Ipomoea batatas*), and mushroom (*Agaricus bisporus*). Enzymes were extracted from stems and leaves of trees, tomato, and barley; bean leaves; potato tuber; and mushroom stalks.

Experimental design. Experiments were replicated 3× or more. Data in figures represent means of typical experiments. Standard errors did not exceed the height of symbols used to depict means. Tissue was collected from randomly selected plants, and no estimate was made of possible variation within or between plants.

Enzyme extraction and assay. Full details of enzyme extraction and assay were reported elsewhere [2,15,16]. In summary, 50 mg of lyophilized tissue, previously passed through the 20-mesh screen of a Wiley mill, were homogenized in 2 ml of 100 mM Tris–HCl, pH 7·5, containing 100 mM sucrose and 0·1% (w/v) fatty acid-free bovine albumin (with additions noted in the text). The pH of buffers was adjusted after EtSH or DTT was added. Homogenates were diluted with 1 ml of extraction buffer that was used to rinse the homogenizer. Supernatants were further diluted with extraction buffer, as necessary, to decrease reaction rates. Extracts were prepared and always maintained at about 5°. G-3-PD and MD, and G-6-PD and 6-PGD activity was determined by reduction of NAD and NADP, respectively. Phenolase was determined by measuring tyrosine oxidation to *o*-quinone and its condensation products in oxygenated buffer.

Estimation of phenolics. Phenolics were estimated by the method of Folin and Ciocalteu [17]. Extracts were treated overnight at 5° with an eq. vol. (1 ml) of 20% (w/v) trichloroacetic acid and centrifuged to remove precipitated protein before analysis. Blanks were similarly prepared by treating extraction buffer alone with trichloroacetic acid. Absorbances were converted to μg phenol per ml by using a linear regression obtained with solutions of phenol. This method is an approximate one, since although phenol itself obeys Beer's law, other plant phenols do not.

Estimation of tannins. A bluish color results when 2 ml of a freshly prepared 100 mM aq. soln of ferrous ammonium sulfate are added to a mixture of 1 ml of aqueous tannin solution and 2 ml of 0·1% (w/v) bovine albumin. Absorbance was measured at 550 nm 15 min after adding the iron salt. Tannin solution alone, in the presence of ferrous ammonium sulfate, did not give a color reaction, nor did simple phenols react with or without bovine albumin present. Data were quantified by use of a curvilinear plot of absorbance against concentration of tannic acid.

Measurement of the apparent phenolase activity decrease caused by EtSH. A solution of phenolase was prepared in glass-distilled water with (soln A) and without (soln B) 100 mM EtSH. Soln B and a portion of soln A were lyophilized, brought to the original volume with glass-distilled water, and assayed for phenolase activity. The remainder of soln A was monitored for phenolase activity in a recording spectrophotometer for several hours until apparent enzyme activity was restored.

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