EFFECT OF IONIC STRENGTH ON RECOVERY OF PLANT ENZYMES IN EXTRACTS PREPARED WITH ANION EXCHANGE RESIN

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SUMMARY

In order to remove inhibitory phenols, enzymes were extracted in the presence of Dowex AG 1-X8 or insoluble polyvinylpyrrolidone (PVP) from germinating flax seeds and branch tissue of a poplar and willow. Dowex treatment resulted in decreased enzyme activity unless ionic strength of the extraction buffer was raised above a threshold value. Apparently, isoenzymes with low isoelectric points are selectively removed from extracts by Dowex under conditions of low ionic strength. Under the best conditions tested, Dowex, in comparison with PVP treatment, did not generally enhance the recovery of enzyme activity from poplar and willow, nor was browning of extracts lessened.

INTRODUCTION

Anion exchange resin has recently been advocated for the removal of phenols from plant extracts in order to prevent denaturation of enzymes (1). Plant extracts, particularly those prepared from woody stems, contain sufficient phenols to quickly inactivate unprotected enzymes. Phenols act directly as denaturants or indirectly after their enzymatic conversion to quinones, which undergo non-enzymatic polymerization to form inhibitory brown pigments (2,3). Thus, browning of an extract is tantamount to complete loss of enzyme activity. Removal of phenols is a good but not singular method to prevent browning (3). However, although enzyme activity may be protected in the presence of phenols, they often interfere with subsequent analytical techniques, again necessitating removal.

Phenols have most commonly been removed from crude extracts by adsorption during tissue homogenization (2). PVP[1/] is the favorite adsorbant, although

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[1/] The following abbreviations are not found in Arch. Biochem. Biophys. 115: 1-12 (1966): PVP, polyvinylpyrrolidone; PR, peroxidase (E.C. 1.11.1.7); Glc-6-PD, glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49); Glyc-3-PD, glyceraldehydephosphate dehydrogenase (E.C. 1.2.1.12); and CS, citrate synthase (E.C. 4.1.3.7).

it is inefficient, frequently delaying but not preventing loss of enzyme activity (2,3). Recently, in experimenting with extracts of flax cotyledons, Lam and Shaw (1) found Dowex 1-X8 superior to PVP. In the comparison, Dowex quantitatively removed phenols, resulting in increased activities of o-diphenol oxidase and PR.

These promising results led us to further explore the feasibility of employing anion exchange resin to remove phenols during the extraction of PR, Glc-6-PD, Glyc-3-PD, and CS from flax and two woody plants. Our results, though generally supporting Lam and Shaw (1), suggest caution in the use of anion exchange resin which, under certain conditions, yielded extracts with lower enzyme activity when compared with insoluble PVP.

METHODS

Branch tissue of dormant aspen (Populus tremuloides) and brittle willow (Salix fragilis), and germinating seeds of flax (Linum usitatissimum) were lyophilized and ground to pass the 20-mesh screen of a Wiley mill. About 50 mg of lyophilized powder was extracted in a glass homogenizer containing 2 ml of buffer, always with 0.1% bovine albumin and 0.1 M sucrose. At times, various amounts of dry, insoluble PVP (Polyclar AT) or Dowex (AG 1-X8) were included. An additional 1 ml of grinding buffer was added to each homogenate prior to centrifugation. All operations were performed at 0 to 5C in a coldroom.

Supernatants were assayed immediately for activity of Glc-6-PD, CS, PR, and, sometimes, Glyc-3-PD. Assays were modified from those of the Worthington Biochemical Corp. and Srere (4).

PR from some crude extracts and a commercial preparation of Glc-6-PD (Leuconostoc messenteroides; Worthington Biochemical Corp.) were partially characterized by vertical-slab acrylamide gel electrophoresis which allowed comparison of 24 samples in a single gel. PR and Glc-6-PD isoenzymes were detected by the methods of (5) and (6), respectively. In addition, pI of the Leuconostoc Glc-6-PD was determined by isoelectric focusing in 110 ml columns, followed by pH measurement and enzyme assay of effluent fractions.

Experiments were repeated at least once with results that did not differ appreciably from those shown.

RESULTS AND DISCUSSION

The first data obtained clearly indicated that either no treatment or PVP treatment yielded higher enzyme activity than did extraction in the presence of Dowex (Table 1).

TABLE 1. Effect of Dowex and PVP on enzyme activity in 0.05M Tris-HCl, pH 7.5, extracts.

Treatment	% (w/v) in buffer	Enzyme	Units per gm dry wt. by species		
			Willow	Aspen	Flax
PVP	0	PR	180.6	238.9	9.6
"	0.6	"	183.9	159.0	8.5
"	1.2	"	232.4	167.0	9.1
"	2.5	"	191.9	185.0	8.9
"	5.0	"	159.3	159.0	9.6
"	0	Glc-6-PD	0	3.4	-*
"	0.6	"	0	10.0	-
"	1.2	"	1.4	9.5	-
"	2.5	"	1.1	10.0	-
"	5.0	"	1.0	11.4	-
"	0	CS	0	3.4	3.4
"	0.6	"	0.3	3.3	2.8
"	1.2	"	0.7	3.4	2.7
"	2.5	"	0.6	3.6	2.9
"	5.0	"	0.8	3.2	3.1
Dowex	0	PR	180.6	238.9	9.6
"	0.6	"	135.4	200.3	7.9
"	1.2	"	111.7	159.9	8.0
"	2.5	"	118.8	119.0	7.6
"	5.0	"	95.2	101.0	7.5
"	0	Glc-6-PD	0	3.4	-*
"	0.6	"	0	1.7	-
"	1.2	"	0	0	-
"	2.5	"	0	0	-
"	5.0	"	0	0	-
"	0	CS	0	3.4	3.4
"	0.6	"	0	2.0	2.7
"	1.2	"	0.2	2.4	2.7
"	2.5	"	0.2	1.9	2.4
"	5.0	"	0	1.7	2.4

*Trace activity.

Under the conditions of this test, Dowex was little or no more effective than PVP in prolonging the onset of browning in brittle willow and aspen extracts. Brittle willow extracts from which phenols were not removed browned during or immediately after homogenization; aspen extracts browned more slowly; flax extracts did not brown, even overnight, at room temperature. If either aspen or brittle willow extracts were prepared several hours before assay, the amount of enzyme activity retained, except for PR, was directly related to the amount of PVP used during homogenization. This indicates that even the highest levels of PVP were only partially effective in removing phenols. We found no indication that too much PVP could be used in extraction of the enzymes studied, except PR, although with more than 10 percent PVP/buffer (w/v) it was difficult to separate homogenates into a liquid and solid phase by centrifugation.

Our results contradicted those of Lam and Shaw (1) who found increased PR activity when flax cotyledons were extracted with 0.05M Tris-glycine, pH 8.3, and 10 percent (w/v) Dowex 1-X8 chloride, as opposed to the same buffer containing 2 gm insoluble PVP per gm fresh weight of tissue. Since our findings might have been tempered by experimental conditions, we explored various possibilities for the noted reductions in enzyme activity in extracts prepared with Dowex.

Extracts prepared with either 0.1M Tris-HCl, pH 7.5, or 0.1M Tris-glycine, pH 8.3, containing Dowex chloride or glycinate yielded results similar to those shown in Table 1. The pH of supernatants (centrifuged immediately after grinding without the additional 1 ml of buffer) was not appreciably lowered. The logical hypothesis remained that Dowex was removing isoenzymes with low isoelectric points, a possible disadvantage of using the resin which was raised by Lam and Shaw (1). Subsequent experiments seemed to verify this hypothesis.

First, the activity of Leuconostoc Glc-6-PD (pI of the two major isoenzymes about 4.2) diminished in proportion to the amount of Dowex to which it was exposed, if the ionic strength of the buffer was low (Table 2). Contrariwise,

TABLE 2. Effect of PVP and Dowex treatment on activity of Leuconostoc Glc-6-PD.

Treatment	% (w/v) in buffer	Buffer molarity*	Units of enzyme	
			Per mg protein	As % of control
PVP	0	0.05	229	-
"	2.5	"	230	100
"	5.0	"	237	103
"	7.5**	"	241	105
Dowex	0	"	229	-
"	2.5	"	205	90
"	5.0	"	181	79
"	7.5	"	165	72
"	10.0	"	145	63
"	12.5	"	137	60
"	0	0.50	229	-
"	2.5	"	225	98
"	5.0	"	225	98
"	7.5	"	225	98
"	10.0	"	217	95
"	12.5	"	225	98

*Tris-HCl, pH 7.5 + 0.1% bovine albumin.
**Higher amounts of PVP were inseparable from enzyme solution.

no such reduction was found when this enzyme was exposed to PVP. If the ionic strength of the buffer was increased, Dowex treatment no longer had a negative effect. Second, the electrophoretic patterns of aspen and brittle willow PR showed selective removal of isoenzymes from extracts prepared with Dowex but not from those prepared with PVP. Restoration of isoenzyme patterns occurred in extracts prepared with Dowex when the ionic strength of the extraction buffer was increased (Fig. 1).

We subsequently found that the activities of enzymes in extracts prepared with PVP and Dowex were about equal if the resin was used in a buffer of high enough ionic strength (Table 3). It is noteworthy, however, that regardless of the ionic strength of the extraction medium used in this experiment, extraction with Dowex consistently removed Glc-6-PD activity from brittle willow extracts. The effect of ionic strength on enzyme activity in extracts prepared with PVP was negligible, except that PR activity was substantially increased at the highest ionic strength tested, probably because bound enzyme was released (7).

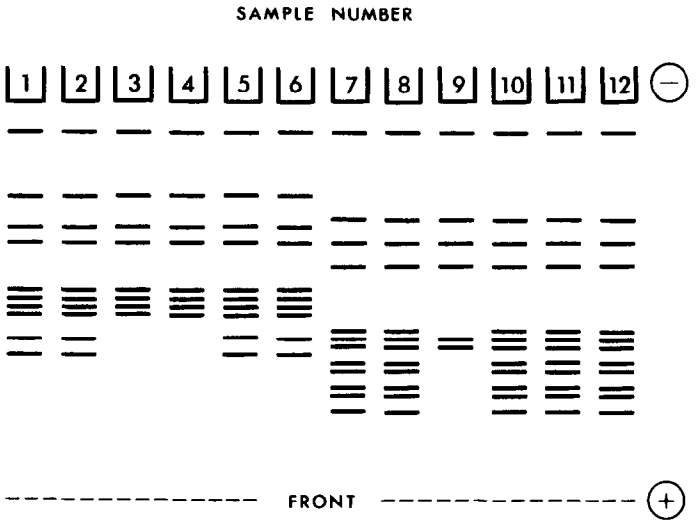

Figure 1. Electrophoretic patterns of peroxidase isoenzymes extracted in buffer of varying ionic strength containing PVP or Dowex.*

*Methods: Stacking gel -- 6% Cyanogum 41 in 0.06M Tris-HCl, pH 6.7. Running gel -- 12% Cyanogum 41 in 0.38M Tris-HCl, pH 8.5. Polymerization catalyzed with N,N,N',N' - tetramethylethylenediamine and ammonium persulfate (0.1% each). 50ul unconcentrated crude extract per sample compartment. Tissue was extracted with pH 7.5 Tris-HCl containing 7.5% (w/v) PVP or Dowex.

Sample identification: Brittle willow (1-6) or aspen (7-12) peroxidases extracted with, in numerical order: 0.05M buffer + no addition; 0.05M buffer + PVP; 0.05M buffer + Dowex; 0.10M buffer + Dowex; 0.15M buffer + Dowex; 0.50M buffer + Dowex.

Note: Intensity of banding patterns increased for Dowex extracts with increasing ionic strength of buffer.

It appears, then, that at least woody tissue extracts prepared with Dowex to remove phenols will maintain short term enzyme activity under conditions of high ionic strength. We cannot conclude, however, that Dowex can be freely substituted for PVP in established extraction techniques, nor that the use of Dowex will necessarily better prevent browning of extracts and thus preserve enzyme activity. Our use of Dowex under conditions of high ionic strength has produced extracts that initially exhibit enzyme activity equal to or greater than that of extracts prepared with PVP, as demonstrated in the present experiments. However, extracts prepared with Dowex brown and

TABLE 3. Effect of ionic strength on enzyme activity in extracts prepared with PVP and Dowex.

Species	Enzyme	Treatment*	Buffer molarity**		
			0.05	0.20	0.50
			Units of enzyme per gram		
Brittle willow	Glc-6-PD	PVP	1.5	1.5	1.3
		Dowex	0.0	0.0	0.0
" "	Glyc-3-PD	PVP	10.2	9.4	10.4
		Dowex	6.5	7.7	7.9
" "	CS	PVP	0.7	1.5	1.1
		Dowex	0.0	0.5	1.0
" "	PR	PVP	278.7	266.8	377.1
		Dowex	127.1	171.2	377.3
Aspen	Glc-6-PD	PVP	8.6	6.6	4.7
		Dowex	0.0	0.0	3.6
"	Glyc-3-PD	PVP	5.8	6.2	7.2
		Dowex	6.4	5.6	9.1
"	CS	PVP	1.8	2.3	3.5
		Dowex	0.8	2.3	2.9
"	PR	PVP	231.1	258.3	305.9
		Dowex	141.5	266.5	355.4

*7.5% Dowex or PVP (w/v) in Tris-HCl, pH 7.5, containing 0.1 M sucrose + 0.1% BSA (w/v).

**Similar results were obtained when NaCl was used to increase ionic strength of 0.05 M Tris-HCl extraction buffer.

lose activity faster than those prepared with PVP, indicating that conditions of high ionic strength lessen the effectiveness of the resin in removing not only proteins but also phenols from solution.

Manipulation of buffer ionic strength and amount of resin, and, particularly, use of a milder resin might yield extracts with higher enzyme activities and lower phenol levels in comparison with PVP treated extracts. The results of Lam and Shaw (1) and our results, especially with aspen PR and Glyc-3-PD (Table 3), give such indications, although proof of that basic tenet awaits further experimentation.

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