Phytochemistry, 1971, Vol. 10. pp. 2337 to 2341. Pergamon Press. Printed in England.

EXTRACTION OF COTTON LEAF ENZYMES WITH BORATE

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(Received 8 August 1970, in revised form 7 December 1970)

Abstract-Extracts of cotton leaves made with borate contain more total protein and more protein species, and exhibit greater enzyme activity than extracts made with insoluble polyvinylpyrrolidone. Borate forms soluble complexes with phenolics which otherwise coprecipitate with proteins during extraction. Because of the wide reactivity of borate, and the high solubility of its complexes, extracted protein must be thoroughly isolated from the extraction medium before reliable data on enzyme activities can be obtained.

INTRODUCTION

NUMEROUS endogenous phenolic compounds in plants are recognized as protein precipitants[1,2] and enzyme inhibitors.[3,4] Many species of plants apparently contain sufficient phenolics to limit the amount of protein extracted by water or dilute buffers, and to inhibit the activity of many of the enzymes that are extractable. Such extracts of cotton leaves generally contain very little protein; enzymic activity, with very few exceptions, is absent.

Various additives to extraction media have been employed to increase protein yield because of their action on phenolic constituents or on the enzymes of phenol metabolism. These include polyethylene glycols,[5,6] reducing agents,[7,8] and polyvinylpyrrolidone (PVP) both soluble[9] and insoluble.[10] Although addition of higher molecular weight polyethylene glycols to extraction media greatly increases protein yields from cotton leaves,[11] it is impossible to remove the glycol from the protein by gel filtration, dialysis or acetone precipitation.[12] Inclusion of reducing agents such as ascorbate, metabisulfite, thioglycollate or dithiothreitol (DTT) produces extracts from cotton leaves with low phenolic content, but concomitantly low protein content.[12] Insoluble PVP has proven moderately successful in increasing protein yields from cotton leaf tissue, but this report shows it adversely affects some enzymes.

Although the reaction of boric acid with polyhydroxy compounds is well known and employed in analytical methods[13] its use in the extraction of protein from phenolic-rich plant tissues is rare. This paper presents comparative data on protein yield and enzyme activity from cotton leaf tissue obtained by the use of insoluble PVP and borate.

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RESULTS

Protein Yield

The results of preliminary experiments showed that insoluble PVP was most effective at acid pH and with high concentration of extraction buffer (Table 1). Protein extraction at acid pH is not customary, but these results are in conformity with the data of Andersen and Sowers[14] who studied the affinity of tobacco phenols for insoluble PVP, and found that increased acidity favored bonding, presumably due to decreased salt formation by phenolic hydroxyl groups in acid media. Increased ionic strength may prevent sorption of protein onto insoluble polymers of the cell, i.e. structural polysaccharides, which may act as ion exchangers.[15]

Borate was mot effective at high concentration ($\geqslant 0{\cdot}2$ M), and the extraction pH was quite critical. Borate was prepared at 0·2 pH intervals over a pH range 7·0–8·0; pH 7·6 extracts were the only ones to yield an acceptable quantity of trichloracetic acid (TCA) precipitate. These extracts yielded twice as much protein per gram of fresh weight tissue as the best extracts made with PVP. Table 1 summarizes data concerning protein yields from buffered PVP and borate; it may be seen that DTT drastically reduced protein content of both media.

In order to compare PVP and borate qualitatively, aliquots of both extracts were subjected to polyacrylamide gel electrophoresis. Included for comparison were samples of freshly homogenized leaf tissue. Although the pattern of protein distribution obtained from borate extracts closely resembled that of the fresh tissue in both number and relative migration (R,) of bands, the pattern of PVP extracts was radically dissimilar with regard to both (Fig. 1).

Enzyme Activity

As may be seen from Table 2, borate and PVP extracts differed greatly in activity among the seven enzymes selected for assay. These enzymes represent varied facets of metabolic

TABLE 1. PROTEIN CONTENT OF COTTON LEAF EXTRACTS*

Buffer pH	TCA precipitated protein (mg/g fresh wt. tissue) 0·5 M phosphate + PVP +5 mM DTT†	0·5 M phosphate + PVP −DTT†	0·2 M borate +5 mM DTT†	0·2 M borate −DTT†
6·0	3.1	14.8		
6.2	1·6	12.5		
6·4	1·6	12.5		
6.6	1·6	7.8		
6.8	< 1	6.2		
7·0	< 1	5·5	0	0
7.2	0	3.9	0	0
7.4	0	3.9	0	< 1
7.6	0	3.9	8·1	30.0
7.8	0	3.9	0	< 1
8·0	0	2·3	0	0

* Method of preparing extracts is detailed in Experimental.
† Dithiothreitol.

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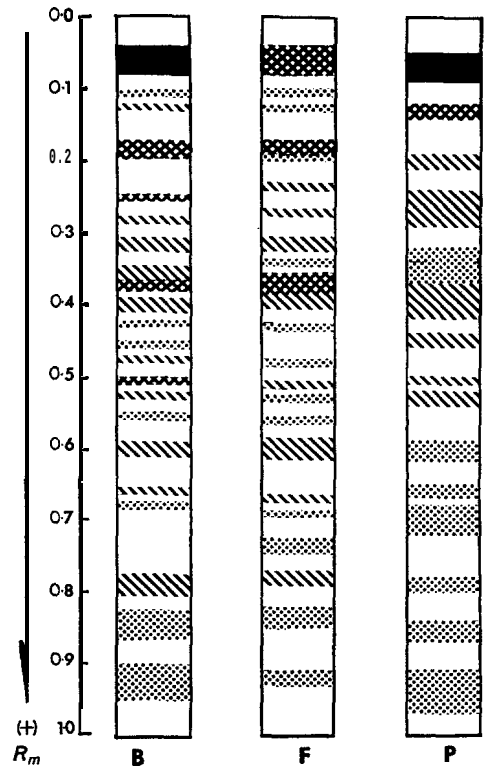

FIG. 1. ELECTROPHORETIC PATTERNS OF PROTEINS FROM EXTRACTS AND HOMOGENIZED TISSUE. B, borate extract; F, freshly homogenized tissue; P, PVP extract; R_m, relative migration. Arrow indicates direction of migration. Constant current of 4 mA/tube was applied to 7 % polyacrylamide gels in 5 mm i.d. x 75 mm tubes. pH of electrophoresis was 9·5.

activity. Although PVP extracts demonstrated activity in only four of the seven assays, borate extracts were active in all. Furthermore, in assays in which both extracts showed activity, borate extracts were from three to 72 times as active as PVP extracts and never less active.

DISCUSSION

Extracts made with borate contained more total protein and more protein species, and exhibited greater enzyme activity than extracts made with PVP. PVP, heretofore the most

TABLE 2. ENZYME ACTIVITIES OF COTTON LEAF EXTRACTS

	Activity (units/g fresh wt. tissue)*						
	Carbonic anhydrase	Catalase	Glutamate-oxalacetate transaminase	Lipoxidase	Malate dehydrogenase	Acid Phosphatase	Phenolase
PVP	0	6.2	2·4	0	22·2	$4{\cdot}4 \times 10^{-4}$	0
Borate	39·0	450.0	9·3	13125	81·0	$9{\cdot}0 \times 10^{-3}$	187·0

* Definitions of units are given in the Experimental.

satisfactory extraction adjuvant for cotton leaf tissue, has been shown to act through hydrogen bonding to yield insoluble complexes with phenolics.[10,14] Its major limitations seem to be that the optimum pH for bonding to phenols lies in the acid range; and inconveniently large quantities are necessary to effectively remove some common plant phenolics (chlorogenic acid, rutin, scopoletin).[14] Low molecular weight (soluble) PVP has been shown to inhibit phenolase[9,16,17] and certain dehydrogenases.[9] Loomis and Battaile[10] report that variable amounts of soluble PVP are formed from insoluble PVP by high speed homogenization (as was used in this work); this could account for some of the missing or low levels of enzyme activity in the cotton leaf preparations.

Like PVP, borate also forms complexes with phenolics; but unlike PVP, the borate–phenol complexes remain soluble. Furthermore, the range of compounds capable of complexing with borate is evidently much wider. To react with borate, a compound must have adjacent *cis* hydroxyl groups, and the reaction intensity is proportional to the number of such groups. A majority of the plant phenolics might be expected to complex with borate, as well as a-hydroxy and aromatic o-hydroxy acids, presumably through hydration of carboxyl groups.[13]

There is no ready explanation for the adverse effect of DTT on protein content of the cotton leaf extracts. The possibility exists that phenolic oxidation products bonded covalently to protein in the extracts made without DTT, reacted with the biuret reagent, and produced erroneously high biuret readings. However, the results of tests made with albumin and oxidized phenols (chlorogenic acid, catechin and rutin) tend to discount this as a consideration. Although the presence of oxidized phenols with albumin did result in biuret readings somewhat higher than those of albumin alone, the color of biuret-protein-phenol complexes was markedly different from that of the purple biuret-protein complex. In the course of the research reported in this paper, albumin standards were run concurrently with plant samples. Any samples which produced a biuret color differing from that of the standard were discarded.

The wide reactivity of borate and the solubility of its complexes make mandatory the complete isolation of the extracted protein from the complexed phenols and borate to obtain reliable data on enzyme activity. Not only do the borate-phenol complexes retain inhibitory properties, but borate alone may inhibit enzymes which require polyhydroxy coenzymes or substrates.[13] The procedure followed in this study seems adequate: gel filtration eluting with borate plus antioxidant, followed by exhaustive dialysis or ultrafiltration against water.

EXPERIMENTAL

Plant ***material.*** Young but fully expanded leaves were taken from cotton plants (*Gossypium hirsutum* L., cult. Stoneville 213) grown in clay soil in the greenhouse. Plants were kept in vigorous growth by regular pruning after their first year.

Preparation of extracts and protein purification. All steps of homogenization, centrifugation, gel filtration and ultrafiltration were carried out at or near 4". Freshly harvested leaf blade tissue was homogenized for 1 min at high speed in a Servall Omni-Mixer at the rate of 8 ml buffer/g tissue in either (a) 0·5 M phosphate, pH 6·0, plus hydrated Polyclar AT (purified according to Loomis and Battaile[10]) at the rate of 1 g (dry wt.) Polyclar/g tissue, or (b) 0·2 M boric acid-sodium borate, pH 7.6. Extracts were filtered through cheesecloth and centrifuged 20 min at 30,000 g. Supernate was loaded on a column of Bio-Gel P-60 and eluted with extraction buffer plus 3 mM DTT. To remove buffer salts and DTT, the protein-containing eluate

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was dialysed and ultratiltered to a concentration of 1 mg/ml H_2O in an Amicon ultrafiltration cell using a PM-10 membrane. Enzyme assays and electrophoresis were performed on these purified protein fractions only.

Assays. Protein in aliquots of the centrifuged extracts was precipitated with cold 10% (w/v) TCA, washed exhaustivelywith methanol, dissolved in dilute NaOH and assayed with biuret reagent[18] and bovine serum albumin standard.

Carbonic anhydrase (E.C. 4.2.1 .1) was assayed by a modification of the Wilbur-Anderson electrometric method[19] as given in the Worthington Enzyme Manual (Worthington Biochemical Corp., Freehold, N.J.). One unit corresponds to the hydration of 55 μmoles CO_2/l./sec.

Catalase (E.C. 1.11.1.6) was assayed by the method of Beers and Sizer.[20] One unit corresponds to the decomposition of 1 μmole H_2O_2/min.

Glutamate-oxaloacetate transaminase (E.C. 2.6.1.1) was assayed according to Arnador and Wacker.[21] One unit is equivalent to ΔA_{340} of 0·001/min.

Lipoxidase (EC. 1.99.2.1) was assayed by monitoring the oxidation of linoleate as described in the Worthington Enzyme Manual. One unit is equivalent to ΔA_{234} of 0·001/min.

Malate dehydrogenase (E.C. 1.1.1.37) was assayed spectrophotometrically as described in the Worthington Enzyme Manual. One unit corresponds to the hydrolysis of 1 pmole substrate/min.

Phenolase (E.C. 1.10.3.1) was assayed by monitoring the oxidation of DL-dopa at 475 nm. One unit is equivalent to ΔA_{475} of 0·001/min.

Electrophoresis. Electrophoresis was carried out in 7% polyacrylamide gels at pH 9·5 according to Davis;[22] proteins were stained with Coomassie blue.[23]

Acknowledgements—The author thanks Dr. A. A. Bell, Research Plant Pathologist, USDA, National Cotton Pathology Laboratory, College Station, Texas, for helpful discussion about the problem of cotton phenolics and protein extraction and for the suggestion that borate be tested. He also thanks Dr. H. C. Lane, Research Plant Physiologist, USDA, State College, Miss., for his interest and encouragement.

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