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A COMPARATIVE INVESTIGATION OF ISOZYME FRACTIONS SEPARATED FROM PLANT TISSUES*

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Abstract—The successful separation by disc electrophoresis of soluble proteins and isozymes of acid phosphatase (E.C. 3.1.3.2), esterase (E.C. 3.1.1.2), malate dehydrogenase (E.C. 1.1.1.37) and peroxidase (E.C. 1.11.1.7) from a variety of plant sources is described. Comparison of these fractions from leaf, petiole, stem, seed, pod, root and tuber tissues is made. The study includes separations from eleven diverse genera of green plants. Although many differences were found in isozyme patterns between the genera, and between the tissues of a plant, some isozymes are of common occurrence.

INTRODUCTION

THE development of an isolation medium and electrophoretic procedure giving excellent separation of protein fractions from *Dianthus* has recently been reported.[1] Since techniques often prove applicable to only a limited range of material we thought it most important to demonstrate the use of these procedures for separating protein from several diverse genera, and from different tissues of the same plant.

RESULTS

Soluble Protein (*Fig.* 1)

The separations shown in Fig. 1 confirm that the procedure gives multiple banded patterns for soluble proteins extracted from several species and tissues. These patterns are consistent from extraction to extraction. The least successful separation was for *Geranium* where it appeared that the oils from the leaf gland cells were a major complicating factor. Considerable streaking of the protein (revealed by staining) down the sides of the gel occurred with *Geranium* extract, and continued from the zone having an R_f 1–10. This region appears to contain much protein which enters the gel with difficulty, probably as a result of its high molecular weight. While modifications to the procedure for each individual tissue most likely would have improved the band resolution of the extracts, we made no changes since the goal was to determine the efficacy of the described method for several tissues.

It is unlikely that each, or possibly even that any, band represents a single protein, since the many hundreds of proteins present in the tissues may be expected to have overlapping electrophoretic mobilities. This situation is clearly demonstrated by the number of isozyme bands detected in the four enzyme assays described here for each tissue. Summation of the bands for the small number of enzymes studied gives a result frequently higher than that for

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[1] B. H. MCCOWN, G. E. BECK and T. C. HALL, *Plant Physiol.* **43**, 578 (1968).

the total number of bands observed on staining for protein with Aniline Blue Black. This may account for the rather fuzzy appearance of some of the bands, especially in the leaf tissues. Because storage tissues (and purified protein standards) have fewer protein components they yield more discrete bands than are obtained from extracts of metabolically complex tissues subjected to identical electrophoretic procedures.

The many proteins present in trace amounts in extracts of complex tissue separate during electrophoresis. This gives rise to a high number of lightly staining proteins which cannot be visually resolved as distinct bands; they therefore appear as a colored background.

A comparison of proteins for a single plant indicated in many cases that protein bands had R_f values which were common to all or to several tissues. Thus bands of R_f 15, 32, 69, 84 of beet are common to leaf, petiole and root extracts. Several other bands were common to beet leaf and petiole (R_f 18, 51, 62, 74) or to petiole and root (R_f 29, 35, 38, 45). Similar comparisons may be made for bean and carrot tissues shown in Fig. 1.

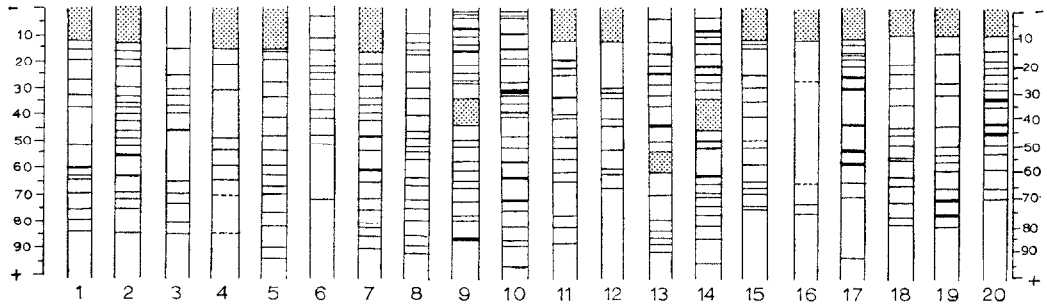

FIG. 1. SOLUBLE PROTEIN. GELS WERE STAINED WITH ANILINE BLUE-BLACK, AND DESTAINED ELECTROPHORETICALLY WITH 7% ACETIC ACID. R_f VALUES FOR EACH BAND DETECTED ON THE GEL ARE SHOWN AS A LINE BESIDE THE DIAGRAM OF THE GEL; THEY MAY BE READ OFF FROM THE SCALES AT EITHER END OF THE FIGURE.

Relative thickness of the lines indicates intensity of staining. All leaf tissues had a protein band running coincident with the front (R_f 100, not shown), and many show darkly staining areas (R_f 0–10, lipoprotein?) near the top of the running gel. Sample numbers correspond to those given in Table 1.

The protein separations for stem and petiole tissues (Fig. 1, samples 2, 5, 8) showed a greater number of bands than for leaf tissue of the corresponding plants. High enzyme activity was readily found to be associated with stem extracts, and these results suggest that the stem may well have a greater involvement in the overall metabolism of the plant than is generally realized. As discussed above, the larger number of easily discernible bands does not necessarily indicate that a greater number of proteins occur in these tissues than in leaves. A study of the soluble proteins of wheat[2] showed fewer bands from a leaf extract than were obtained from dormant and germinated seed extracts.

Acid Phosphatase (Fig. 2)

At least eleven isozymes of acid phosphatase were shown to be present in the tissues of certain of the plants assayed. Bean stem and pod tissue (Fig. 2, samples 8 and 10) were prolific in acid phosphatase isozymes, and many were common to the two tissues. The several beet, carrot and potato tissues compared also showed similarities within the species. An active

[2] V. MACKO, G. R. HONOLD and M. A. STAHMANN, *Phytochem.* **6**, 465 (1967).

isozyme at R_f 37 was present in fifteen of the twenty tissues tested, and was present in at least one tissue of all species assayed except dogwood and geranium. Several bands were also common to a number of plants: R_f 24 occurred in samples 1–3, 5, 8, 15–17, 20; R_f 48 in 1–3, 8–10, 16, 18; R_f 66 in 4, 6, 10. Acid phosphatase isozymes have been found in similar positions in wheat extracts.[2]

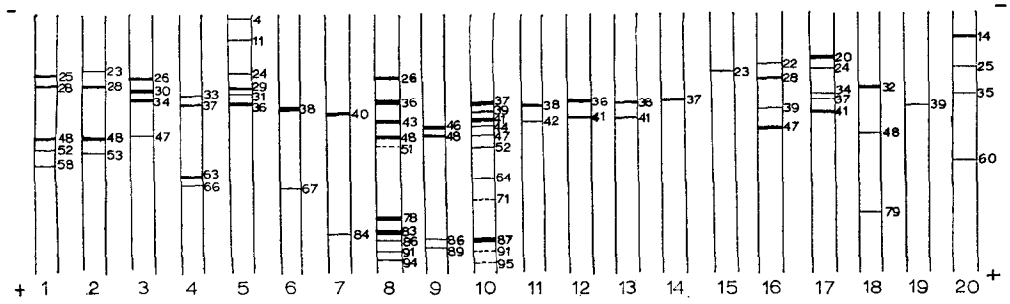

FIG. 2. ACID PHOSPHATASE. POSITIVE REACTIONS ARE SHOWN IN THE GEL DIAGRAMS AND CORRESPONDING R_f VALUES ARE SHOWN ALONGSIDE.

Thickness of line denotes relative intensity of color reaction. Sample numbers correspond with those given in Table 1.

Esterase (*Fig.* 3)

The larger number (more than ten) of bands showing enzyme activity in the esterase test was to be expected, since any enzyme capable of breaking the ester bond of α-naphthyl acetate will give a positive reaction. As with other enzymes the rate at which the reaction occurs depends upon the optimum conditions for the specific isozyme,[3] and thus banding patterns frequently increase with time. In this study the reaction was permitted to continue for 30–45 min at room temperature. Many bands were common to the various tissues of one plant (e.g. R_f 21 for *Beta*, 31 for *Daucus* and 93 for *Solanum*) although they varied from genus to genus. Several corresponding bands did, however, occur in the genera tested, notably those

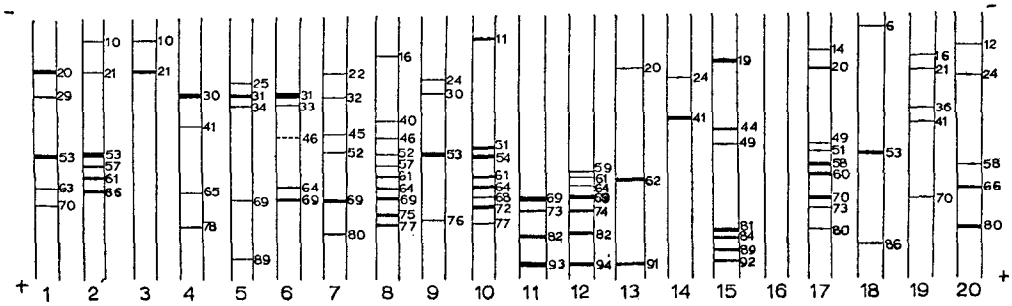

FIG. 3. ESTERASE. POSITIVE REACTIONS ARE SHOWN IN THE GEL DIAGRAMS, AND CORRESPONDING R_f VALUES ARE SHOWN ALONGSIDE.

Thickness of line denotes relative intensity of color reaction. Sample numbers correspond with those given in Table 1.

[3] H-U. BERGMEYER, E. BERNT and B. HESS, in *Methods of Enzymatic Analysis* (edited by H-U. BERGMEYER), p. 736, Academic Press, New York (1965).

having R_f values of 21, 53 and 69. Multiple esterase isozyme bands have been reported for maize,[4] potato,[5] wheat,[2] legumes[6, 7] and other crop plants.[8]

Malate Dehydrogenase (*Fig.* 4)

Malate dehydrogenase was detected in all tissues. In contrast to the other enzymes assayed there were very few isozymes, less than six being detected for any one tissue. One band, having an R_f of approximately 37 was present in the majority of genera tested. Two isozymes of malate dehydrogenase were described for barley seedlings,[9] and at least ten have been detected in wheat varieties.[10]

Rather interesting were sharply defined areas or "anti bands" which did not take up any color. These were found frequently between R_f 40–46 (samples 7–9, 11, 14–17) and also at other positions for other samples. We have observed these bands in several dehydrogenase tests (6-phosphogluconate dehydrogenase, glutamate dehydrogenase, lactate dehydrogenase). They are particularly clear if the gel is permitted to react long enough for non-specific staining to occur; "anti bands" have also been observed during staining for total protein and in gels tested for catalase (Fig. 6).

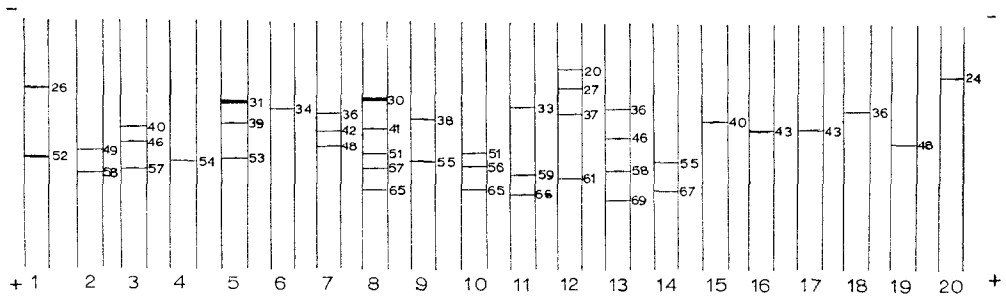

FIG. 4. MALATE DEHYDROGENASE. POSITIVE REACTIONS ARE SHOWN IN THE GEL DIAGRAMS, AND CORRESPONDING R_f VALUES ARE SHOWN ALONGSIDE.

Thickness of line denotes relative intensity of color reaction. Sample numbers correspond with those given in Table 1.

Peroxidase (*Fig.* 5)

The assay selected was relatively unspecific for any one substrate, and more than nine active isozyme bands were detected. Peroxidase activity was rapid compared with the other enzymes studied, the reaction being instantaneous upon placing the gel into the reaction mixture. Particularly frequent in occurrence were isozyme bands at R_f 12 (samples 1–4, 8, 10, 11, 15–18, 20), 25 (samples 4–7, 9, 12–15, 18–20), 53 (samples 2, 9–13, 17), 57 (samples 2, 3, 8, 10, 12, 13, 17) and 65 (samples 2, 3, 7, 8, 11–14). In connection with the comment made earlier, that any particular band may not represent a single protein, it is of importance that acid phosphatase, esterase and peroxidase were present at position R_f 52 for bean pod (sample 10, Figs. 2, 3, 5). This tissue gave a soluble protein band at R_f 52; also malate dehydrogenase was detected at R_f 51.

[4] D. SCHWARTZ, *Proc. Natl Acad. Sci.* **48,** 750 (1962).
[5] S. DESBOROUGH and S. J. PELOQUIN, *Phytochem.* **6,** 989 (1967).
[6] T. N. FRANKEL and E. D. GARBER, *Botan. Gaz.* **126,** 221 (1965).
[7] P. F. FOTTRELL, *Phytochem.* **7,** 23 (1968).
[8] H. M. SCHWARTZ, S. I. BIEDRON, M. M. VON HOLDT and S. REHM, *Phytochem.* **3,** 189 (1964).
[9] S. B. YUE, *Phytochem.* **5,** 1147 (1966).
[10] G. R. HONOLD, G. L. FARKAS and M. A. STAHMANN, *Cereal Chem.* **43,** 517 (1966).

Three isoperoxidase peaks were detected in extracts from maturing leaves during a study on bean isozymes,[10] and these peaks may correspond with the bands shown in Fig. 5, sample 7. Three isozymes of malate dehydrogenase were observed on the gels (Fig. 4, sample 7) in contrast with two in the study of Racusen and Foote.[11]

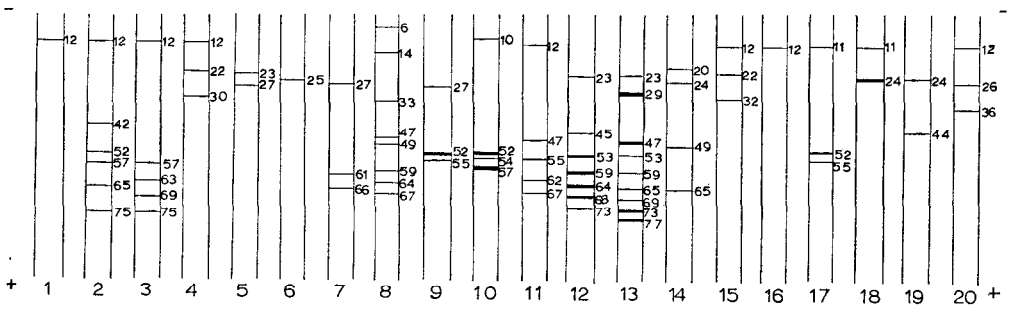

FIG. 5. PEROXIDASE. POSITIVE REACTIONS ARE SHOWN IN THE GEL DIAGRAMS, AND CORRESPONDING R_f VALUES ARE SHOWN ALONGSIDE.

Thickness of line denotes relative intensity of color reaction. Sample numbers correspond with those given in Table 1. Bubbles present at the top of several gels are believed to be the result of catalase activity (see text).

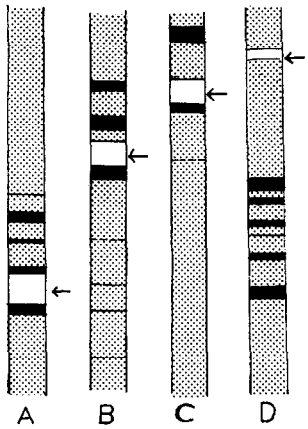

FIG. 6. REPRESENTATIVE GELS OF GREEN TISSUE EXTRACTS WITH "ANTI BANDS" (SEE TEXT) AT DIFFERENT R_f VALUES.

A: malate dehydrogenase test; B: soluble protein in basic gel; C: soluble protein in acid (urea) gel; D: catalase test.

Bubbles were released in localized areas of the gel by many extracts during the peroxidase assay, and this is thought to be catalase activity, since a positive reaction for catalase occurs using the method of Scandalios.[12] This test requires the addition of starch in the acrylamide gel, and negative staining occurred coincident with this position. Further staining with iodine darkened this area indicating absence of amylase activity.[2] Additional evidence for the presence of catalase was the bands in this area reacting in the peroxidase test, probably indicating peroxidative activity by catalase.

[11] D. RACUSEN and M. FOOTE, *Can. J. Botany* **44**, 1633 (1966).
[12] J. G. SCANDALIOS, *Proc. Natl Acad. Sci.* **53**, 1035 (1965).

DISCUSSION

Mechanical grinding of the tissue greatly facilitates the preparation of extracts, especially in the case of woody or leathery tissue. Although we have detected a difference between hand and mechanical grinding on only one occasion (a single isoperoxidase band was absent from mechanically ground extract) it remains highly advisable to compare results from both methods if a study of a particular isozyme is being undertaken.

During the isolation and concentration of extracts from leaf tissues our liquid preparations remained green. If the level of protective reducing agent became too low, or if insufficient polyvinylpyrrolidone was used in early stages of extraction, then browning due to oxidation and phenolic tanning occurs, the proteins are degraded and electrophoretic resolution is severely decreased. Green plant tissue extracts which turn amber,[13] or give yellowish bands on electrophoresis,[14] may possibly have undergone oxidative denaturation, although coloration will be higher in extracts obtained by procedures not including polyvinylpyrrolidone than in those involving its use, since it removes much of the carotenoids. For non-green material, notably dormant storage tissues, simpler extraction procedures[15] are valid. We have confirmed that extracts of such tissues prepared by either technique give identical results, but that the procedure of McCown *et al.*[1] is essential for less stable material.

The electrophoretic separations reported here indicate that proteins may be reliably isolated from many plant tissues. We have found the procedure to yield reproducible results from extract to extract for any particular tissue. The proteins remain stable, provided they are adequately protected by dithiothreitol.[16] Extracts from plants grown under differing environmental conditions show marked changes,[17] and protein response to physiological stress together with details of isozyme component changes following freezing of plant tissues and extracts will be described in a future communication.

EXPERIMENTAL

The plant tissues used for extraction were collected from plants in the greenhouse immediately prior to use. Table 1 lists the tissues investigated, and the numbers allocated in this table have been used in Figs. 1–5 to denote the plant and tissue.

The only change from the extraction procedure reported previously[1] was the substitution of mechanical for hand grinding. It was essential that the material be kept below 4° and in an inert (nitrogen) atmosphere. Plant material was chopped into about 20 ml extraction slurry contained in an extraction flask with a side-arm through which N_2 was passed. The flask stood in an ice bath. Grinding time was kept to a minimum; three 20-sec bursts at full speed with a "Virtis-45" grinder usually completely macerated the tissue.

All separations were in 7·5% acrylamide gels using the buffer system described previously.[1] Gels used for acid phosphatase and peroxidase assay were transferred to 1 M acetate buffer, pH 5, and rinsed twice with acid buffer before being placed in the substrate mixture. Gels for malate dehydrogenase and esterase reactions were similarly rinsed with 0·1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, Calbiochem) buffer, pH 7·4. These steps were essential, and failure to correctly adjust the pH of the gels resulted in inhibition of the enzymes. The methods followed for acid phosphatase and peroxidase assays were essentially those of Macko *et al.*;[2] for malate dehydrogenase that of Honold *et al.*;[10] and for esterase that of Desborough and Peloquin.[5] Benzidine was supplied as hydrogen donor for the peroxidase reaction. Enzyme reactions were usually complete within 60 min, and most required a shorter reaction time.

R_f values were calculated for the bands taking the distance traveled by the bromophenol blue marker dye from the top of the running gel as R_f 100. Since other factors besides the concentration of protein applied to the gel had some effect on the separation of the extracts R_f values usually have an accuracy of about ± 2.

[13] C. W. WRIGLEY, H. L. WEBSTER and J. F. TURNER, *Nature* **209**, 1133 (1966).
[14] D. RACUSEN and N. CALVANICO, *Anal. Biochem.* **7**, 62 (1964).
[15] S. DESBOROUGH and S. J. PELOQUIN, *Phytochem.* **5**, 727 (1966).
[16] W. W. CLELAND, *Biochemistry* **3**, 480 (1964).
[17] G. E. HART and C. R. BHATIA, *Can. J. Genet. Cytol.* **9**, 367 (1967).

TABLE 1. PLANTS AND TISSUES INVESTIGATED

Sample No.	Species	Tissue
	Crop plants	
1	*Beta vulgaris*	Leaf
2	*B. vulgaris*	Petiole
3	*B. vulgaris*	Root
4	*Daucus carota*	Leaf
5	*D. carota*	Petiole
6	*D. carota*	Root
7	*Phaseolus vulgaris*	Leaf
8	*P. vulgaris*	Stem
9	*P. vulgaris*	Seed
10	*P. vulgaris*	Pod
11	*Solanum microdontum*	Leaf
12	*S. microdontum*	Stem
13	*S. microdontum*	Tuber
	Ornamentals	
14	*Begonia argenteo-guttata*	Leaf
15	*Cornus stolonifera*	Leaf
16	*Cyrtomium falcatum*	Leaf
17	*Dianthus caryophyllus*	Leaf
18	*Geranium hortorum*	Leaf
19	*Philodendron hastatum*	Leaf
20	*Phoenix dactylifera*	Leaf

20 g fr. wt. tissue was used for samples 2, 6, 8, 13, 20; 15 g for samples 3, 10, 12; 10 g for the others. Extracts were concentrated to about 3 mg protein/ml and 500–700 μg protein applied/gel.