Phytochemistry, Vol. 25, No. 2, pp. 329–332, 1986.
Printed in Great Britain.

0031-9422/86 $3.00 + 0.00

DETECTION OF POLYPHENOLOXIDASE ISOENZYMES BY ELECTROBLOTTING AND PHOTOGRAPHY

MARYCOLLETTE HRUSKOCY and WILLIAM H. FLURKEY*

Department of Chemistry, Indiana State University, Terre Haute, IN 47809, U.S.A.

(*Received* 25 *February* 1985)

Key Word Index—Angiosperms; fungi; polyphenoloxidase; electrophoresis; electroblotting; photography.

Abstract—Crude extracts of polyphenoloxidase from plant, fruit and fungal sources were subjected to polyacrylamide gel electrophoresis under denaturing and partially denaturing conditions. Enzymes present in the gels were transferred electrophoretically (electroblotting) to nitrocellulose membranes and air dried. The nitrocellulose membranes were stained for polyphenoloxidase activity using catechol and dopa as substrates. These enzyme-stained membranes were dried and photographed, either directly or using a backlighting technique. Electroblotting coupled with photography results in a reliable method for detection, identification, and recording of polyphenoloxidase isoenzyme forms found in a variety of sources.

INTRODUCTION

Polyphenoloxidase (*o*-diphenol: O_2 oxidoreductase; EC 1.14.18.1; PPO) catalyses the *ortho*-hydroxylation of monophenols as well as the oxidation of these phenols to *ortho*-quinones and has been studied for many years with regard to its multiple forms and isoenzyme patterns by electrophoresis [1–5]. Electrophoresis followed by specific enzyme staining has been used to characterize banana PPO isoenzymes, the multiplicity of spinach root PPO, the activation of spinach chloroplast PPO by aging and frost, the gibberellic acid mediated activation of wheat PPO, and PPO in peaches [6–13]. Isoenzyme patterns have also been used to study the nuclear inheritance of PPO in *Nicotiana*, to study PPO in organized and unorganized tissues of *Solanum melongena*, and to observe changes in PPO in *Nicotiana glutinosa* infected with tobacco mosiac virus [14–16]. A search through the literature revealed that most, if not all, of these isoenzyme patterns were hand drawn representations of enzyme stained polyacrylamide gels. The analysis of PPO isoenzyme patterns from stained gels is based upon a subjective visual evaluation of the coloured products and their positions unless scanning densitometry is used. These enzyme product stained gels are faint in colour, fade with time, and are difficult to photograph; thus, it is difficult to obtain a permanent record of the data using methods other than hand drawn representations. We have tried to alleviate some of the problems by electrophoretically transferring PPO isoenzymes from polyacrylamide gels to nitrocellulose membranes (i.e. electroblotting). The nitrocellulose membranes can be stained for enzyme and photographed using a backlighting technique. This method is suitable for storing data, is relatively inexpensive, is very sensitive, is less prone to subjective evaluation of the number and intensities of PPO isoenzyme forms separated by electrophoresis, and can be adapted for many other proteins transferred to nitrocellulose.

RESULTS AND DISCUSSION

Mushroom PPO was chosen as a standard to determine appropriate conditions for electrophoretic transfer of the enzyme from polyacrylamide gels onto nitrocellulose membranes because it is commercially available, contains few isoenzyme forms, and stains well using catechol or DOPA as substrates. Various amounts of the enzyme were electrophoresed in 7% acrylamide gels and electroblotted onto nitrocellulose. The nitrocellulose sheet was stained for enzyme and photographed directly. Enzyme catalysed staining on the membrane was barely detectable under normal lighting conditions; however, backlighting the same membrane resulted in the appearance of bands of intense staining (Fig. 1a). A duplicate gel stained directly for isoenzyme forms could not be photographed because the bands were not dark enough (data not shown). In fact, we have not been able to photograph any isoenzyme patterns in polyacrylamide gels unless the bands were extremely intense. The intensity of the bands on the nitrocellulose appeared to be correlated with the amount of enzyme applied and individual isoenzyme forms were readily discernible. We have consistently observed that when coloration was not readily apparent under normal conditions, the enzyme patterns could be easily detected by backlighting the membrane. Thus backlighting the nitrocellulose sheet enhances the detection and visualization of PPO isoenzymes.

Mushroom PPO was electroblotted for 1 hr and used to determine the minimum amount of enzyme that could be detected using the technique described above (Fig. 1b). The same amount of sample was subjected to a spectrophotometric assay for comparison. Enzyme activity equivalent to 0.0004 A/min (0.1 μg protein) could be detected. As little as 0.4 μg of protein could be detected even after 10 min of transfer (data not shown). The intensity of the bands of enzyme staining increased in proportion to the

*To whom correspondence should be addressed.

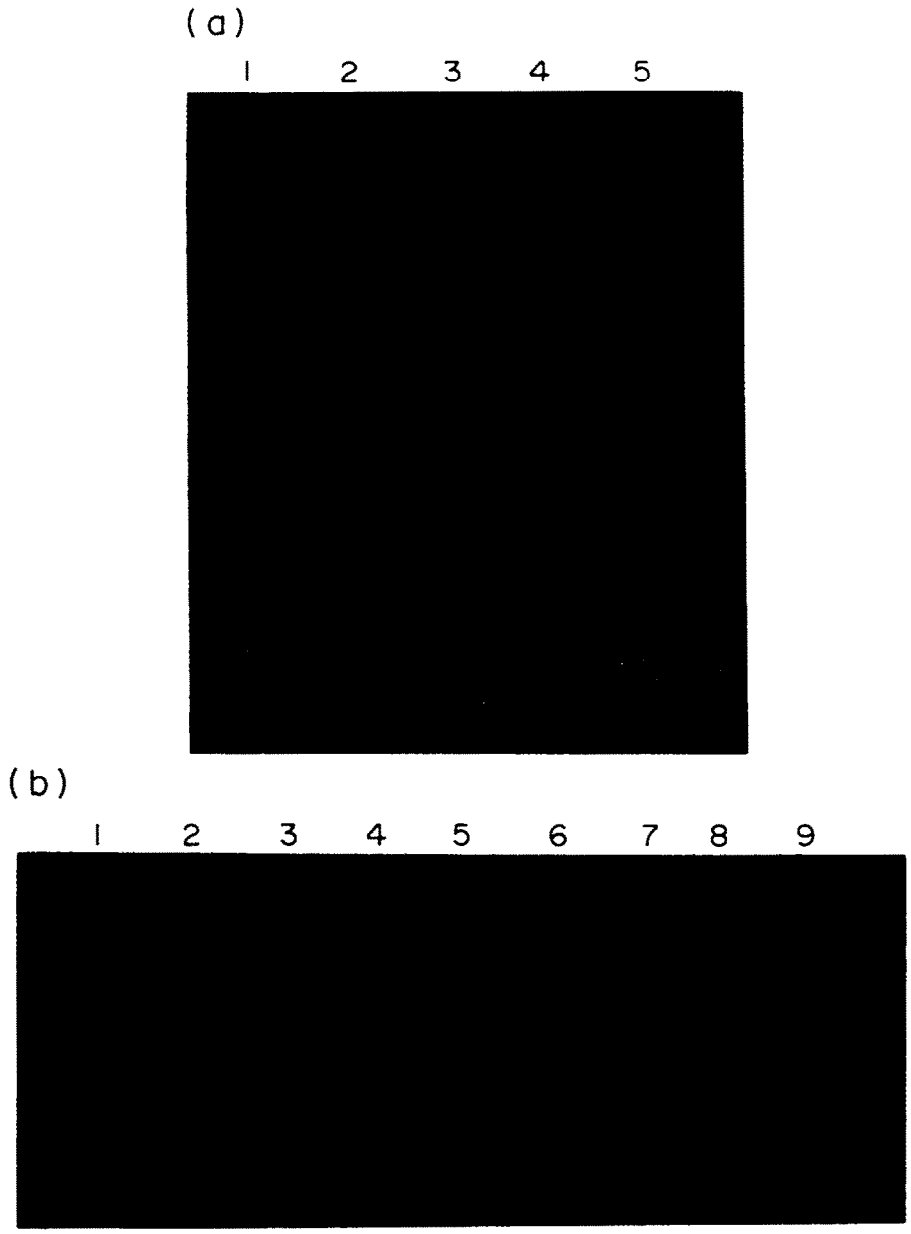

Fig. 1. Backlighting photograph (a) of a nitrocellulose membrane stained for mushroom PPO using catechol and L-DOPA as substrates. Mushroom PPO (Sigma T-7755) was electrophoresed and electroblotted for 1 hr then stained for enzyme as described in the Experimental section. Lanes 1–5 represent 2, 1, 0.5, 0.25 and 0.1 μg of protein respectively. Direction of migration was from top (−) to bottom (+). Backlighting photograph (b) of various amounts of mushroom PPO electrophoresed, and then electroblotted for 1 hr. Lanes 1–9 represent 1.6, 1.4, 1.2, 1.0, 0.8, 0.6, 0.4, 0.2 and 0.1 μg of protein respectively. This corresponds to 0.017, 0.015, 0.011, 0.01, 0.008, 0.005, 0.003, 0.001 and 0.0005 absorbance unit changes per min at 410 nm using a spectrophotometric assay.

amount of enzyme up to a maximum of *ca* 0.01 *A*/min of activity and remained relatively constant thereafter. Staining of gels after transfer always showed that a small amount of enzyme remained within the gel. Transfers of duration longer than 2 hr resulted in enzyme being transferred through the nitrocellulose membranes. This phenomenon was observed using PPO from other sources and suggests that individual PPO isoenzymes may differ in binding to the membrane.

To demonstrate the usefulness of this technique when analysing PPO isoenzymes in other tissues, crude extracts from mushrooms, pears, cherries, ornamental peppers and leaves from tomatoes, broad beans and mung beans were subjected to electroblotting. Since activated and latent PPO can be detected when electrophoresed in the presence of 0.1% SDS [1], samples were electrophoresed in the presence of SDS, electroblotted, stained and photographed. Intense bands of enzyme staining were present in extracts from tomato and broad bean leaves (Fig. 2). Pear extracts contained two isoenzyme bands of different but lesser intensity. Crude extracts from cherries, mung bean leaves and ornamental peppers showed two to

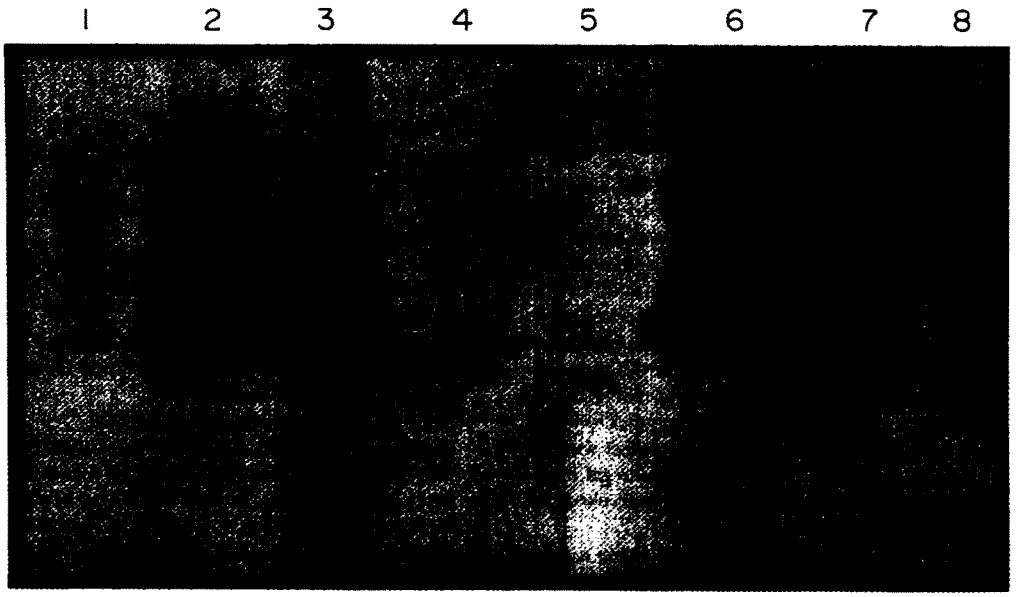

Fig. 2. Backlighting photograph of PPO extracts electrophoresed in the presence of SDS, electroblotted for 1 hr and stained for enzyme. Lanes 1–4 correspond to extracts from mushrooms, tomato leaves, partially purified broad bean PPO and pears. Lanes 5–8 correspond to crude extracts from Bing cherries, broad bean leaves, ornamental peppers and mung bean leaves.

three isoenzymes of lesser intensity. Thus, electroblotting can be used to record PPO isoenzymes which vary in the intensity of enzyme staining. Previously, this has been difficult when trying to photograph PPO isoenzymes with such variations. These results confirmed earlier observations by Angleton and Flurkey [1] and suggest that no overall changes occurred in the isoenzyme profiles after electroblotting.

It appeared that the variation in intensity of PPO bands depended upon the brand of nitrocellulose used (data not shown). This is not surprising since Ribero *et al.* [17] showed that large variations existed in the binding of [^{3}H]-leucine labelled proteins to different bands of nitrocellulose, mixed esters and nylon supports when transferred electrophoretically. The duration of transfer and the type of substrate can be manipulated to obtain reproducible and multiple replicas from a single gel. Transfer for 10 min is sufficient to detect mushroom PPO isoenzymes. Thus, a series of transfers (i.e. 10, 20, 30 min) can provide many copies of a single electrophoretic separation. These copies can be assayed independently using a different substrate for each copy. This should allow a direct correlation between the various isoenzyme forms and their substrate specificity. This is not possible using a spectrophotometric assay unless each isoenzyme form is purified and assayed separately. A similar approach was used by McLellan and Ramshaw who used serial electrophoretic transfers onto DEAE cellulose paper to detect phosphatases, esterases, kinases and dehydrogenases [18]. Kakefuda and Duke have also shown that electrophoretic transfers can be used to identify plant amylolytic enzymes [19].

Photographic records of PPO isoenzymes separated by electrophoresis have been difficult to obtain. Some of this difficulty can be attributed to products which are faint in colour and fade with time. In addition, background colouration becomes darker with the length of staining. These same problems are also encountered when subjecting enzyme stained gels to scanning densitometry.

EXPERIMENTAL

Enzyme extracts. Extracts were prepared by blending the tissue in 10 vols of 0.05 M NaPi buffer (pH 6.5) as described in ref. [1]. The homogenate was centrifuged at 10000 *g* for 10 min at 4°. The supernatant was used as the source of soluble PPO enzyme. The crude extracts were frozen and stored at −15° until analysed by electrophoresis. Polyphenoloxidase was assayed as described earlier [1]. Protein content was measured using the BioRad protein assay. The following plant, fungal and fruit sources were used: mushroom (*Agaricus bisporus*), broad bean leaves (*Vicia faba* L. Moensch cv Long Pod), tomato leaves (*Lycopersicon esculentum* Mill cv Big Boy), pear (*Pyrus communis* L. cv D'Anjou), cherry (*Prunus avium* cv Bing Cherry), ornamental pepper (*Capsicum* cv Holiday Time), mung bean leaves (*Vigna radiata* L. Wilczek cv Berken).

Electrophoresis and electroblotting. Slab gel electrophoresis was performed according to the method of ref. [20] in the presence or absence of SDS as described [1]. Electrophoresis was carried out at 50 V until the tracking dye had entered the separating gel at which time the voltage was increased to 100 V. Electrophoresis was stopped when the tracking dye had migrated near the bottom of the gel. The polyacrylamide gel (7%) was removed and placed on top of filter paper presoaked in electrophoresis buffer minus SDS. A 'sandwich' was prepared which consisted of a synthetic sponge pad, Whatman No. 1 filter paper, polyacrylamide gel, nitrocellulose membrane, Whatman No. 1 filter paper, followed by a second sponge pad. The sandwich was supported between two plastic sheets as recommended by the manufacturer (BioRad). All pads, filter paper and membranes were presoaked in electrophoresis buffer minus SDS. The entire sandwich was placed between parallel electrodes oriented so proteins were travelling to the anode and subjected to 30 V (0.1 A) for 1 hr at 4° in a BioRad Transblot ^{TM}Cell. In some

experiments a second piece of nitrocellulose or DEAE cellulose paper was placed behind the first nitrocellulose membrane. Great care was taken to exclude any air bubbles that formed during the sandwich preparation. These bubbles could be removed by using a pipette and rolling out any trapped air between the gel and the nitrocellulose sheet.

Enzyme staining. After 1 hr of electroblotting, the sandwich was removed and the nitrocellulose membrane air dried. The side of the membrane facing the polyacrylamide gel was placed face upward. A piece of filter paper (Whatman No. 40 or 42), soaked in 0.01 M NaPi buffer (6.5) containing 1 mM catechol and 1 mM L-DOPA, was placed on top of the nitrocellulose and rolled out to exclude any air bubbles. Alternatively, the nitrocellulose membrane could be dipped in or lightly overlaid with the above buffer plus substrates and allowed to dry. Depending on the sample, yellow-orange bands appeared within minutes to hours. The filter paper soaked in substrate was left in contact with the nitrocellulose sheet for *ca* 1 hr. The polyacrylamide gel in the sandwich was stained with the same substrate soln to check for any enzyme remaining in the gel.

Photography. A light box was constructed by lining a cardboard box with Al foil. A glass plate was fitted into a hole cut in the side of the box. A frosted acetate sheet and a light blue cellophane sheet were taped on the back of the glass plate. The dried nitrocellulose sheet was attached to the front of the glass plate with a piece of cellophane tape. Two pieces of matte black construction paper were cut into L-shaped pieces and fitted around the nitrocellulose sheet to mask stray light. A halogen light, placed behind the box at a distance of 1 m, was used to illuminate the nitrocellulose sheet. A 35 mm camera containing Kodak Tri-X ASA 400 film was placed in front of the nitrocellulose sheet at a distance of 1 m and a photograph taken.

Acknowledgements—We would like to thank P. Archer and M. Whitlock for typing this manuscript and Robert Lantz for the photography. This research was supported by Indiana State University Research Funds.

REFERENCES

1. Angleton, E. and Flurkey, W. H. (1984) *Phytochemistry* **23**, 2723.
2. Butt, V. S. (1980) *Biochemistry of Plants*, Vol. 21, p. 585. Academic Press, New York.
3. Mayer, A. M. and Harel, E. (1979) *Phytochemistry* **18**, 193.
4. Thomas, P., Delincee, H. and Diehl, J. F. (1978) *Analyt. Biochem.* **88**, 138.
5. Vaughn, K. C. and Duke, S. O. (1984) *Physiol. Plant.* **60**, 106.
6. Mowlah, G., Tankano, K., Kamoi, I. and Obara, T. (1982) *Lebensm. Wiss u Technol.* **15**, 207.
7. Sato, M. (1982) *Phytochemistry* **21**, 1229.
8. Lieberei, R. and Biehl, B. (1978) *Phytochemistry* **17**, 1427.
9. Meyer, H. U. and Biehl, B. (1980) *Phytochemistry* **19**, 2267.
10. Berry, M. and Sachar, R. C. (1982) *Phytochemistry* **21**, 585.
11. Flurkey, W. H. and Jen, J. J. (1978) *J. Food Sci.* **43**, 1826.
12. Flurkey, W. H. and Jen, J. J. (1980) *Biochem. Physiol. Pflanzen* **175**, 637.
13. Flurkey, W. H. and Jen, J. J. (1980) *J. Food Biochem.* **4**, 29.
14. Lax, A. R., Vaughn, K. C. and Templeton, G. E. (1984) *J. Hered.* **75**, 285.
15. Grosso, E. and Alicchio, R. (1981) *Z. Pflanzenphysiol.* **102**, 467.
16. Conti, G. G., Bocci, A. M. and Sprocati, M. T. (1982) *Riv. Patol. Veg.* **18**, 183.
17. Ribeiro, P. L., Mitra, R. and Berstein, I. A. (1984) *Biotechniques* March/April, 69.
18. McLellan, T. and Ramshaw, J. A. M. (1981) *Biochem. Genet.* **19**, 647.
19. Kakefuda, G. and Duke, S. H. (1984) *Plant Physiol.* **75**, 278.
20. Laemmli, U. K. (1970) *Nature* **227**, 680.