Short Communications

Assay of Plant Tyrosinase by Agar-Gel Double Diffusion and Electro-osmophoresis

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Phenolase activity in plant tissues is generally measured by spectrophotometry or manometry (Bendall & Gregory, 1963). Manometric methods are laborious, and spectrophotometric methods are unsuitable for crude phenolase preparations, such as extracts from various plant organs, besides being limited to one enzyme-substrate combination at a time. We therefore adapted agar-gel double diffusion and electro-osmophoresis as rapid and sensitive assay methods for tyrosinase, a commonly occurring phenolase. In these methods we have used the observation that tyrosinase can oxidize a variety of phenols as well as tyrosine (Keilin & Mann, 1938), leading to the formation of coloured products. These different substrates may be tested simultaneously with very small quantities of the reactants.

Agar-gel double diffusion. The 1% agar gels in 0.05M-NaH₂PO₄-Na₂HPO₄ buffer, pH 6.5, with wells capable of holding 0.1 ml. of reactants were prepared on glass slides by the method described by John (1965). The enzymes and substrates were placed in opposite wells 1-2 cm. apart. The agars used were Davis Girard T and DEAE-Bacto (Ragetli & Weintraub, 1965), Difco-Bacto and Difco-Noble. The enzyme solutions used were commercial tyrosinase (freeze-dried Worthington Biochemical Corp., Freehold, N.J., U.S.A.) and crude extract obtained by grinding plant tissues in distilled water (1:2, w/v) in a mortar and expressing the sap through two layers of cheesecloth. The substrates were chlorogenic acid, DL-dihydroxyphenylalanine (DOPA) (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), catechol (Fisher Scientific Co., Montreal, Quebec, Canada) and L-tyrosine (Calbiochem, Los Angeles, Calif., U.S.A.).

When tyrosinase preparations and substrate were applied to the wells a coloured band appeared between them. Since this band marked the interaction of the enzyme and substrate it is referred to below as a reaction band. There was considerable variation in the intensity of colour and time of appearance of the reaction band, depending on the

substrate and type of agar used. David Girard T agar was found to be well suited for detecting commercial tyrosinase in the presence of catechol. The lowest concentration of tyrosinase detectable by this method was 2.5 µg./0.1 ml. with an excess of catechol (33 µg./0.1 ml.). The bands appeared, within 30 min. after the addition of the reactants, as faint yellow lines that gradually became dark brown. The intensity of these bands was directly proportional to the concentration of enzyme.

In Davis Girard T agar, dihydroxyphenylalanine and tyrosine reacted with commercial tyrosinase, producing dark-brown reaction bands in about 120 min. In Difco-Bacto and Difco-Noble agars, catechol and dihydroxyphenylalanine produced bands with commercial tyrosinase in 120 min. or longer. These bands were reddish at first, then turned dark brown. Chlorogenic acid could not be detected definitely with these agar media, although with Bacto and Noble agars there was a transient faint-yellow line.

When crude enzyme preparations were applied to Girard T, Bacto and Noble agars, a general coloration of the area in between the reactant wells due to the coloured materials already present in the preparations prevented a clear identification of the reaction band. However, in DEAE-agar clear bands were obtained with crude extracts from *Nicotiana glutinosa* leaves, mushroom and tobacco root, especially when these preparations were added to the wells 1-4 hr. before the addition of catechol or dihydroxyphenylalanine. Chlorogenic acid produced an ill-defined reaction band, whereas tyrosine did not visibly react in this agar.

Electro-osmophoresis. This method is analogous to immuno-osmophoresis used in the study of viral antigens and antibodies (Ragetli & Weintraub, 1964, 1965, 1966). It involves the active anodic movement of tyrosinase and the passive cathodic movement of the substrate due to the endosmotic liquid flow in all agar gels except DEAE-agar (Ragetli & Weintraub, 1966). The set-up was similar to that described by John (1965), except that the agar bed was placed on a surface cooled by

running tap water during electro-osmophoresis. The buffer in the electrode compartment was replenished at the rate of about 6 ml./min. and the excess removed by a suction device (Ragetti & Weintraub, 1964).

In Girard T agar, which was the most suitable medium for this assay method, a dark-brown reaction band was produced within 15–30 min. at about 6 v/cm. The intensity of the band increased for some time after the termination of electro-osmophoresis. Most of the coloured interfering materials in the crude enzyme preparation moved away from the reaction band, and the remaining pigments could be leached out by immersing the agar bed in water for 30 min. after electro-osmophoresis. This treatment made the bands more conspicuous and rendered the slide ideal for photography.

With catechol (33 μ g./0.1 ml.) as substrate the reaction bands produced by commercial tyrosinase and crude extracts of mushroom, potato tuber and leaves of *N. glutinosa* appeared in Girard T agar by 20 min. after the start of electro-osmophoresis. Since the crude extract generally disrupted the agar during electro-osmophoresis, it was either suitably diluted or, where the enzyme concentration was low, centrifuged at 5000g for 10 min. At extremely low enzyme concentrations as in *N. glutinosa*, the reaction band may not become visible during electro-osmophoresis, but will develop on standing. Catechol was found to be the ideal substrate for the various enzyme preparations tested. Chlorogenic acid, with its anodic movement, was unsuitable as a substrate.

Electro-osmophoresis was 8 times as sensitive as double diffusion (the lowest amounts of commercial tyrosinase detectable by the two methods being 0.31 μ g. and 2.5 μ g./0.1 ml. respectively) and also more rapid. If only a semi-quantitative evaluation is desired, then the dilution end point can be visually determined with either method. By electro-osmophoresis the tyrosinase in a minimum of 0.25 mg. of mushroom tissue could be detected; for potato tuber and leaves of *N. glutinosa* 2.5 mg. and 24 mg. were required respectively. Indeed, mushroom extract could be used as a standard since it produced similar reaction bands to those of commercial tyrosinase. For quantitative evaluation, the density of the reaction band can be measured. By using a Joyce automatic recording densitometer (Joyce, Loebel and Co. Ltd., Newcastle upon Tyne) the concentration of the enzyme in the range

Table 1. *Density of reaction bands produced by commercial tyrosinase and catechol in Girard T agar*

The densities are expressed as the height of peaks (cm.) of the reaction band measured by using the Joyce automatic recording microdensitometer, 30 min. after electro-osmophoresis at 6 v/cm.

Tyrosinase (μ g./0.1 ml.)... Expt. no.	Density of reaction band				
	0.625	1.25	2.5	5.0	10.0
1	0.4	1.1	2.4	4.0	7.1
2	0.6	1.1	2.1	3.7	6.0
3	0.6	1.3	2.0	4.5	7.6
4	0.4	1.1	3.5	4.8	6.8
Average	0.5	1.15	2.5	4.25	6.87

0.6–5.0 μ g. was found to be proportional to the density of the reaction band (Table 1). With crude extracts a blank was run with buffer in place of the substrate and the density of the blank was subtracted from that obtained with the substrate. The enzyme concentration was then read from the correlation graph. Alternatively, a reference tyrosinase concentration may be run with each set of samples to be assayed and its density can serve as a basis for the calculation of the unknown. The tyrosinase concentrations per mg. fresh wt. of mushroom, potato tuber and leaves of *N. glutinosa* were found to be 4.3 μ g., 0.31 μ g. and 0.055 μ g. respectively.

The methods described above might well be extended to other enzyme–substrate interactions, especially where they give suitably coloured insoluble reaction products. A micro-technique analogous to micro-immuno-osmophoresis (John, 1965) may be applied where screening of a large number of samples is required.

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