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A FLUOROMETRIC PROCEDURE FOR DETERMINATION OF *o*-DIPHENOL OXIDASE*

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Abstract—A new rapid fluorometric technique has been developed for the measurement of *o*-diphenol oxidase activity. The change in concentration of a fluorescent substrate such as chlorogenic acid is measured directly by recording loss of fluorescence as the substrate is converted to the quinone. Using this method good linearity was obtained in Lineweaver–Burk determinations. Mushroom, tobacco leaf, tomato leaf, and tomato callus were used as enzyme sources in the development of the method.

INTRODUCTION

o-DIPHENOL oxidase (tyrosinase, polyphenol oxidase, catecholase) catalyzes the oxidation of mono or *o*-diphenols to *o*-quinones.[1-2] The polymerization of these quinones causes the typical brown or black color of plant tissue injured by mechanical treatment or disease.[3-6] There is increasing evidence that *o*-diphenol oxidase plays a major role in disease resistance. The exact nature of the resistance mechanism is not known, but it may involve production of fungitoxic quinones.[7-10]

There are many methods reported in the literature for the assay of *o*-diphenol oxidase activity. These involve the measurement of O_2 uptake, the coupled oxidation of an added reducing agent, or the formation of a product. Some of these methods have been compared by Mayer *et al.* who stated that measurement of O_2 uptake by a polarographic oxygen electrode is the method of choice.[11] As they noted, however, few of the available methods allow the activity to be expressed in μmoles substrate changed as recommended by the International Union of Biochemistry. Adachi and Halprin recently developed a fluorometric technique for the determination of mammalian tyrosinase; it is very sensitive but

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requires several steps and does not allow continuous monitoring of the reaction.[12] The fluorometric procedure described here directly measures the change in substrate concentration and, in addition, allows continuous monitoring of the reaction. In this assay the loss of fluorescence of a substrate as it is converted to the non-fluorescent *o*-quinone is recorded against time. The amount of substrate changed is then directly determined from a standard curve.

METHOD

Chlorogenic acid at 1 μmole/ml in 0·05 M phosphate buffer, pH 6·5, was used as the stock substrate solution. It was made up fresh before each use because there was significant auto-oxidation even when stored at 0°. A standard curve of fluorescence vs. concentration was made from this stock. The reaction mixture contained from 0·05 to 0·20 μmole of chlorogenic acid and from 0·05 to 0·20 ml of enzyme extract, made from acetone powders of mushroom, tobacco leaf, tomato leaf and callus, in a total volume of 4·0 ml of phosphate buffer, pH 6·5.

Measurements were made with a Turner 111 fluorometer equipped with a water-cooled door and a Heath recorder. The door was maintained at room temperature with an ambient temperature bath. A long wave, 366 nm, light source was used with a 7-60 primary filter and 2A + 47-B as the secondary filter. The latter formed a narrow pass filter with maximum transmission at about 440 nm which is very close to the emission peak of chlorogenic acid. Sensitivity was set at 1x.

The enzyme solutions had different native fluorescence so the fluorometer was arbitrarily adjusted to read 50 units with enzyme blanks for all determinations. The fluorometer was adjusted by manipulating the door to read 50 plus the fluorescence of the substrate to be added as determined from the standard curve. With the recorder running, substrate was then injected with a syringe into a cuvette sealed with Parafilm containing enzyme and buffer. The reactants were mixed by inverting and the cuvette was immediately placed in the fluorometer. The drop in fluorescence as the chlorogenic acid was oxidized was thus recorded against time.

RESULTS AND DISCUSSION

The drop in fluorescence of the substrate upon the addition of the enzyme was rapid and linear for 15–30 sec and then became slower. This was expected as there is significant product inhibition in this reaction; Mayer *et al.* reported the reaction to be linear for 1 min or less.[11] Also some of the polymerization products of the quinones formed could be fluorescent. A typical recorder trace of the reaction is seen in Fig. 1.

A straight line was drawn from time = 0 tangent to the recorded curve to obtain initial activity. This is expressed as rate of drop in fluorescence, which can be converted to μmoles of chlorogenic acid oxidized per minute by using the standard curve.

A plot of enzyme concentration versus activity for a fixed substrate concentration should be linear if the assay procedure is accurately measuring the results of enzyme action. With the fluorometric technique described herein, good linearity was obtained with all enzyme preparations used.

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Two additional criteria for determining if a procedure actually measures enzyme activity are linearity in a Lineweaver–Burk plot and consistent values for K_m with varying enzyme concentrations. Results from this procedure also meet these requirements. With two levels of mushroom *o*-diphenol oxidase and chlorogenic acid as a substrate. The K_m determined from such plots is $1{\cdot}67 \times 10^{-4}$ M. This is similar to the value of $2{\cdot}2 \times 10^{-4}$ M obtained by Sisler and Evans who used measurement of the coupled oxidation of ascorbic acid as the assay method.[13] Lineweaver–Burk determinations were also done for the tomato leaf enzyme; these too were linear and gave consistent values of K_m.

In developing this technique, chlorogenic acid was used as the substrate. It is a common substrate in *o*-diphenol oxidase determinations[13-16] and is widespread in higher plants.[17] Also, the build-up of chlorogenic acid around an infection site is a common response in some host–parasite interacitons, indicating that chlorogenic acid may play a key role in disease resistance.[4, 10, 18, 19]

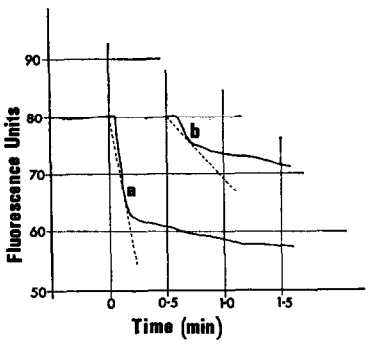

FIG. 1. RECORDINGS OF FLUOROMETRIC ASSAYS OF *o*-DIPHENOL OXIDASE FROM MUSHROOM SHOWING SLOPE OF THE INITIAL VELOCITY.

Trace "a" is of 0·5 ml enzyme added to 0·2 μmole of chlorogenic acid at T = O. Trace "b" is of 0·2 ml enzyme added to 0·2 μmole of chlorogenic acid at T = 0·5.

The consistency of the results obtained by this method with theoretically linear plots demonstrates its validity as a measure of *o*-diphenol oxidase activity. In addition to its direct measurement of substrate changed, the inherent sensitivity of fluorometry and the relative speed and ease of the assay make this procedure a valuable new technique for the study of this enzyme. Its application is limited to fluorescent substrates but as most of the common *o*-diphenols of plant origin are naturally fluorescent this is not a severe limitation to its usefulness in phytochemistry.

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EXPERIMENTAL

Acetone powders were prepared from fresh mushroom, tobacco leaf, tomato leaf and tomato callus and stored in desiccators at −15°. Crude enzyme extracts were prepared by homogenizing 5 mg of powder/ml of ice-cold 0·05 M phosphate buffer, pH 6·5. This homogenate was centrifuged at 20,000 *g* for 20 min at 0°, and the supernatant was used as the enzyme solution. Acetone powders that had lost their activity due to exposure to air at room temperature were used as controls.