Direct spectrophotometric determination of chlorogenic acid oxidase activity

Several methods have been described for the determination of polyphenol oxidase activity[1,2,3]. Most of the methods are indirect and are applicable to different polyphenolic substrates including chlorogenic acid. Since the absorption of chlorogenic acid at 326 mμ decreases markedly upon oxidation, chlorogenic acid oxidase activity may be conveniently assayed by measurement of absorbance changes at this wave length.

The absorption spectrum of chlorogenic acid has been reported by SHIROYA *et al.*[4] and HULME[5], but these workers present no evidence indicating the relative proportion of the oxidized to reduced compound in their samples. The absorption spectrum of a $2.8 \cdot 10^{-5}$ *M* solution of chlorogenic acid (prepared from a sample of c.p. compound obtained from the Delta Chemical Company, New York, N.Y.) was determined by use of a Beckman D. U. spectrophotometer and is presented in Fig. 1. Evidence to be presented indicates that 93% of this sample is in the reduced form. The curve for chlorogenic acid exhibits a peak at 326 mμ and a trough with a minimum at 265 mμ. The spectrum of the product obtained from oxidation of chlorogenic acid also is presented in Fig. 1. For this determination, sufficient purified tobacco-root extract (1) to cause an absorbance change of 0.200/min was added to the cuvette containing chlorogenic acid and buffer and also to the reference cuvette containing buffer. When the decrease in absorbance at 326 mμ ceased, the reaction was considered complete and the spectrum of the product of chlorogenic acid oxidation was recorded. The values were corrected for the dilution resulting from the addition of enzyme. It is apparent from these determinations that the spectra recorded by HULME[5] and by SHIROYA *et al.*[4] are representative of the reduced form of chlorogenic acid.

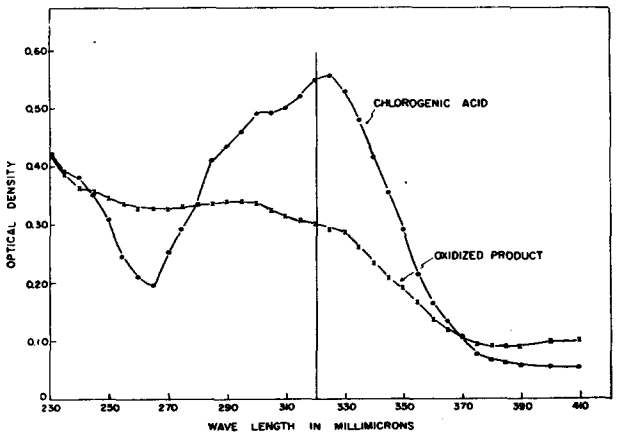

Fig. 1. Absorption spectra of chlorogenic acid and of the oxidation product formed in presence of a tobacco-root extract. The spectrum for chlorogenic acid was determined with $2.8 \cdot 10^{-5}$ *M* solution of the compound dissolved in 0.1 *M* potassium phosphate buffer, pH 7.0. The spectrum of the oxidized product was determined after the same solution reached equilibrium in presence of a purified tobacco polyphenol oxidase[1]. Silica cuvettes of 1-cm light path were used. The reference cuvette for the chlorogenic acid spectrum contained phosphate buffer alone and that for the oxidized product contained phosphate buffer and tobacco polyphenol oxidase. The vertical line at 320 mμ represents the point where the tungsten lamp was replaced by a hydrogen-discharge lamp.

The addition of about 1 mg ascorbic acid to 3 ml $2.8 \cdot 10^{-5}$ *M* chlorogenic acid resulted in a 3.5% increase in optical density, but ascorbate and buffer failed to increase the optical density at this wave length. The molar absorptivity (extinction coefficient) of the reduced chlorogenic acid solution after ascorbate addition was calculated to be $2.04 \cdot 10^4$. This may be compared with the value of $1.97 \cdot 10^4$ at 326 mμ reported by HULME[5]. A molar absorptivity of $1.04 \cdot 10^4$ at 326 mμ was calculated for the product of chlorogenic acid oxidation, assuming that the concentration of the product at equilibrium was $2.8 \cdot 10^{-5}$ *M*. The difference in molar absorptivities of the oxidized and reduced compounds is $1 \cdot 10^4$. By use of these values, it was concluded that the $2.8 \cdot 10^{-5}$ *M* solution of the c.p. chlorogenic acid in 0.1 *M* potassium phosphate buffer contained 7% of an oxidized form that could be reduced upon the addition of ascorbate.

A reaction mixture containing chlorogenic acid and tobacco-root extract (identical with that described for the oxidized product of Fig. 1) turned brown in color as the reaction proceeded. As indicated by the data plotted in Fig. 2, the addition of ascorbic acid to the reaction mixture 4 min after the reaction was initiated resulted in an increased optical density at 326 mμ. Undoubtedly the increase was caused by the reduction of the oxidized product formed from the oxidation of chlorogenic acid. Also as shown in Fig. 2, the chlorogenic acid formed from the ascorbate reduction was further oxidized in the presence of the extract. When the chlorogenic acid was allowed to reach

equilibrium and stand an appreciable period of time thereafter (approximately 3 min) the brown color failed to disappear upon the addition of ascorbic acid. Apparently this is due to the polymerization of the oxidized product which prevents reversal of the oxidation.

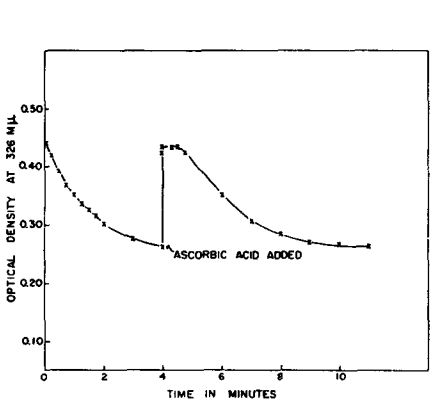

Fig. 2. Reversal of the enzymic oxidation of chlorogenic acid by ascorbic acid. The reaction mixture was identical with that described for the determination of the spectrum of the oxidized product of Fig. 1. 4 min after the reaction was initiated sufficient ascorbate was added to make the reaction $3 \cdot 10^{-5}$ *M*. The absorbance (optical density) determinations were corrected for dilution.

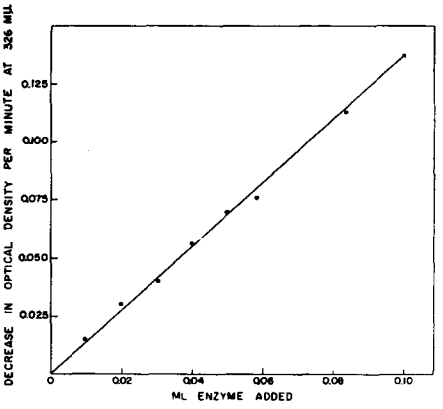

Fig. 3. Proportionality of enzyme activity with enzyme concentration. The assay procedure described in the text was utilized with variation in concentration of enzyme as indicated and also with the exception that rates were calculated on the basis of the rates for the first 15-sec interval. The crude extract was prepared by homogenizing one weight of tobacco roots in three weights of 0.1 *M* phosphate buffer, pH 7, and then removing the particulate material by centrifugation at 25,000 × *g* for 20 min[1].

The assay of the oxidase activity is carried out in a silica cuvette of 1.2-ml volume. The reaction mixture contains 0.1 *M* potassium phosphate buffer, pH 7.0; $5.7 \cdot 10^{-5}$ *M* chlorogenic acid; and 10^{-3} *M* ethylenediaminetetraacetate. The reaction is initiated by the addition of sufficient enzyme to cause a decrease of absorbance of approximately 0.020 in 15 sec. The absorbance is recorded at each 15-sec interval for 75 sec. One unit of activity is defined as that amount causing an absorbance change of 0.001 for the interval between 15 and 75 sec after the reaction was initiated. Since a small fraction of the substrate is utilized at this rate of oxidation, the reaction appears to be linear with time. As shown in Fig. 3 the initial enzyme activity is proportional to the enzyme concentration throughout a relatively broad range. It has been demonstrated that the reaction is dependent upon oxygen.

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