The Suppression of Catechol Oxidase Activity during the Enzymic Hydroxylation of *p*-Coumaric Acid by Spinach Leaf Phenolase

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The hydroxylation of *p*-coumaric acid to caffeic acid can be effected with a purified spinach leaf phenolase, which acts in the reaction as a mixed-function oxidase with a number of electron donors, notably ascorbate, NADH and 6,7-dimethyl-5,6,7,8-tetrahydropteridine. The enzyme also exhibits catechol oxidase activity (Vaughan & Butt, 1967, 1968). Molecular oxygen is utilized in both the hydroxylation of *p*-coumaric acid and the oxidation of caffeic acid to its *o*-quinone, which is reduced again by any of the reductants above. The further oxidation of caffeic acid is demonstrated by an oxygen consumption in excess of that required for its production.

When 10 μ moles of ascorbate was used in the hydroxylation of up to 10 μ moles of *p*-coumaric acid, oxygen uptake was linear with time but caffeic acid production, although initially equivalent to oxygen consumption, became slower. This decline was probably not due to an unfavourable competition between *p*-coumaric and caffeic acids, for the addition of 1 μ mole of caffeic acid, although increasing oxygen consumption by 80%, decreased only slightly the hydroxylation reaction.

The greater stability of catechol oxidase activity was further demonstrated with aged preparations, which formed caffeic acid more slowly without affecting the rate of oxygen consumption. The addition of bovine serum albumin restored the hydroxylation activity to that of the fresh preparations without altering oxygen uptake.

By contrast, with 2, 3 or 5 μ moles of dimethyltetrahydropteridine, oxygen consumption and caffeic acid production remained exactly equivalent throughout the reaction; the rates of each fell similarly as hydroxylation progressed. The addition of further quantities of the reductant restored the rates of caffeic acid production and oxygen consumption equally. A marked divergence between them was observed only when dimethyltetrahydropteridine was present in excess of *p*-coumaric acid.

With NADH, caffeic acid production fell below oxygen consumption as soon as hydroxylation commenced, even when non-catalytic protein was added. When dimethyltetrahydropteridine was added with NADH, however, catechol oxidase activity was completely suppressed.

It is suggested that hydroxylation requires some specific enzymic organization, which is unstable.

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Its maintenance during the reaction depends upon the nature of the electron donor and the relative concentration of *p*-coumaric acid. Catechol oxidase activity does not require the retention of this arrangement and survives the partial or complete loss of hydroxylase activity.

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An Alkaline Deoxyribonuclease from the Regenerating Liver of the Rat

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Alkaline deoxyribonucleases are found in a variety of mammalian tissues (Shugar & Sierakowska, 1967) but there is no evidence of structural identity between these enzymes (Laskowski, 1961). In rat liver the enzyme was shown to be associated with the mitochondria (Beaufay, Bendall, Baudhuin & de Duve, 1959) and proved to be a non-specific nuclease that degrades both RNA and denatured DNA (Curtis, Burdon & Smellie, 1966). A second alkaline deoxyribonuclease that preferentially hydrolyses native DNA can also be obtained from rat liver (O'Connor, 1968) and is described here.

The source of enzyme was the liver removed from Wistar rats 36 hr. after partial hepatectomy (Higgins & Anderson, 1931). The tissue was frozen on solid carbon dioxide, and then thawed and homogenized in potassium phosphate buffer, pH 8.0. From this homogenate the enzyme was purified at least 60-fold by a procedure that employed freeze-thawing, centrifugation at 105 000g, fractionation at pH 5, ammonium sulphate precipitation and chromatography on hydroxyapatite.

The purified enzyme was active over the range pH 7.0–8.0 but showed optimum activity in glycine-NaOH buffer, pH 8.0. In the presence of Mg^{2+} , activity was maximal at concentrations from 4–7 mM but decreased at higher concentrations. The cations Mn^{2+} , Zn^{2+} and Ca^{2+} all stimulated activity, but only to 50% of that shown with Mg^{2+} . For maximum activity the enzyme required a substrate with a high degree of secondary structure. It was three times more active with native DNA than with denatured DNA and was sensitive to the presence of partially denatured DNA. Although the preparation contained ribonuclease activity it was not associated with the deoxyribonuclease activity in

the elution profile obtained after hydroxyapatite chromatography.

Analysis of the products of limited digests of native DNA by Sephadex-gel filtration and by DEAE-cellulose chromatography indicated that the hydrolysis was almost exclusively endonucleolytic. The exonucleases from snake venom and bovine spleen (Razzell, 1963) were used to show that the oligonucleotides produced by the liver deoxy-ribonuclease bear a 5'-phosphate terminus.

The enzyme can be distinguished from the alkaline nuclease of the mitochondrial fraction by its preference for native DNA, its specificity for DNA and its activity in the presence of different bivalent cations. Preliminary experiments suggest a further distinction, in that the activity is associated with the nucleus rather than the cytoplasm and is located in the chromatin acidic protein fraction.

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A Simple Method for the Isolation of Very Large Numbers of Leaf Protoplasts by using Mixtures of Cellulase and Pectinase

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Takebe, Otsuki & Aoki (1968) described a method for the isolation of leaf protoplasts in which cells isolated from the leaf by prior treatment with pectinase (Macerozyme) are subsequently treated with cellulase (Onozuka P1500) to digest away the cell wall (cf. Cocking, 1960). Work in this Laboratory has now shown that this method can be greatly improved and made much simpler by adding a mixture of the cellulase and pectinase directly to the leaf material. Very large quantities of protoplasts can be isolated by using mixtures of these commercially available enzymes.

Pieces, approx. 4cm.², from fully expanded leaves of 50-70-day-old tobacco plants (*Nicotiana tabacum* var. *Xanthi*), from which the lower

epidermis had been removed (cf. Takebe *et al.* 1968), were treated with a mixture of pectinase [0.5% (w/v) Macerozyme, Kinki Yakult Manuf. Co. Nishinomiya, Japan, or 0.5% (w/v) Pectinase, Sigma Chemical Co. Ltd., St Louis, Mo., U.S.A.; or 5% (w/v) Pectinol R10, Rohm & Haas Co., Philadelphia, Pa., U.S.A.] and cellulase [5% (w/v) Onozuka P1500 from *Trichoderma viride*, Kinki Yakult Manuf. Co.; or 5% (w/v) Meicelase P, from *Trichoderma viride*, Meiji Seika Kaisha Ltd., Tokyo, Japan] in 20% (w/v) sucrose adjusted to pH 5.6 with 2N-HCl. After an initial vacuum infiltration for 15min. the pieces of leaf were incubated in this enzyme mixture for 4hr. without shaking. The incubation with the enzymes was terminated by gently swirling the incubation mixture, pouring it through a coarse wire gauze and washing with a modified White's medium containing 20% (w/v) sucrose (Pojnar, Willison & Cocking, 1967). The very large quantities of spherical green isolated protoplasts obtained were washed free from contaminating enzymes by repeated flotation in this medium.

These isolated leaf protoplasts are stable for several days in this medium but unless they are subsequently transferred to a more suitable cell-wall regeneration medium (cf. Pojnar *et al.* 1967) progressive bursting takes place, often with release of the large central vacuole.

These isolated leaf protoplasts should be particularly suitable for investigations of pinocytic uptake of viruses, and, after cell-wall regeneration, for elucidating early stages in the virus infection of isolated plant cells (cf. Cocking & Pojnar, 1969).

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The Partial Inhibition of Yeast Invertase by Iodine

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Iodine partially inhibits invertase (Euler & Landergren, 1922); cysteine, glutathione, ascorbic acid and sodium amalgam do not reactivate the enzyme (Myrbäck & Willstaedt, 1957). We have investigated the reaction of iodine with purified, homogeneous invertase (Waheed & Shall, 1967).

The extent of inactivation is dependent on the iodine concentration; maximal inhibition of about