Cellular Localization of *t*-Cinnamic Acid 4-Hydroxylase Produced in Sweet Potato in Response to Cut Injury

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Sweet potato root tissue produces polyphenols in response to cut injury¹⁾ and the synthesis is markedly increased by incubation in ethylene.²⁾ Under these conditions, activities of phenylalanine ammonia-lyase (PAL) and *t*-cinnamic acid 4-hydroxylase (4-hydroxylase) are dramatically developed principally following the same pattern.³⁾ Where the polyphenols are produced in sweet potato tissue cells is not known. In some plants, histochemical observation showed that polyphenols were localized in vacuoles or cellular organelles.^{4,5)} Furthermore, *o*-diphenol oxidase, which catalyzes the oxidation of *o*-diphenol to produce the brown polymeric pigment when cells are damaged, is present in cytoplasm. The facts suggest that the polyphenols are compartmentalized in the cell. Possibly, some enzymes, which are positioned in the later stage of the polyphenol biosynthetic pathway than PAL, are localized in the vacuolar membrane and upon production of polyphenols they are excreted into the vacuoles. Following this aspect, we studied cellular localization of 4-hydroxylase by differential centrifugation and sucrose density gradient centrifugation.

Slices (3 mm thick) were prepared from the parenchymal root tissue of sweet potato (*Ipomoea batatas* Lam. cv. Kokei 14), and incubated at 30°C for 2 days on a polyethylene net in a styrol box containing wet paper. The slices were homogenized with a grater in 50 mM Tris-HCl buffer, pH 8.5, containing

0.7 M sorbitol, 1 mM EDTA, 1% (w/v) potassium isoascorbate and Polyclar AT (0.2× fresh weight of tissue). The homogenate was squeezed through four layers of cotton gauze and centrifuged at 1,000×*g* for 10 min. The supernatant solution was passed through a column of Sephadex G-25 (coarse) which had been equilibrated with 50 mM Tris-HCl buffer, pH 8.5, containing 0.7 M sorbitol and 1 mM EDTA. The protein fraction thus obtained was retained for further fractionation. All procedures were performed below 4°C. Activities of 4-hydroxylase, NADPH-cytochrome *c* reductase, antimycin A insensitive NADH-

FIG. 1. Cellular Fractionation by Differential Centrifugation.

Crude enzyme solution was centrifuged at 10,000×*g* for 15 min to yield the precipitate (fraction I). The supernatant was again centrifuged at 24,000×*g* for 30 min to yield the precipitate (fraction II). The supernatant was last centrifuged at 105,000×*g* for 90 min to obtain the precipitate (fraction III) and supernatant fraction. The ordinate indicates activity per mg of protein in a given subcellular fraction. The abscissa shows the percent of protein content in each subcellular fraction relative to the sum of fraction I, II and III. Roman numerals at the upper side of the figure indicate numbers of the subcellular fractions. A: 4-hydroxylase activity (×10³ cpm per hr per mg protein). B: NADPH-cyt. *c* reductase activity (×10² ΔO.D.₅₅₀ per min per mg protein). C: NADH-cyt. *c* reductase activity (×10² ΔO.D.₅₅₀ per min per mg protein). D: cyt. *c* oxidase (×10⁴ ΔO.D.₅₅₀ per min per mg protein).

cytochrome c reductase, and cytochrome c oxidase were assayed with some modifications by the methods of Russell,⁶⁾ Masters,⁷⁾ Mackler⁸⁾ and Smith,⁹⁾ respectively.

Differential centrifugation was performed to analyze the cellular fractionation of activities of the above four enzymes. Distribution of the activities are shown in Fig. 1. Mitochondrial marker enzyme, cytochrome c oxidase was sedimented faster than the other enzymes. The two microsomal marker enzymes, NADPH-cytochrome c reductase and antimycin A insensitive NADH-cytochrome c reductase, showed very similar distribution and were sedimented the slowest. 4-Hydroxylase was sedimented slower than the mitochondrial marker enzyme and faster than the microsomal marker enzymes.

FIG. 2. Cellular Fractionation by Sucrose Density Gradient Centrifugation.

Sweet potato tissue slices were incubated at 30°C for 48 hr. Membrane sample was isolated by centrifugation at $105,000 \times g$ for 60 min and suspended in 5 mM Tris-HCl buffer, pH 8.5, containing 0.5 M sucrose and 2 mM $MgCl_2$. The suspension was layered on the linear 17.1~45% sucrose gradient which contained 5 mM Tris-HCl buffer, pH 8.5, and 2 mM $MgCl_2$. Sucrose density gradient centrifugation was performed with a Beckman SW 25.3 rotor at 25,000 rpm for 3 hr. 4-Hydroxylase activity (●) was assayed by incubation for 1 hr at 30°C in a total volume of 0.15 ml. NADPH-cyt. c reductase activity (□) was assayed and protein (×) was determined.

A membrane sample was isolated from the above protein fraction by centrifugation at $105,000 \times g$ for 60 min and fractionated by linear sucrose density gradient centrifugation. When the sample was fractionated on a sucrose gradient (17.1~60%), 4-hydroxylase activity was hardly detectable in the mitochondrial fraction and its distribution was not in accord with that of NADPH-cytochrome c reductase activity. The obvious difference in distribution of activity between 4-hydroxylase and NADPH-cytochrome c reductase was also confirmed by fractionation on a sucrose gradient (17.1~45%), which indicated that 4-hydroxylase activity was localized in a denser fraction of the gradient than NADPH-cytochrome c reductase activity (Fig. 2).

These results indicate that 4-hydroxylase is bound to a specific microsomal membrane or to some unknown organelle. Further investigation of localization of the enzymes positioned later in the pathway than 4-hydroxylase should be interesting.

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