Note

An Enzyme in Sweet Potato Root which Catalyzes the Conversion of Chlorogenic Acid, 3-Caffeoylquinic Acid, to Isochlorogenic Acid, 3,5-Dicaffeoylquinic Acid

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When sweet potato root tissue is sliced and incubated, much synthesis of chlorogenic acid (3-caffeoylquinic acid) and isochlorogenic acid (isomers of dicaffeoylquinic acid) takes place.^{1~4)} Our earlier tracer experiment⁴⁾ suggested that chlorogenic acid was directly converted to isochlorogenic acid through a one-step reaction in injured sweet potato root.

This communication deals with the detection of the enzyme activity in sweet potato root which catalyzes the conversion of chlorogenic acid to isochlorogenic acid (3,5-dicaffeoylquinic acid).

Acetone powder was prepared from sweet potato (*Ipomoea batatas* Lam, cv, Norin 1) root slices, 0.5 cm thick, which had been incubated at 25°C for 48 hr. The powder (3 g) was extracted with 30 ml of 0.1 M phosphate

buffer, pH 7.0, containing 0.1% mercaptoethanol. The extract was brought to 70% saturation with ammonium sulfate and the resultant precipitate was collected by centrifugation at $3.2 \times 10^4 g$ for 15 min. The precipitate was dissolved in 2 ml of 25 mM phosphate buffer, pH 6.4, and put on a column of Sephadex G-25 (2×24 cm) pre-equilibrated with the same buffer. The void volume fraction from the column was assayed for its activity in the conversion of chlorogenic acid to isochlorogenic acid by the following procedures.

A reaction mixture consisted of 6 μ mol of chlorogenic acid, 8 μ mol of magnesium chloride, 7.5 μ mol of phosphate (pH 6.4) and a certain amount of enzyme in a final volume of 540 μ l. The mixture was incubated at 30°C for a certain period under nitrogen gas to inhibit the oxidation by contaminating polyphenoloxidase. The reaction was terminated by the additions of 3 ml of ethanol and 2 ml of acetone. The mixture was left at room temperature for 30 min and centrifuged at $2 \times 10^3 g$ for 10 min. The supernatant was condensed under reduced pressure at 30°C, put onto a column of Develosil ODS 5 (4 mm $\times$ 25 cm) and eluted with 25% acetonitrile containing 1% acetic acid at a flow rate of 0.5 ml/min. The amount of the enzyme reaction product (3,5-dicaffeoylquinic acid) was determined from the area of the peak at 9 ml of elution volume.

The enzyme reaction was done on a large scale to prepare the reaction product for identification. The reaction mixture after termination by additions of ethanol and acetone was chromatographed on paper (Whatman 3 MM) using 5% acetic acid (AW). The fluorescent band at R_f 0.23 was cut off and eluted with a mixture of ethanol and water (1:1, v/v). The eluate was condensed with a rotary evaporator and used for the liquid chromatography described above. The main peak fractions at 9 ml of elution volume were pooled and used for identification.

FIG. 1. ¹H-NMR Spectrum of Enzyme Reaction Product (500 MHz, in CD₃OD).

TABLE I. MOLAR RATIO OF ENZYME REACTION PRODUCTS

A reaction mixture consisting of 7.6 μ mol of chlorogenic acid, 10 μ mol of magnesium chloride, 9.4 μ mol of phosphate (pH 6.4) and 820 μ g protein of enzyme in a final volume of 680 μ l was incubated at 30°C for 30 min. The reaction was terminated and used for liquid chromatography to isolate 3,5-dicaffeoylquinic acid, caffeic acid, and quinic acid, as described in text. The peak fraction of each product was collected and assayed. 3,5-Dicaffeoylquinic acid and caffeic acid were measured by the optical density at 326 μ m and the quinic acid was measured by the PT reaction. It was assumed that caffeic acid was produced by hydrolysis of the substrate, chlorogenic acid.

	Amount of enzyme reaction products (nmol)		
	3,5-Dicaffeoylquinic acid	Quinic acid	Caffeic acid
Gross amount in the reaction mixture after incubation	18.0	25.0	5.9
Amount of quinic acid produced concomitantly with caffeic acid production	—	5.9	—
Net amount of quinic acid due to the enzyme reaction producing 3,5-dicaffeoylquinic acid	—	19.1	—
Molar ratio of 3,5-dicaffeoylquinic acid and quinic acid produced by the enzyme reaction	1 (18.0)	1.1 (19.1)	—

FIG. 2. Proposed Enzymatic Reaction for Production of 3,5-Dicaffeoylquinic Acid.

The reaction product showed the same UV-absorption spectrum as chlorogenic acid; UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm: 245, 326. The reaction product and authentic chlorogenic acid (Aldrich Chemical Co.) were hydrolyzed in 0.5N NaOH at 30°C under nitrogen gas for 75 min. The hydrolyzates were neutralized by HCl and chromatographed on paper using three solvent systems; AW, *n*-butanol-acetic acid-water, 4:1:2, v/v (BAW) and *n*-butanol-pyridine-water, 14:3:3, v/v (BPW). The chromatographed papers were examined first by a UV lamp to detect the aromatic moiety and then cut into 1-cm pieces, on which color was developed⁵⁾ with periodic acid and 2-thiobarbituric acid (PT reaction) which is positive with quinic acid and deoxysugar, to detect the non-aromatic moiety. The hydrolyzate of the enzyme reaction product showed the same two spots as those of chlorogenic acid on every chromatogram. The *R_f* value of the fluorescent spot coincided with that of authentic caffeic acid and that of the pink spot by PT reaction with that of authentic D-quinic acid. The *R_f* values of spots of the aromatic and non-aromatic moieties of the product and chlorogenic acid on chromatograms developed by various solvent systems were as follows: 0.33, 0.95 (AW); 0.85, 0.30 (BAW); and 0.51, 0.02 (BPW), respectively.

The structure of the reaction product was 3,5-dicaffeoyl-

quinic acid^{6,7)} from the following evidence of the spectrum of ¹H-NMR (Fig. 1). First, the spectrum indicated that it consisted of one molecular quinic and two molecular caffeic acids. Second, the hydroxyl groups at the 3 and 5 positions of quinic acid must be acylated with caffeic acid since the signals at 5.38 and 5.53 ppm which were assigned to H-3 and H-5, respectively, by spin-spin decoupling analysis ($J_{2e,3} = 5$ Hz, $J_{2a,3} = J_{3,4} = 10$ Hz, $J_{4,5} = J_{5,6a} = J_{5,6e} = 3$ Hz) appeared more than 1.0 ppm lower in the field than those of quinic acid.

The enzyme reaction mixture contained only chlorogenic acid, 3-caffeoylquinic acid, as a substrate. As mentioned above, the enzyme produced 3,5-dicaffeoylquinic acid as one of the reaction products. Consequently, it was expected that one molecule of quinic acid was produced concomitantly with production of one molecule of 3,5-dicaffeoylquinic acid, and it was (Table I).

The amount of 3,5-dicaffeoylquinic acid produced by the enzyme reaction increased with incubation time and enzyme concentration (data not shown). On the other hand, no 3,5-dicaffeoylquinic acid was detected with any of the following reaction mixtures; the complete reaction mixture containing active enzyme of which reaction was terminated at zero time incubation, the complete reaction mixture which was incubated with boiled enzyme, or the

reaction mixture without chlorogenic acid which was incubated with active enzyme.

The results in this communication indicated the occurrence of an enzyme in sweet potato root which catalyzes the reaction shown in Fig. 2. It is noteworthy that the enzyme produced only one isomer, 3,5-dicaffeoylquinic acid, among three possible isomers of dicaffeoylquinic acid.^{6~9)}

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