Short Communication

Sweet Potato Root Enzyme which Catalyzes the Formation of Chlorogenic Acid from 1-*O*-Caffeoyl-D-glucose and D-Quinic Acid

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Received August 20, 1984

Chlorogenic acid, 3-*O*-caffeoyl-D-quinic acid, is ubiquitous in the plant kingdom.¹⁾ Its biosynthetic pathway, however, still remains obscure, although some evidence that hydroxycinnamoyl-CoA thioesters are intermediates in its biosynthesis has been reported.^{2~4)}

Previously, we proposed the following biosynthetic pathway of chlorogenic acid based on the results of tracer experiments using injured sweet potato root.^{5~7)} *t*-Cinnamic acid is converted to 1-*O*-*t*-cinnamoyl-D-glucose in the first step, then two hydroxyl groups are introduced into the benzene ring of 1-*O*-*t*-cinnamoyl-D-glucose and finally, *trans*-esterification takes place between the D-glucose moiety and D-quinic acid, producing 3-*O*-caffeoyl-D-quinic acid, chlorogenic acid.

In our preceding papers,^{8~10)} we described enzymes in sweet potato root which catalyze the formation of 1-*O*-*t*-cinnamoyl-D-glucose and the *trans*-esterification between 1-*O*-*p*-coumaroyl-D-glucose and D-quinic acid, producing *p*-coumaroylquinic acid.

This communication deals with the detection of the enzyme in sweet potato root which catalyzes the *trans*-esterification between 1-*O*-caffeoyl-D-glucose and D-quinic acid producing 3-*O*-caffeoyl-D-quinic acid, chlorogenic acid.

1-*O*-Caffeoyl-D-glucose was chemically synthesized following a procedure similar to that for synthesis of 1-*O*-*p*-coumaroyl-D-glucose.⁹⁾ Diacetylcaffeoylchloride and β -D-glucose-2,3,4,6-tetraacetate were conjugated to give 1-*O*-diacetyl-caffeoyl-2,3,4,6-tetraacetyl- β -D-glucose which was finally deacetylated to form 1-*O*-caffeoyl-D-glucose. The product was purified by counter-current distribution between water and ethyl acetate, then lyophilized. Purity of the product was examined by a liquid chromatograph equipped with a column of Develosil ODS5 (4 mm $\times$ 25 cm) using 20% methanol containing 10 mM acetic acid. When the product was chromatographed on paper (Whatman No. 1) using solvent systems of *n*-butanol-pyridine-water, (14:3:3, v/v) (BPW) and 5% acetic acid (AW), it showed *R_f* values of 0.66 and 0.70, respectively. These *R_f* values agreed with those reported for 1-*O*-caffeoyl-D-glucose.¹¹⁾ The identification of the product was confirmed by UV, IR and NMR spectrophotometries; UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm: 245,333. IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3350, 1700, 1600, 1510. NMR (CD₃OD), δ : 3.30~3.50 (4H, m), 3.70 (1H, dd, *J*=5, 13 Hz), 3.86 (1H, dd, *J*=3, 13 Hz), 5.58 (1H, d, *J*=8 Hz), 6.30 (1H, d, *J*=16 Hz), 6.79 (1H, d, *J*=8 Hz), 6.97 (1H, dd, *J*=2, 9 Hz), 7.06 (1H, d, *J*=2 Hz), 7.66 (1H, d, *J*=8 Hz).

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 24 hr. The powder (6 g) was extracted with 38 ml of 0.1 M phosphate buffer, pH 7.0. The extract was fractionated with ammonium sulfate and the precipitate between 40 and 70% saturation was collected by centrifugation at 3.2×10^4 g for 15 min. The precipitate was dissolved in 2 ml of 25 mM phosphate buffer, pH 6.3, containing 13 mM β -mercapthoethanol (buffer A). The solution was applied to a column of Sephadex G-25 (1.5 $\times$ 14 cm) pre-equilibrated with buffer A. The void volume fraction from the column was assayed for its activity in the *trans*-esterification between 1-*O*-caffeoyl-D-glucose and D-quinic acid by the following procedures.

A reaction mixture consisted of 2.5 μmol of 1-*O*-caffeoyl-D-glucose, 17.5 μmol of D-quinic acid, 8 μmol of magnesium chloride, 14 μmol of phosphate, pH 6.3, and 408 μg protein of enzyme in a final volume of 660 μl . 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 with the additions of 3 ml of ethanol and 2 ml of acetone. The mixture was left at room temperature for 45 min and centrifuged at 2×10^3 g for 5 min. The supernatant was condensed by an evaporator and applied onto a paper (Whatman No. 1). The paper was developed with a solvent system of BPW. The enzyme reaction product was detected as a fluorescent band at *R_f* 0.23 under UV-lamp. The fluorescent band was cut off and eluted with a mixture of ethanol and water (1:1, v/v). The eluate was condensed with a rotary evaporator, applied to a liquid chromatograph equipped with a column of Develosil ODS 5 (4 mm $\times$ 25 cm), and eluted with 35% methanol containing 10 mM acetic acid. The amount of the product was determined from the area of the peak at 7 ml of elution volume which agreed with that of authentic chlorogenic acid (Aldrich Chemical Co.).

The enzyme reaction product and chlorogenic acid showed the same retention time on high performance liquid chromatography as described above. On paper chromatographies, they also showed the same *R_f* values: 0.3 (BPW); 0.65 (*trans*-form) and 0.78 (*cis*-form) (AW); and 0.51 (KFW=methyl isobutyl ketone-formic acid-water (14:3:2, v/v). Furthermore, when the enzyme reaction product was co-chromatographed with chlorogenic acid, they showed exactly the same behavior on all these chromatographies. Thus, the enzyme reaction product was identified as chlorogenic acid, 3-*O*-caffeoylquinic acid.

As shown in Table I, the amount of chlorogenic acid produced by the enzyme reaction increased with incubation time. On the other hand, no enzyme reaction product (chlorogenic acid) was detected with any of the fol-

TABLE I. CHARACTERIZATION OF *trans*-ESTERIFICATION REACTION BETWEEN 1-*O*-CAFFELOYL-D-GLUCOSE AND D-QUINIC ACID BY THE ENZYME FROM SWEET POTATO ROOTS

The enzyme activity was assayed by the methods described in text. The incubation period was 40 min unless otherwise stated. Boiled enzyme was prepared by heating enzyme solution at 100°C for 10 min.

Enzyme reaction	Chlorogenic acid produced (nmol)
Active enzyme, complete reaction mixture	
0 min of incubation	0
20 min of incubation	55.0
40 min of incubation	109.6
Bioled enzyme, complete reaction mixture	0
Active enzyme, reaction mixture without D-quinic acid	0

lowing 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 D-quinic acid which was incubated with active enzyme. This indicated that chlorogenic acid was synthesized by the action of the enzyme which catalyzes *trans*-esterification between 1-*O*-caffeoyl-D-glucose and D-quinic acid.

D-Quinic acid has four hydroxyl groups at the 1-, 3-, 4-, and 5-positions, all of which could be involved in conjugation with the carboxyl group of the caffeoyl moiety. However, we detected only 3-*O*-caffeoylquinic acid, chlorogenic acid, as the enzyme reaction product. Therefore, the enzyme seems to have a preference for the hydroxyl group at the 3-position of D-quinic acid in catalyzing the *trans*-esterification reaction with caffeoyl-D-glucose.

In our preceding paper,¹⁰⁾ we reported the enzyme in sweet potato root which catalyzes the *trans*-esterification between 1-*O*-*p*-coumaroyl-D-glucose and D-quinic acid, producing *p*-coumaroylquinic acid. We don't know yet whether or not these two *trans*-esterification reactions are catalyzed by a sin-

gle enzyme.

This communication indicates the occurrence of an enzyme activity in sweet potato which catalyzes the *trans*-esterification between 1-*O*-caffeoyl-D-glucose and D-quinic acid, producing 3-*O*-caffeoylquinic acid, chlorogenic acid. The existence of this enzyme strongly supports the operation of the pathway which we previously proposed for chlorogenic acid biosynthesis in the sweet potato.^{5~7)}

Acknowledgment. The authors are grateful to Dr. T. Kondo, of Nagoya University for his advice and measurement of the NMR spectrum.

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