Short Communication

Detection of the Enzyme in Sweet Potato Root Which Catalyzes *trans*-Esterification between 1-*O-p*-Coumaroyl-D-glucose and D-Quinic AcidMineo KOJIMA and
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Received April 25, 1984

Chlorogenic acid, 3-*O*-caffeoyl-D-quinic acid, is one of the most ubiquitous polyphenols in the plant kingdom.¹⁾ It has been shown that the aromatic moiety of chlorogenic acid is derived mainly from *t*-cinnamic acid produced from L-phenylalanine.^{2,3)} The pathway beyond *t*-cinnamic acid, however, still remains obscure, although some evidence that hydroxycinnamoyl-CoA thioesters are intermediates in chlorogenic acid biosynthesis has been reported.^{4~6)}

Previously, we proposed the following biosynthetic pathway of chlorogenic acid based on the results of tracer experiments using injured sweet potato root.^{7~9)} *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. Recently, we reported the occurrence and the properties of the enzymes in sweet potato root which catalyze the formation of 1-*O-t*-cinnamoyl-D-glucose.^{10,11)}

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

According to our proposed biosynthetic pathway of chlorogenic acid,⁸⁾ the *trans*-esterification between hydroxycinnamic acid glucose ester and D-quinic acid should occur not after the introduction of one hydroxyl group into the benzene ring, that is, the formation of 1-*O-p*-coumaroyl-D-glucose, but after the introduction of two hydroxyl groups, that is, the formation of 1-*O*-caffeoyl-D-glucose. We, however, tried to detect an enzyme which catalyzes the *trans*-esterification between hydroxycinnamic acid glucose ester and D-quinic acid by using 1-*O-p*-coumaroyl-D-glucose as a substrate because the extract of sweet potato root shows a very high activity of *ortho*-diphenoloxidase¹²⁾ and could rapidly oxidize caffeoyl-D-glucose which is *ortho*-diphenol, and also some enzymes involved in the secondary metabolism show broad substrate specificities.^{11,13)} 1-*O-p*-Coumaroyl-D-glucose was synthesized and identified by the method reported in our preceding paper.¹¹⁾

Sweet potato (*Ipomoea batatas* Lam, cv. Norin 1) roots were cut into slices, 0.5 cm in thickness, and incubated at 25°C for 24 hr. The slices (60 g) were homogenized in 80 ml of 0.1 M phosphate buffer, pH 7.0, containing 35 mM sodium isoascorbate, 4 g of polyvinylpyrrolidone, and 16 g of sea sand with a mortar and pestle. The homogenate was squeezed through cheesecloth and centrifuged at 8×10^3 g for 5 min at 4°C. The supernatant fraction was applied to a column of Sephadex G-25 (3.5 × 7 cm), which had been equilibrated with 25 mM phosphate buffer, pH 7.0, containing 13 mM β-mercaptoethanol (buffer A). The fraction eluted at the void volume of the column was pooled and applied to a column of DEAE-Toyopearl 650 M (0.9 × 6 cm) pre-equilibrated with buffer A. After sample addition, 6 ml of buffer A containing 75 mM and 100 mM NaCl were washed through the column. Then the sample was eluted from the column with 6 ml of buffer A containing 150 mM NaCl. The eluted fraction was applied to a column of Sephadex G-25 (2 × 24 cm) pre-equilibrated with buffer A to remove NaCl. The effluent fraction from the column

was assayed for its activity in the trans-esterification between 1-*O-p*-coumaroyl-D-glucose and D-quinic acid by the following procedures.

A reaction mixture consisting of 0.64 μmol of 1-*O-p*-coumaroyl-D-glucose, 10 μmol of D-quinic acid, 32 μmol of phosphate, pH 7.0, and 600 μl of enzyme solution, in a final volume of 1000 μl , was incubated at 30°C for 3 hr. 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 45 min and centrifuged at 2×10^3 g for 5 min. The supernatant was condensed by a rotary evaporator and applied onto a paper (Whatman No. 1). The paper was developed with a solvent system of *n*-butanol-pyridine-water, 14:3:3, v/v (BPW). After development, the chromatograms were examined with a UV-lamp.

The enzyme reaction product was detected as a quenching spot at R_f 0.3 with a complete reaction mixture which was incubated with the active enzyme. On the other hand, no enzyme reaction product was detected with any of the following reaction mixtures; the complete reaction mixture containing active enzyme of which the reaction was terminated at zero time incubation, the complete reaction mixture which was incubated with boiled enzyme, or the reaction mixture without quinic acid which was incubated with active enzyme. These results indicated that the product was synthesized by the action of the enzyme which depended on the presence of both *p*-coumaroyl-D-glucose and D-quinic acid.

The enzyme reaction was carried out on a large scale to prepare the reaction product for identification. The quenching band of the enzyme reaction product on paper (Whatman 3 MM) chromatographed by BPW 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 Hitachi model 635 liquid chromatograph equipped with a column of Develosil ODS 5 (4 mm $\times$ 25 cm) and eluted with 25% methanol containing 10 mM acetic acid. The main peak fraction,

which appeared at 25 ml of elution volume, was collected and used for identification.

The product showed a maximum absorption at 320 nm and it shifted to 355 nm in alkaline conditions. These results suggested the presence of a free phenolic hydroxyl group in the product. When the product was chromatographed on Whatman No. 1 paper using solvent systems of BPW, methyl-isobutyl ketone-formic acid-water, 14:3:2, v/v (MFW), and 5% acetic acid (AW), it showed R_f values of 0.3, 0.59, and 0.68 (*trans*-form) and 0.78 (*cis*-form), respectively. These R_f values agreed with those reported for *p*-coumaroyl-quinic acid.¹⁴⁾ The chromatographed papers were cut into 1-cm pieces, which were put into test tubes containing 300 μl of 0.5 N NaOH. They were incubated at 40°C for 4 hr to hydrolyze the product and subjected to the color-developing reaction¹⁵⁾ with periodic acid and 2-thiobarbituric acid (PT reaction) which are positive with quinic acid and deoxysugar, but not with glucose. A pink color developed only at the position of a quenching spot under a UV lamp with every chromatogram.

The product was hydrolyzed in 0.5 N NaOH at 35°C for 2 hr. The hydrolyzate was neutralized by HCl and chromatographed on paper using three solvent systems; AW, BPW, and *n*-butanol-acetic acid-water, 4:1:2, v/v, (BAW). The chromatographed papers were examined first by UV lamp to detect the aromatic moiety of the product and then cut into 1-cm pieces, which were subjected to PT reaction to detect the non-aromatic moiety.

On every chromatogram two spots were detected; the R_f value of the quenching spot coincided with that of authentic *p*-coumaric acid and that of the pink spot with that of authentic D-quinic acid. R_f values of the spots of the aromatic and non-aromatic moieties of the product on the chromatograms developed by various solvent systems were as follows: 0.39, 0.78 (AW); 0.62, 0.03 (BPW) and 0.88, 0.29 (BAW). The molar ratio of *p*-coumaric acid and quinic acid was shown to be almost 1 to 1 by the determinations of *p*-coumaric acid

content by UV absorption of the hydrolyzate and quinic acid contents by PT reaction. The examination by anthrone-H₂SO₄ reagent¹⁶⁾ showed the absence of any reducing sugar such as D-glucose in the product. Thus, the reaction product was identified as *p*-coumaroyl-quinic acid. At present, we however have no definite information about which of the hydroxyl groups in the D-quinic acid moiety is involved in the conjugation with *p*-coumaric acid moiety.

The present communication indicates the occurrence of an enzyme in sweet potato root which catalyzes *trans*-esterification between 1-*O-p*-coumaroyl-D-glucose and D-quinic acid, producing *p*-coumaroylquinic acid. The existence of this enzyme in sweet potato root supports the operation of the pathway which we previously proposed for chlorogenic acid biosynthesis.^{7~9)} In addition, it constitutes evidence of the notion that phenolic glucose esters are the activated forms of phenolic acids and essential intermediates in their metabolism in plants.

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