Identification and Accumulation of 1-*O*-*trans*-Cinnamoyl-*β*-D-glucopyranose in Developing Strawberry Fruit (*Fragaria ananassa* Duch. Cv. Kent)

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Strawberry fruit (*Fragaria ananassa* Duch. cv. Kent) were discovered as the first food source of 1-*O*-*trans*-cinnamoyl-*β*-D-glucopyranose. Isolation was performed by preparative HPLC of a solid phase desorbate. Identification was carried out by comparing chromatographic properties, NMR spectroscopy data, and specific *β*-glucosidic cleavage. The accumulation was monitored by HPLC. A sharp rise of the concentration of 1-*O*-*trans*-cinnamoyl-*β*-D-glucopyranose was observed 21 days after anthesis and was related to general phenylpropanoid metabolism. 1-*O*-*trans*-Cinnamoyl-*β*-D-glucopyranose is discussed as a metabolic link between phenylalanine ammonia-lyase-mediated cinnamic acid and 1-*O*-*p*-coumaroyl-*β*-D-glucopyranose formation. The cinnamoyl ester may be the true precursor of volatile cinnamates in strawberry flavor.

Keywords: *Strawberry; Fragaria ananassa; 1-O-trans-cinnamoyl-β-D-glucopyranose; phenylpropanoid metabolism; glucoconjugate; flavor; HPLC*

INTRODUCTION

Plants synthesize large amounts of phenylpropanoid acids, mainly (hydroxy)cinnamic acids, which widely occur in conjugated forms, such as glycosides. Since an early comprehensive study (Harborne and Corner, 1961), glucose esters of hydroxycinnamic acids have been identified in numerous plants. However, little attention was paid to the glycoconjugates of the ring unsubstituted precursor, cinnamic acid. Stimulated by feeding of exogenous *trans*-cinnamic acid, 1-*O*-*trans*-cinnamoyl-*β*-D-glucopyranose (Figure 1) was discovered in cut leaves of *Raphanus sativus* and tuber disks of *Solanum* species (Harborne and Corner, 1961), in sliced roots of *Ipomoea batatas* (Kojima and Uritani, 1972; Moriguchi et al., 1988), and in cell lines of *Nicotiana tabacum* (Berlin and Witte, 1982). Without any addition of precursors, natural occurrence was reported in *Bauhinia manca* (Achenbach et al., 1988), and some chemical data were given on *Salix sachalinensis* (Mizuno et al., 1989).

The respective 2-*O*- and 6-*O*-esters were isolated from *Rheum* species (Kashiwada et al., 1984, 1987); however, 1-*O*-acyl glucosides are of special interest, since they are discussed as a pathway alternative to CoA thioesters in plant secondary metabolism (Barz et al., 1985; Glässgen and Seitz, 1992). Disregarding the above feeding studies, the present paper furnishes first proof of 1-*O*-*trans*-cinnamoyl-*β*-D-glucopyranose in a food plant. This cinnamoyl ester may be the natural progenitor of methyl and ethyl cinnamate, widely occurring aroma volatiles in various fruits (Maarse et al., 1994). Methyl cinnamate and ethyl cinnamate were reported in strawberry fruit (Hirvi and Honkanen, 1982), strawberry wine (Schreier and Drawert, 1981), and strawberry jam (Barron and Etiévant, 1990). A recent study demonstrated the release of cinnamic acid from strawberry extracts upon hydrolysis with a commercial pectinase enzyme which is known to contain a series of hydrolytic side activities (Wintoch et al., 1991). Thus, the presence of a cinnamic acid conjugate in strawberries seemed likely.

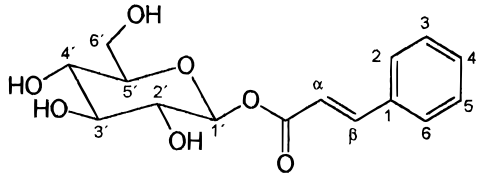

Figure 1. 1-*O*-*trans*-Cinnamoyl-*β*-D-glucopyranose.

EXPERIMENTAL PROCEDURES

Solvents. HPLC grade methanol was obtained from J. T. Baker B. V. (Deventer, Holland); methanol for preparative HPLC (Bayer AG, Leverkusen, Germany) was glass distilled before use. Distilled water was obtained from a Heraeus Destamat system (Heraeus, Hanau, Germany).

Fruits. Fresh strawberries (*Fragaria ananassa* Duch. cv. Kent), from regional cultivation of 2-year-old plants, were collected at eight developmental stages in May/June 1995. Anthesis was defined as the point of time at which 60% of the buds were in full bloom. After removal of the calyx, each fruit sample was sealed in two inserted food grade polyethylene bags and was kept at −70 °C until used for analysis. The freezer did not contain any other materials or chemicals.

Preparation of an Extract of Medium-Polar Compounds. To inhibit enzymatic degradation reactions, whole frozen strawberries (250 g) were blended with 250 mL of a 20:50 (v/v) methanol−0.1 M perchloric acid mixture in a Rotor blender (Rotor AG, Uetendorf, Germany) for 5 min (14000 rpm) at ambient temperature. The homogenate (pH 1.5) was then centrifuged for 45 min (2900*g*, 1 °C). After the supernatant was collected, the residue was extracted again with 100 mL of methanol−perchloric acid mixture and centrifuged as above, and the supernatants were combined.

Lewatit Extraction. Thirty grams of dry Lewatit OC 1064 styrene−divinylbenzene resin (Bayer AG, Leverkusen, Germany) was swollen in 200 mL of methanol (1 h) and slurry-packed into a 3 cm i.d. × 50 cm glass column (Krings and Berger, 1995), which was then rinsed with 5 volumes of 200 mL of distilled water and loaded with the combined supernatants. In a batch process, the resin beads were slurried by means of upstreaming air until decolorization of the turbid supernatant occurred. After the column was drained, it was rinsed three times with 200 mL of distilled water to eliminate sugars, acids, and other water-soluble compounds. Resin-bound medium-polar compounds were then eluted completely

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with 5 volumes of 100 mL of acetone. The resulting deep red extract was concentrated to 50 mL under oil pump vacuum (rotary evaporator, 30 °C maximum bath temperature) and was devoid of acetone.

Preparative High-Performance Liquid Chromatography (HPLC). The Lewatit extract equivalent to 1 kg of fully ripe strawberries was further concentrated as above at 30 °C to a final volume of 10 mL.

Reversed Phase Fractionation. Preparative chromatography was carried out using a Pye Unicam system (Pye Unicam Ltd., Cambridge, England) consisting of an LC-XP gradient programmer, an LC XPD pump (0.28 mL preparative pump heads), an LC-UV detector (preparative flow cell), a Foxy fraction collector (Isco Inc., Lincoln, NE), and a Model 500 chart recorder (Linear Instruments Corp., Irvine, CA). Manual injection (RH 7125 injection valve, Rheodyne, Berkeley, CA) was carried out using a 2 mL loop onto a reversed phase ODS-Hypersil, 5 μm, 16 mm i.d. × 250 mm column (Knauer, Berlin, Germany). The mobile phase consisted of (A) distilled water and (B) distilled water−methanol 20:80 (v/v). The elution program was 100% solvent A isocratic for 5 min, followed by a 0−100% linear gradient with solvent B for 94 min at room temperature. The chromatogram was monitored at 287 nm with a flow rate of 10.0 mL/min; fractions were cut in 2 min intervals. Reversed phase fractions R1−38 were then monitored by HPLC with photodiode array detection (see Quantitative HPLC). Fractions R24 and R25 (retention time = 46−50 min) contained a compound with a UV spectrum similar to that of cinnamic acid, but with a bathochrome shift of 10 nm.

Hydrophilic Phase Purification. Fractions obtained during five repeated runs were pooled and concentrated under oil pump vacuum at 30 °C to a final volume of 4 mL. For further separation, a hydrophilic Hibar LiChrosorb Diol, 7 μm, 25 mm i.d. × 250 mm column (Merck, Darmstadt, Germany) was employed. Isocratic elution was performed with distilled water, followed by a final methanol rinse. Preparative equipment and other conditions were the same as described for the ODS separations. The fractions eluting after 22 and 24 min (fractions H12 and H13) were pooled and concentrated under vacuum, and the remaining residue was freeze-dried.

Finally, 13 mg of a white amorphous substance (purity monitored at 287 nm was > 95%) was dissolved in 500 μL of D_2O for NMR spectroscopy.

Quantitative HPLC. The analytical equipment consisted of a Gynkotek 250 B gradient former and a Series 300 high-precision pump (Gynkotek, Germering, Germany) and a Shimadzu SPD-6AV UV−vis detector and C-R3A integrator (Shimadzu Co., Kyoto, Japan). Monitoring of preparative HPLC fractions and confirmation of peak purity were assessed using a Shimadzu SPD-M6A UV−vis photodiode array detector with computerized data handling. Manual injection (Rheodyne RH 7125 injection valve) was carried out with a 20 μL loop onto a reversed phase Nucleosil 120-5-C_{18}, 5 μm, 4 mm i.d. × 250 mm column (Macherey-Nagel, Düren, Germany), fitted with a 4 mm i.d. × 11 mm guard column of the same material. The mobile phase consisted of (A) 0.2 M disodium phosphate/citric acid (pH 4) buffer−methanol 80:20 (v/v) and (B) methanol, with the following linear gradient: 100% solvent A was increased to 100% solvent B within 35 min at a flow rate of 1.0 mL/min at room temperature. UV detection was performed at 287 nm.

Polyamide Extraction. For further purification prior to quantitative HPLC a Chromabond PA column (Macherey-Nagel) was conditioned with 5 bed volumes of methanol and subsequently rinsed with the same amount of distilled water. Ten milliliters of the acetone-free medium-polar extract was poured through the prewashed column at a low flow rate. Subsequently, 15 mL of distilled water was passed through the column and pooled with the collected eluate to yield a final volume of 25 mL.

Quantification. Dilutions of cinnamic acid (Roth, Karlsruhe, Germany) in methanol were used to fit an external standard curve at the 277 nm maximum (area vs concentration in micromoles per liter, linear regression). The absorbance of 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose was measured at its 287 nm UV maximum (10 nm bathochrome shift), and calculation was based on the cinnamic acid standard curve. For confirmation, alkaline hydrolysis of the cinnamoylglucose ester was carried out. Cinnamic acid-based analysis and calculation demonstrated that results corresponded exactly.

Enzymatic and Alkaline-Mediated Hydrolysis. For enzymatic hydrolysis, 1 mg of the isolated and freeze-dried compound was dissolved in 50 mL of 0.1 M disodium phosphate/citric acid (pH 5) buffer. In 1 mL of the same buffer, 0.85 mg (30.6 units) of lyophilized almond β-glucosidase (EC 3.2.1.21, chromatographically purified, essentially salt-free, Sigma Chemical Co., St. Louis, MO) was dissolved. For the assay, 1 mL of sample solution was added and the mixture incubated for 15 min at 30 °C. The blank (1 mL) was treated in the same way with 1 mL of buffer (pH 5) instead of enzyme solution. For alkaline hydrolysis, samples were adjusted to pH 12 with 2.0 M sodium hydroxide solution at ambient temperature.

NMR Spectroscopy. NMR spectra were recorded in deuterium oxide (D_2O, 99.96 atom % D, MSD Isotopes, Montreal, Canada) at 300 MHz on a Bruker AN 300 spectrometer. Instead of using an internal shift reference, ^{1}H-NMR scale calibration was referenced to the HO signal of remaining H_2O or HDO; ^{13}C scale normalization was performed by zero adjustment to an external TMS signal. Proton decoupling of ^{13}C-NMR spectra was performed by DEPT procedure (strawberry compound) and APT technique (authentic reference compound). Two-dimensional heteronuclear ^{1}H−^{13}C coupling and homonuclear ^{1}H−^{1}H COSY were applied.

Absorbance Measurement. For assessment of anthocyanin accumulation, the acetone-free Lewatit extract was filtered and then diluted with distilled water to fit the measuring range of a Pye Unicam SP8-500 spectrophotometer. After adjustment to pH 3, absorbance was recorded at 510 nm. Results were expressed as absorbance per 1 L of aqueous solution obtained from 1 kg of strawberries.

Reference Compound. 1-*O*-*trans*-Cinnamoyl-β-D-glucopyranose was synthesized by the reaction of *trans*-cinnamoyl chloride with 8-hydroxyquinoline, followed by transesterification with excess β-D-(+)-glucose (Plusquellec et al., 1986). To promote formation of the anomeric hydroxyl-bond product, transesterification was carried out at 0 °C, leaving the reaction mixture for 4 days in the cold. Purification of the crude product was performed with preparative reversed phase HPLC under the conditions cited above.

RESULTS AND DISCUSSION

^{13}C/DEPT spectra of the isolated strawberry compound revealed the presence of 15 carbon atoms with 14 directly attached protons (1 × CH_2, 2 × C, 12 × CH). Analysis of chemical shift values (Table 1) suggested the presence of a glucopyranosyl moiety in the upfield range (six resonances) and a downfield cinnamoyl group consisting of nine carbon atoms. The ^{1}H-NMR spectrum displayed a similar distribution of nuclei. Five aromatic protons indicated that the cinnamoyl moiety was unsubstituted. The presence of *trans*-olefinic protons was confirmed by proton resonance at δ 6.59 and 7.49 (each 1H, d), coupled with a characteristic constant of J = 16.0 Hz. In the heteronuclear ^{1}H−^{13}C spectrum, a strong cross peak was observed between the proton resonance at δ 5.69 (1H, d) and the 1′-carbon signal (δ 96.91) of the glucopyranose moiety. This peak was assigned to the anomeric proton. The coupling constant (J = 7.3 Hz) was characteristic for the vicinal protons $H_{1'}$ and $H_{2'}$ in *trans*-position in the glucopyranose cycle. Thus, the anomeric configuration was concluded to be β, and the strong low-field shift of the anomeric proton (δ 5.69) indicated a β-glucosidic linkage. Due to strong overlap, neither ^{1}H−^{13}C coupling nor ^{1}H−^{1}H COSY allowed detailed assignment of the remaining glucopyranose protons (δ 3.38−3.89, m, $H_{2'-6'}$). Definitive evidence of the proposed structure followed from ^{1}H-NMR and ^{13}C/APT (*a*ttached *p*roton *t*esting) spectroscopy of synthe-

Table 1. ^{1}H and ^{13}C NMR Assignments for 1-*O*-*trans*-Cinnamoyl-β-D-Glucopyranose, Isolated from Strawberries

assignment	δ^1H, *mult.*	signal, *J* (Hz)	δ^{13}C	DEPT signal[a]
	Cinnamoyl Moiety			
−COO−			170.08	C
α	6.59, *d*	1H (16.0)	118.72	CH
β	7.86, *d*	1H (16.0)	150.72	CH
1			136.43	C
2, 6	7.66, *m*	2H	131.30	CH (2C)
3, 4, 5	7.49, *m*	3H	131.84 and 133.99	CH (2C) CH (C_4)
	Glucose Moiety			
1′	5.69, *d*	1H (7.3)	96.91	CH
2′	3.38−3.98, *m*[b]	1H	74.76	CH
3′	3.38−3.98, *m*[b]	1H	78.18	CH
4′	3.38−3.98, *m*[b]	1H	71.89	CH
5′	3.38−3.98, *m*[b]	1H	79.52	CH
6′	3.38−3.98, *m*[b]	2H	63.12	CH_2

[a] The DEPT technique (*d*istortionless *e*nhancement by *p*olarization *t*ransfer) reveals the number of protons attached to the respective carbon atom. [b] Signals of $H_{2'}$ to $H_{6'}$ overlapped to form a single complex multiplet, comprising six H atoms.

sized 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose. Scale expansion of the glucopyranose multiplet demonstrated that ^{1}H-NMR spectra were fully superimposable.

^{13}C-NMR shift values were corresponding. Regardless of slight differences in detailed chemical shift values, NMR data for the strawberry compound were identical with published data for 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose (Plusquellec et al., 1986; Mizuno et al., 1989).

Further proof of the β-glucosidic linkage was given by selective cleavage. As expected, after employment of highly purified almond β-glucosidase, cinnamic acid (retention time under conditions = 18.17 min, λ_{max} = 277 nm) was detected by analytical HPLC instead of the 1-*O*-glucopyranose ester (retention time = 14.39 min, λ_{max} = 287 nm). The same result was obtained by alkaline hydrolysis. Cochromatography of the isolated compound and the synthesized ester showed no differences in retention time, and UV spectra were precisely superimposable.

Time course analysis of 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose accumulation during fruit ripening was performed by analytical HPLC, preceded by final purification of the respective Lewatit extracts on polyamide. The cinnamoyl ester was not retarded on polyamide, whereas flavonoids interfering during chromatography were adsorbed. The recovery of authentic 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose during the two-stage Lewatit and polyamide extraction procedure was >98%.

Fruits of Kent strawberries grew slowly for 17 days and then rapidly gained weight, reaching 14.8 g (average of n = 50) by 32 days after anthesis (DAA). Green fruits were pink at 24 DAA and were red-ripe at 32 DAA. In addition, anthocyanin content was assessed by measuring absorbance of the acetone-free Lewatit extracts at 510 nm (Figure 2). Due to interference from chlorophyll (Cheng and Breen, 1991), low absorbance was recorded in premature fruit. As expected, anthocyanins began to accumulate at the pink stage (24 DAA).

Accumulation of 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose succeeded fruit growth and seemed to precede anthocyanin formation (Figure 3). On a weight-dependent basis, its content seemed to decrease in ripe fruit. However, as single fruits still gained weight, apparently there was a continuous increase up to 1 mg per fruit

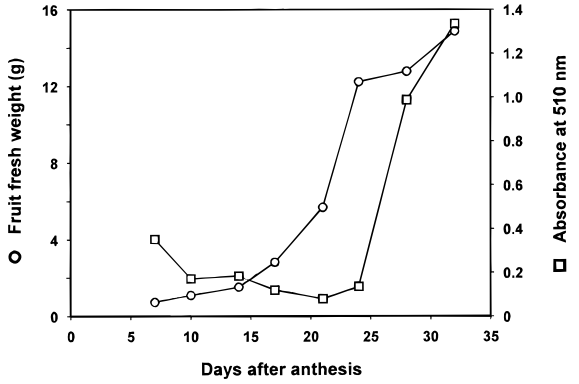

Figure 2. Average fruit fresh weight (n = 50) as a function of time after anthesis (circles) and absorbance at 510 nm per 1 L of aqueous solution (pH 3) obtained from 1 kg of strawberries (squares).

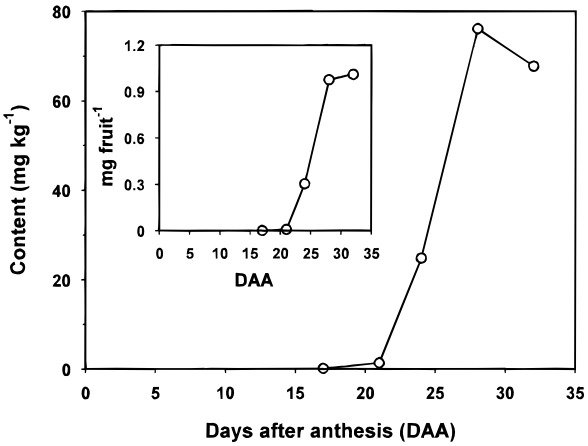

Figure 3. Content of 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose in strawberry fruit (mg kg^{-1}) as a function of time (days after anthesis). (Insert) Content of 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose (mg) in a *single* strawberry fruit as a function of time.

(67.6 mg kg^{-1}) as seen in Figure 3, though minor deviations due to experimental variation cannot be ruled out completely.

From an overall point of view, a relationship to phenylalanine ammonia-lyase (PAL) activity throughout the development of the fruit seems likely. Cheng and Breen (1991) monitored PAL activity in strawberry fruit cv. Tillikum from anthesis to ripeness and demonstrated maximum activity at 27 DAA. PAL mediated conversion of L-phenylalanine to *trans*-cinnamic acid is the initial step toward numerous phenylpropanoid structures. 1-*O*-*trans*-Cinnamoyl-β-D-glucopyranose may be the transport metabolite and physiological precursor of the subsequently formed hydroxycinnamoyl esters. Schuster and Herrmann (1985) detected 1-*O*-*p*-coumaroyl-, 1-*O*-caffeoyl-, and 1-*O*-sinapoyl-β-D-glucopyranose in several strawberry cultivars. Therefore, 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose could be the missing link between PAL-mediated cinnamic acid generation and 1-*O*-*p*-coumaroyl-β-D-glucopyranose, the first hydroxylated derivative.

It remains to be determined why strawberry fruit accumulate these considerable amounts of 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose (about 0.1% of total dry weight). Volatile cinnamoyl esters, mainly the methyl and ethyl derivatives, were found in comparably low concentrations (95.2 and 12.6 μg kg^{-1}, respectively, at 32 DAA; unpublished results), and no spontaneous formation of methyl cinnamate could be observed in a

0.1% methanolic solution of 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose (1 mg L^{-1}, buffered to pH 4) when heated to 60 °C.

As a result, enzymatic action appears to be needed for transesterification of the glucopyranose moiety with endogenous alkanols. In quantitative terms, volatilization of cinnamic acid is a pathway of very minor importance in strawberry fruit.

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