Evidence for Covalently Attached p-Coumaric Acid and Ferulic Acid in Cutins and Suberins

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ABSTRACT

p-Coumaric acid (4-hydroxycinnamic acid) and ferulic acid (4-hydroxy-3-methoxycinnamic acid) have been identified as constituents of cutin. Their reduction products were isolated from a phenolic fraction released from the cutin of the fruits of apple, peach, pear, and two varieties of tomato and apple leaf by treatment with LiAlH₄ or LiAlD₄. They were identified by combined gas chromatography and mass spectrometry. p-Coumaric acid was present in all samples of cutin (0.07-0.33% by weight), whereas only peach and pear cutin contained measurable amounts of ferulic acid (0.007% and 0.035%, respectively). Both p-coumaric acid and ferulic acid were identified as constituents of the insoluble material recovered after partial hydrolysis (12-42% loss) of cutin in 1 M NaOH at 80 C. A significant part (48%) of the p-coumaric acid contained in tomato cutin was contained in the insoluble material recovered after partial degradation (7.4%) of this cutin with 0.01 M NaOH. These data indicate that these phenolic components are tightly (possibly covalently) bound to cutin. Similar analysis of the phenolic fractions from the suberin of potato, sweet potato, turnip, rutabaga, carrot, and red beet revealed that they contained only ferulic acid (0.05-0.22%). Ferulic acid was identified as a constituent of the insoluble material recovered after partial hydrolysis of potato and beet suberins (34% and 32% loss, respectively) in 1 M NaOH at 80 C. A major part (65%) of the ferulic acid contained in potato suberin was contained in the insoluble material recovered after partial (26.8% loss) degradation of this suberin with 0.01 M NaOH. Ferulic acid appears to be tightly (probably covalently) bound to suberin.

Cutin, the structural component of plant cuticle, is a polymer of hydroxy fatty acids (8). The common major components of this polymer are palmitic acid, 16-hydroxypalmitic acid, 10,16-dihydroxypalmitic acid, or its positional isomers, oleic acid, 18-hydroxyoleic acid, 18-hydroxy-9,10-epoxystearic acid, and 9,10,18-trihydroxyoleic acid. The composition of the aliphatic components of suberin from the underground parts of plants is somewhat similar to that of cutin; however, there are significant quantitative differences between the composition of these two types of protective polymers (5-8). One major difference appears to be that suberin preparations contain quite large proportions of phenolic materials, while cutin preparations contain very little phenolic compounds, although the phenolics which might be present in cutin preparations have not been examined. In this paper we report experimental evidence which strongly suggests that p-coumaric acid and or ferulic acid are covalently attached to cutin and suberin.

MATERIALS AND METHODS

Isolation of Phenolic Compounds from Cutin and Suberin after LiAlH₄ and LiAlD₄ Degradation. Powdered cutin from apple leaf and fruits of apple, tomato, peach, and pear were obtained as previously described (11). A 500 mg sample of each cutin was degraded in refluxing tetrahydrofuran (25 ml) with an excess of LiAlH₄ for a period of 24 to 48 hr. The excess LiAlH₄ was decomposed by the slow addition of the reaction mixture to 50 ml of H₂O and the mixture was acidified with concentrated HCl. The aqueous layers were extracted with ethyl ether (2 x 100 ml) and the combined ether layers were extracted with 5% aqueous NaOH (2 x 50 ml). The basic layers were combined, acidified with concentrated HCl, and extracted with ether (2 x 100 ml). The phenolic compounds from the ether extract were purified by TLC (systems A and B) and identified as their di-TMS derivative, or diacetate derivatives by combined GC-MS. The same procedure was used for the identification of LiAlD₄ reduction products. A 250-mg sample of carrot suberin and 500-mg samples of the suberin from potato, sweet potato, turnip, red beet, and rutabaga, isolated as previously described (7), were degraded with LiAlH₄ and the phenolic compounds from these samples were isolated by the methods described above for cutin samples. The diacetates were purified by TLC (system C) and identified by combined GC-MS. A 500-mg sample of potato suberin was similarly analyzed after reduction with LiAlD₄.

Partial Degradation of Cutin. Either 25 ml of hot (80 C) aqueous 1 M NaOH or 50 ml of 0.01 M NaOH at room temperature were added to 500-mg samples of cutin or suberin, and the mixtures were stirred at this temperature for various periods of time. Following this treatment, the residue was recovered by filtration and thoroughly washed with H₂O followed by CHCl₃/CH₃OH (2:1, v/v). The residue was reduced with LiAlH₄, and the isolated phenolic compounds were converted directly to their acetate derivatives for quantitative analysis by GLC-MS as described above.

Preparation of Derivatives. TMS ether derivatives of dihydro-p-coumaryl alcohol and dihydroconiferyl alcohol were made by treating the alcohols with an excess of N,O-bis(trimethylsilyl)-

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2 Abbreviation: di-TMS: di-trimethylsilyl.
acetamide at 90 C for 15 min. The excess N,O-bis(trimethylsilyl)-acetamide was removed under a stream of nitrogen and samples of the silylated alcohols in chloroform were analyzed by GLC-MS. Diacetates were made by treating the above alcohols overnight at room temperature with a 2:1 (v :v) mixture of acetic anhydride and pyridine. The crude diacetates were purified by TLC (system C) and analyzed by GLC-MS.

**Thin Layer Chromatography.** Samples spotted on 1 mm thick Silica Gel G plates (20 × 20 cm) were routinely developed for a distance of 15 cm. The phenolic compounds were detected by spraying the plates with a 10% aqueous NaOH solution of the diazonium salt of sulfanilic acid (9). The diacetates of dihydro-p-coumaryl alcohol and dihydroconiferyl alcohol were visualized under UV light after spraying with a 0.1% solution of 2',7'-dichlorofluorescein. The following solvent systems were used: system A, benzene-methanol-glacial acetic acid (90:16:8, v :v :v); system B, hexane-ethyl ether-methanol (50:40:20, v :v :v); and system C, hexane-ethyl ether (70:30, v :v).

**Synthesis of Dihydro-p-Coumaryl Alcohol and Dihydroconiferyl Alcohol.** Samples of p-coumaric acid and ferulic acid (Aldrich Chemical Co.) were reduced in refluxing tetrahydrofuran containing an excess of LiAlH4 or LiAlD4. The products were poured into H2O, acidified (concentrated HCl), and the aqueous layers were extracted with chloroform. The crude alcohols from the chloroform extract were purified by TLC (system B).

**Quantification of Bound Phenolic Compounds in Cutin and Suberin.** The phenolic compounds isolated from the hydrolygenolysis of cutin and suberin were immediately converted to their diacetate derivatives to prevent oxidative degradation of the phenols. The diacetates were purified by TLC (system C) and subjected to GLC-MS analysis as previously described. 2-Phenoxyethylacetate was used as a standard for the quantitative GLC analysis.

**RESULTS AND DISCUSSION**

Cutin from the fruits of apple, peach, pear, and tomato (scout and starfire varieties), and apple leaf were degraded with LiAlH4 and LiAIH4, and p-coumaric acid and ferulic acid were identified as di-TMS and diacetate derivatives of their reduction products, dihydro-p-coumaryl alcohol and dihydroconiferyl alcohol (Fig. 1). The same procedure was used to identify ferulic acid in potato, turnip, carrot, sweet potato, rutabaga, and red beet suberins. TLC of the crude phenolic fractions isolated from the hydroxyalcoholysis products of cutins and suberins gave spots whose RF values were identical with synthetic samples of dihydro-p-coumaryl alcohol and dihydroconiferyl alcohol. Dihydro-p-coumaryl alcohol was isolated from the crude phenolic fractions by TLC (system A, RF = 0.38; system B, RF = 0.31) and further purified by TLC as its diacetate derivative (system C, RF = 0.15). Dihydroconiferyl alcohol was isolated and purified in a similar manner (system A, RF = 0.34; system B, RF = 0.1; diacetate, system C, RF = 0.1). GLC-MS analysis of purified dihydro-p-coumaryl alcohol and dihydroconiferyl alcohol as di-TMS and diacetate derivatives gave peaks whose retention times were identical to those of synthetic standards (dihydro-p-coumaryl alcohol:di-TMS, 5.2 min, 145 C; diacetate, 3.3 min, 165 C; dihydroconiferyl alcohol:di-TMS, 5.8 min, 145 C; diacetate, 6.8 min, 165 C). A typical ex-
ample is represented by the gas chromatogram of the acetylated phenolic fraction obtained from peach cutin (Fig. 2). The mass spectrum of component 2 (Fig. 3) was identical to that of authen-
tic dihydro-p-coumaryl alcohol diacetate and the mass spectrum of component 3 (Fig. 4) was identical to that of authentic dihy-
droconiferyl alcohol diacetate. The mass spectra of the di-TMS derivatization of component 2 and component 3 were identical to those of authentic TMS derivatives of dihydro-p-coumaryl alcohol and dihydroconiferyl alcohol respectively (data not shown). Dihydro-p-coumaryl alcohol and dihydroconiferyl alcohol were the only phenolic components that were detectable on the thin layer chromatograms of any of the crude phenolic fractions of cutins or suberins examined. GLC-MS analysis of silylated and acetylated TLC fractions, taken from positions above and below these components, revealed no other detectable phenolic com-
pounds.

If dihydro-p-coumaryl alcohol and dihydroconiferyl alcohol obtained by the hydrogenolysis technique were in fact derived from p-coumaric acid and ferulic acid in cutin and suberin, then 3 deuterium atoms should be incorporated into these compounds when cutin or suberin is subjected to degradation with LiAlD₄. LiAlH₄ reduction of apple cutin produced dihydro-p-coumaryl alcohol containing 3 deuterium atoms. Mass spectral analysis revealed that 2 deuterium atoms were on C-1, which originally bore the carboxyl group and the other deuterium atom was equally distributed between the carbon atoms which originally contained the conjugated double bond. This can best be seen in the shift of critical ions in the mass spectra. For example, the molecular ion of the diacetate derivative of dihydro-p-coumaryl alcohol (Fig. 3) shifted from m/e 236 in the undeuterated com-
pound to m/e 239 in the deuterolysis product. The base peak at m/e 134 shifted to 137 and a peak at 107 shifted to 108. Ion frag-
ments of derivatives are listed in Scheme I. Identical shifts were observed between these ions in the mass spectra obtained from the reduction of synthetic p-coumaric acid with LiAlH₄ and LiAlD₄. LiAlD₄ reduction of cutins and suberins produced dihydroconiferyl alcohol containing 3 deuterium atoms which were similarly distributed. Major ion fragments for the derivat-
ives of dihydro-p-coumaryl alcohol and dihydroconiferyl alcohol are listed in Scheme I.

The identification of the structures of dihydro-p-coumaryl alcohol and dihydroconiferyl alcohol obtained from the LiAlH₄ and LiAlD₄ reduction of cutins and suberins indicates that they were in fact present as p-coumaric acid and ferulic acid in cutin and suberin.

Quantitative analysis by combined GLC-MS showed that all of the cutin samples examined contained p-coumaric acid. Both varieties of tomatoes contained the most p-coumaric acid (<0.5%) and peach contained the least (~0.07%). Only peach and pear cutins contained detectable amounts of ferulic acid. Pear contained the most ferulic acid (~0.03%) and peach the least (<0.01%) (Table I).

Suberin samples isolated from potato, sweet potato, turnip, rutabaga, carrot, and red beet were also subjected to analysis for phenolic components in a manner similar to that used for cutin. In all of these cases, dihydroconiferyl alcohol was the only phenol

![Figure 2](image_url)

**Figure 2.** Gas chromatogram of the acetylated phenolic fraction of peach cutin.

![Figure 3](image_url)

**Figure 3.** Mass spectrum of component 2 shown in Fig. 2.
PHENOLIC ACIDS IN CUTINS AND SUBERINS

which could be detected by the present methods, strongly suggesting that all of these suberin samples contained ferulic acid. Quantitative analysis showed that sweet potato suberin contained the highest amount of ferulic acid (Table I).

The phenolic compounds identified could have been either physically adsorbed to cutin and suberin, or they might be covalently attached to these polymers. In an attempt to distinguish between these two possibilities, cutin and suberin samples were first treated with 0.01 M NaOH at room temperature for 4 hr. Under these conditions, varying amounts of degradation of the polymer occurred. The residual polymeric material contained substantial portions of the phenolic materials. For example, even

![Chemical structure](attachment:chemical_structure.png)

**Fig. 4.** Mass spectrum of component 3 shown in Fig. 2.

**Scheme I.** Major ion fragments in the mass spectra of the derivatives of dihydro-p-coumaryl alcohol and dihydroconiferyl alcohol.

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\begin{align*}
\text{Dihydro-p-coumaryl alcohol (di-TMS derivative): } & \quad m/e \ 296(M^0) , \ 281(M^0-CH_3), \\
& \quad 206 [\text{base peak, } M^0-\text{HOSi}(CH_3)_3] , \text{ and } 191 [M^0-CH_3-\text{HOSi}(CH_3)_3]. \\
\text{Dihydro-p-coumaryl alcohol-d_3 (di-TMS derivative): } & \quad m/e \ 299 (M^0), \ 284 (M^0-CH_3), \\
& \quad 209 [\text{base peak, } M^0-\text{HOSi}(CH_3)_3] , \text{ and } 194 [M^0-CH_3-\text{HOSi}(CH_3)_3]. \\
\text{Dihydro-p-coumaryl alcohol (diacetate derivative): } & \quad m/e \ 236 (M^0), \\
& \quad 194 (M^0-\text{ketene}), \ 176 (M^0-\text{ketene-H}_2\text{O}), \ 134 (\text{base peak, } O=\begin{array}{c} \text{CH}_2-\text{CH}=\text{CH}_2 \end{array}), \\
& \quad 107 (\begin{array}{c} \text{CH}_3-\text{C}=\text{O} \end{array}), \ 43 (\text{CH}_3-\text{C}=\text{O}). \\
\text{Dihydro-p-coumaryl alcohol (diacetate derivative-d_3): } & \quad m/e \ 239 (M^0), \\
& \quad 197 (M^0-\text{ketene}), \ 179 (M^0-\text{ketene-H}_2\text{O}), \ 137 (\text{base peak, } O=\begin{array}{c} \text{C}_3\text{H}_2\text{D}_3 \end{array}), \\
& \quad 108 (\begin{array}{c} \text{CH}_3-\text{CD} \end{array}), \ 43 (\text{CH}_3-\text{C}=\text{O}). \\
\text{Dihydroconiferyl alcohol (di-TMS derivative): } & \quad m/e \ 326 (M^0), \ 311 (M^0-CH_3), \\
& \quad 236 [M^0-\text{HOSi}(CH_3)_3], \ 206 [\text{base peak, } M^0-2\text{CH}_3-\text{HOSi}(CH_3)_3]. \\
\text{Dihydroconiferyl alcohol (diacetate derivative): } & \quad m/e \ 266 (M^0), \\
& \quad 224 (M^0-\text{ketene}), \ 164 (\text{base peak, } O=\begin{array}{c} \text{CH}_2-\text{CH}=\text{CH}_2 \end{array}), \\
& \quad 137 (\begin{array}{c} \text{CH}_3-\text{C}=\text{O} \end{array}), \ 43 (\text{CH}_3-\text{C}=\text{O}). \\
\text{Dihydroconiferyl alcohol-d_3 (diacetate derivative): } & \quad m/e \ 269 (M^0), \\
& \quad 227 (M^0-\text{ketene}), \ 167 (\text{base peak, } O=\begin{array}{c} \text{C}_3\text{H}_2\text{D}_3 \end{array}), \ 138 (H-\begin{array}{c} \text{CHD} \end{array}).
\end{align*}
\]
through 18.6% of apple leaf cutin was removed by the mild treatment with alkali, virtually all the ferulic contained in the original material was recovered from the insoluble residue. In this case, there is a possibility that the cutin sample could have contained small amounts of lignin arising from the vascular tissue of the leaf. Similar treatment of starfire tomato fruit cutin preparation, which could not have been contaminated with lignin, resulted in almost 10% loss of the polymer, but at least one-half of the total amount of p-coumaric acid contained in the cutin was still attached to the insoluble material recovered after the alkali treatment. Similarly, treatment of potato suberin with 0.01 M NaOH resulted in solubilization of over one-fourth of the polymer, but about two-thirds of the ferulic acid contained in the suberin preparation remained attached to the insoluble material recovered after this treatment. Treatment of cutin and suberin samples with 1 M KOH at 80°C for 5 to 15 min resulted in 10 to 42% loss in weight. In all cases, the insoluble material recovered even after such treatments contained p-coumaric acid and or ferulic acid. It appears that p-coumaric acid and or ferulic acid are tightly attached to cutin and suberin. It is tentatively concluded that they are covalently attached to these protective polymers.

The purpose of the present investigation was to examine the possibility of covalent attachment of phenolics to cutin and suberin and procedures to maximize the recovery of the phenolic compounds have not been developed. In an attempt to determine the possible loss of the phenolics during the analytical procedure, we found that only about 52% of the added dihydroconiferyl alcohol could be recovered during the procedures used after the hydrogenolysis step. It is clear that the amounts of p-coumaric acid and or ferulic acid present in cutin and suberin are at least twice that indicated in Table I. Phenolics bound to cutin and suberin via ether linkage would obviously not have been detected by the present methods. p-Coumaric acid and ferulic acid are components of lignin (1). In more recent years such phenolic acids have been found to be bound to other polyphenols such as cell walls (3, 4, 12), the aleurome layers (2), and p-hydroxyphenyl naphthics of diesters have been shown to be major components of carnauba wax (10). The present report strongly suggests that such phenolic acids are tightly bound to the protective polymers, cutin and suberin. Probably, such acids are esterified to the hydroxy aliphatic groups of the polymer. Until dimers or oligomers containing the phenolic esters can be isolated, it is difficult to determine the precise nature of the linkage between the aromatic and the aliphatic components of cutin and suberin.

**LITERATURE CITED**


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**Table 1. Concentrations of p-Coumaric Acid and Ferulic Acid in Various Cutins and Suberins**

<table>
<thead>
<tr>
<th>Source</th>
<th>p-Coumaric Acid (mg/g of cutin or suberin)</th>
<th>Ferulic Acid (mg/g of cutin or suberin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple</td>
<td>2.1</td>
<td>ND</td>
</tr>
<tr>
<td>Apple leaf</td>
<td>1.2</td>
<td>ND</td>
</tr>
<tr>
<td>Peach</td>
<td>0.7</td>
<td>0.07</td>
</tr>
<tr>
<td>Pear</td>
<td>1.3</td>
<td>0.34</td>
</tr>
<tr>
<td>Tomato (starfire)</td>
<td>5.1</td>
<td>ND</td>
</tr>
<tr>
<td>Tomato (scout)</td>
<td>5.3</td>
<td>ND</td>
</tr>
<tr>
<td>Suberins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potato</td>
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</tr>
<tr>
<td>Turnip</td>
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</tr>
<tr>
<td>Carrot</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Rutabaga</td>
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<td>1.6</td>
</tr>
<tr>
<td>Red beet</td>
<td>ND</td>
<td>0.5</td>
</tr>
</tbody>
</table>

1 Not detectable under these experimental conditions.