Flavan-3-ol Biosynthesis

THE CONVERSION OF (+)-DIHYDROMYRICETIN TO ITS FLAVAN-3,4-DIOL (LEUCODELPHINIDIN) AND TO (+)-GALLOCATECHIN BY REDUCTASES EXTRACTED FROM TISSUE CULTURES OF GINKGO BILOBA AND PSEUDOTSUGA MENZIESII

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ABSTRACT

Extracts of callus or cell suspension cultures from petioles of Ginkgo biloba catalyzed the production of (+)-gallocatechin (2,3-trans-3,5,7,3',4',5'-hexahydroxy-flavan) from (+)-dihydromyricetin (5'-hydroxy-dihydroquercetin) along with the expected 3,4-cis-diol intermediate, leucodelphinidin, in a NADPH-dependent double-step reductase reaction at pH 7.4. The latter diol, isolated from the above incubation mixture, produced (+)-gallocatechin in a NADPH-dependent reaction. Extracts from tissue cultures derived from needles of Pseudotsuga menziesii (Douglas fir) also produced significant amounts of the 3,4-diol from dihydromyricetin. (+)-Dihydromyricetin, purified via paper chromatography from leaves of Leptarrhena pyrolifolia, was reduced by NaBH₄ to the presumed 3,4-trans-diol and acid epimerized to the 3,4-cis-diol.

Two NADPH reductases, acting in sequence to reduce DHQ² first to its 3,4-cis-diol (leucocyanidin) and then to (+)-catechin (diphenolic B-ring), were previously reported in extracts derived from cell suspension cultures of Douglas fir (10, 11). A similar set of NADPH-dependent reductases converting DHM to (+)-gallocatechin (triphenolic B-ring) via a 3,4-diol (leucodelphinidin) intermediate is now demonstrated in tissue cultures of Ginkgo biloba (Fig. 1). The latter were chosen for such a study as they contain significant amounts of the flavan-3-ol, (+)-gallocatechin, and their proanthocyanidins have a high ratio of prodelphinidins to procyanidins (H. Stafford and H. Lester, unpublished data). Extracts from tissue cultures of Douglas fir, however, also contain DHM reductase activities even though the cultures accumulate nondetectable or only trace amounts of prodelphinidins or (+)-gallocatechin (9; H. Stafford and H. Lester, unpublished data).

MATERIALS AND METHODS

Analysis of products was done by HPLC and by one-dimensio- nal, descending paper chromatography in a butanol-pH 6.8 phosphate buffer solvent; the Prussian Blue Reagent was used as a spray for paper chromatography and in a test-tube assay (11, 12). Unlike recent papers (11, 12), 5% acetic acid (v/v) was substituted for phosphate buffer in the HPLC solvents as it produced sharper peaks without altering Vₑ values; however, ethyl acetate extracts were still forced into pH 6.8 phosphate buffer prior to injection since ethyl acetate interfered with Vₑ values (12).

Standard Reductase Extraction Procedure. Callus and cell suspension cultures of Ginkgo biloba were derived from petioles in a manner similar to that for Douglas fir cultures from needles (8). Extracts were made as in previous studies (11). About 300 mg of acetone powder from frozen cells were homogenized in a pH 8.8 borate buffer plus ascorbate. After centrifugation at 500g, the crude extract was desalted and converted to a pH 7.4 Tris buffer on a small Sephadex G-25 column. Proteins were estimated by the Coomassie Blue method.

Standard Reductase Assay. The two reductases were routinely assayed in a double step starting with DHM or DHQ in a complete medium shown in Table II. After incubation for 3 to 4 h, the mixtures were extracted with ethyl acetate and applied to paper for one-dimensional chromatography (11). The amounts of Prussian Blue positive products were estimated by visual comparison with a concentration series of catechin standards and expressed as catechin equivalents. The absorption coefficients for the various products have not yet been determined due to the lack of purified forms of the diols and gallocatechin.

Substrate and Product Standards. DHQ, (+)-catechin, and (+)-gallocatechin were the same as used before (9–12). We are very grateful for a small amount of DHM (a sample made by Hergert from Pinus contorta (4) from R. G. Rickey, Olympic Research Division, ITT Rayonier Inc., Shelton, WA, and for both DHM and the dimer, gallocatechin4αα8α–8α–catechin, from Dr. Henrik Outtur, Department of Brewing Chemistry, Carlsberg Research Laboratory, Copenhagen, Denmark.

Preparation of Substrate Amounts of DHM. Small amounts of DHM, added to the incubation mixtures as paper-bound sources, were purified from leaves of Leptarrhena pyrolifolia (D. Don) R. Br. (5) from plants supplied by David Palmer, Director of the Berry Botanic Garden, Portland, OR. About 1.5-g leaves were homogenized in 70% methanol (v/v). After evaporation of the methanol under vacuum, the aqueous phase was extracted with ethyl acetate, concentrated, and applied as a narrow strip to Whatman No. 1 sheets for one-dimensional descending paper chromatography in 5% acetic acid (v/v).

The DHM band was eluted in 50% methanol (v/v) and, after evaporation of the methanol, the aqueous phase was extracted with ethyl acetate. The latter was rechromatographed in the butanol-phosphate solvent. The relatively specific Zn-HCl test for 3-hydroxyflavanones (1) was very useful in distinguishing the UV absorbing band of DHM (blue-purple color) from that of possible DHQ (red-purple color); however, Leptarrhena extracts did not contain detectable amounts of the latter aglycone. The DHM band was cut into small squares and analyzed for purity by HPLC and for quantity by the Prussian Blue test-tube assay (12) and expressed as catechin equivalents. This DHM prepara-
This dimer was produced either entirely chemically or partly enzymically. For the chemical preparation, DHM, eluted from paper squares in 50% methanol (v/v) was concentrated to dryness, dissolved in ethanol, combined with catechin, NaBH₄, and H₂O, and the pH adjusted to between 4.5 and 5 for leucodelphinidin-catechin condensation as described by Delcour et al. (3) in a double-step starting with DHQ.

A similar double-step production of the dimer was made from the diol synthesized enzymically with protein extracts from Douglas fir cultures. After a regular 3-h incubation with DHM in a complete medium, 50 µg of (+)-catechin were added and the pH lowered immediately with HCl to about 1 and held at room temperature for 30 min (2), or with acetic acid to pH 5 for 1 h (3). The ethyl acetate extracts of the above incubation mixtures were chromatographed on paper; the major Prussian Blue positive product migrated to the expected dimer area. In a 3x scale experiment with the pH 1 method, the dimer product, collected by HPLC and chromatographed on paper, again migrated to the expected R₁ value.

**RESULTS AND DISCUSSION**

**Identification of Products** (Table I). Activity with DHM was determined in a complete system containing NADPH at pH 7.4, similar to that used for the DHQ reductases (10, 11). In addition to the residual DHM, two distinct Prussian Blue positive spots were observed when ethyl acetate extracts of the incubation mixture were chromatographed on paper. The major spot at the lower R₁ value was considered to be the 3,4-cis-diol (leucodelphinidin), in analogy to the enzymic product from DHQ; the upper, minor spot migrated to an area similar to that of a gallocatechin standard.

HPLC analyses of comparable ethyl acetate extracts showed a relatively complex set of UV absorbing peaks at 280 nm. Two of these, however, had Vₚ values of the expected 3,4-cis-diol and gallocatechin (Table I). The suspected diol peak was collected, extracted into ethyl acetate, and evaporated to dryness; when heated at 95°C in butanol-HCl reagent, the blue-purple color characteristic of delphinidins was observed (9). When a known amount of gallocatechin was chromotographed with an aliquot of the ethyl acetate extract of an enzymic mixture, a symmetrical, additive peak was obtained when compared to the peak from an aliquot with no added gallocatechin.

**DHM Reductase Activity in Ginkgo Extracts** (Table II, Fig. 1). The production of a presumed 3,4-cis-diol and its reduction product, gallocatechin, was completely dependent on the presence of NADPH and did not occur with boiled enzymes. The amount of both products formed was linear with enzyme concentration and was similar at the range between 25 to 100 µg DHM (calculated as catechin equivalents). More of the diol intermediate accumulated than the final flavan-3-ol; this was also the case with DHQ as substrate with enzyme extracts from both Ginkgo and Douglas fir.

**Comparison with Activities in Extracts from Douglas Fir Cell Suspension Cultures.** When DHM was added to a complete incubation mixture with Douglas fir as the enzyme source, significant amounts of the above 3,4-cis-diol (leucodelphinidin) were detected on paper chromatograms, but only trace amounts of gallocatechin were found; the amounts of diol formed were only about one-fourth to one-half of the diol (leucocyanidin) formed from DHQ.

Intact Douglas fir needles contained gallocatechin and about a 1:1 mixture of prodelphinidins to procyanidins (8), whereas the tissue cultures derived from young needles contained only trace or nondetectable amounts of either of the triphenolic types. Therefore, it was not expected that extracts of Douglas fir cell suspension cultures would possess significant reductase activity capable of converting DHM to its diol and some traces of

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**Fig. 1.** Conversion of (+)-DHM to (+)-gallocatechin (triphenolic B-ring) via a 3,4-diol (leucodelphinidin) intermediate by NADPH-dependent reductases.

**Preparation of the Dimer, Gallocatechin-(4α→8)-catechin.**
(+-)GALLOificateCN BIOSYNTHESIS

Table I. Chromatographic Characteristics of 3-Hydroxyflavanols, Flavan-3,4-diols, and Flavan-3-ol Monomers and Dimers

<table>
<thead>
<tr>
<th>Diphenolic B-ring</th>
<th>Triphenolic B-ring</th>
<th>Dimers</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHQ</td>
<td>3,4-Diols</td>
<td>DHM</td>
</tr>
<tr>
<td>HPLC solvents</td>
<td>cat</td>
<td>trans</td>
</tr>
<tr>
<td>0:100</td>
<td>27.0</td>
<td>11.0</td>
</tr>
<tr>
<td>5:95</td>
<td>20.4</td>
<td>17.3</td>
</tr>
<tr>
<td>10:90</td>
<td>15.0</td>
<td>12.8</td>
</tr>
<tr>
<td>20:80</td>
<td>32.0</td>
<td>8.3</td>
</tr>
</tbody>
</table>

Paper chromatography BP solvent: 0.86 0.72 0.65 0.55 0.75 0.48 0.42 0.26 0.44 0.15

*cat, catechin; gc, gallocatechin; BP, 1-butanol saturated with 0.01 m (pH 6.8) phosphate buffer.  
b Pump speed at 2 ml/min.  
c Methanol:5% acetic acid (v/v).  
d One dimensional descending.  
e RF value.

Table II. Reductase Activities in a Two-Step Reaction Starting with DHM or DHQ in Extracts from Callus Cultures of Ginkgo or Cell Suspension Cultures of Douglas Fir (f = 2)

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Products</th>
<th>3,4-cis-diol Galloactive Catechin</th>
<th>μg/incipulation mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginkgo DHM</td>
<td></td>
<td>Completeb</td>
<td>10</td>
</tr>
<tr>
<td>Complete</td>
<td>Incompletec</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DHQ Complete</td>
<td></td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Douglas fir DHM</td>
<td></td>
<td>Complete</td>
<td>9</td>
</tr>
<tr>
<td>DHQ Complete</td>
<td></td>
<td>18</td>
<td>0</td>
</tr>
</tbody>
</table>

*The diol is leucodelphinidin with DHM and leucocyanidin with DHQ.  
b Complete medium contained in 1 ml (pH 7.4) Tris buffer, 50 μl DHM or DHQ, 1 μmole NADPH plus a NADPH regenerating system (10), and 300 to 400 μg protein derived from acetone powders.  
c Incomplete media: (a) minus NADPH; (b) boiled enzyme; or (c) enzyme only.

gallocatechin. The presence of DHM reductase activity in the Douglas fir cultures raises the possibility that the same reductases can act with either DHQ or DHM. Purification studies will be necessary to demonstrate whether this is true or not. The limiting factor in the Douglas fir tissue cultures could be at the level of DHM synthesis.

Comparison with the Nonenzymic Reduction of DHM by NaBH4. When an ethyl acetate extract of a NaBH4 reduction mixture was analyzed by paper chromatography, the major product was observed at an Rf value below that of DHM, at a distance comparable to that between DHQ and its 3,4-trans-diol. When examined with HPLC, two major peaks were observed, the larger one was DHM and the smaller was suspected to be its 3,4-trans-diol. Collection, concentration, and recycling of this latter peak in 5% acetic acid (v/v) produced a peak second considered to be the acid epimerization product, 3,4-cis-diol (11, 12).

This same peak was obtained when the ethyl acetate extract of an acid epimerization mixture (11, 12) of NaBH4 reduction products was analyzed by HPLC. After paper chromatography, a lower Rf product was observed, again at a distance comparable to that obtained with DHQ and its 3,4-cis-diol. This acid epimerization product had identical HPLC and paper chromatographic properties as the product in the enzymic incubation mixtures.

The major NaBH4 reduction product of DHM, therefore, is considered to be the 3,4-trans-isomer. Dr. Henrik Outrup of the Carlsberg Laboratory, Copenhagen, has confirmed this with NMR (6). Since only two isomers are expected, we assume that the acid epimerization product of the 3,4-trans-diol must be the 3,4-cis-isomer.

Diol Reductase Activity in Ginkgo Extracts. Direct evidence that the diol product produced enzymically during the reduction of DHM is the precursor of the gallocatechin formed was obtained by first isolating by paper chromatography the diol produced in the first step, and then feeding it as a paper-bound substrate to Ginkgo enzymes in a second, complete incubation mixture. Identification by paper chromatography showed that about 5 μg of gallocatechin were formed from about 20 μg of diol in 3 h.

Condensation of the Diol with (+)-Catechin to Form a Dimer. The presence of a 3,4-diol product in the enzymic incubation mixture with DHM was determined indirectly by a nonenzymic condensation of the diol (or its carbocation or quinone methide intermediate) with added (+)-catechin to form the all-trans dimer of gallocatechin (4α–8)-catechin. The production of dimer was much greater at pH about 1 with HCl (10) than at pH 5 with acetic acid; this was not expected according to recent work with DHQ (3). Chromatographic analyses indicated that the dimer was identical to that synthesized nonenzymically and to the dimer standard obtained from H. Outrup. While such a condensation does not indicate which 3,4-isomer was present, since both give rise to the same condensation product, it provides indirect evidence of the presence of a leucodelphinidin intermediate capable, upon conversion to a carbocation or quinone methide, of adding to an electrophilic group such as catechin (12).

CONCLUSIONS

Extracts from tissue cultures of G. biloba and Pseudotsuga menziesii (Douglas fir) contained two NADPH-dependent reductases capable of reducing DHM to its 3,4-diol and then to its flavan-3-ol, (+)-gallocatechin, in a manner similar to that obtained with DHQ (11, 12). With both dihydroflavonols, the stereochemistry of the major 3,4-diol reduction product differed depending on whether the initial reduction was enzymic or chemical with NaBH4; i.e. the chemical reduction product was the 3,4-trans-isomer (6, 7), while the enzymic product was the 3,4-cis-diol. The acid epimerization product of the chemically produced 3,4-trans-diol was identical to the enzymic product.
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