ENZYMIC SYNTHESIS OF LOGANIN BY CARBOXYL GROUP
METHYLATION OF LOGANIC ACID*

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1. Introduction

We have found that mevalonic acid-2-14C is incorporated into the aglucone moiety of the iridoid glucoside, loganic acid (fig. 1), in both flowering Vinca rosea and germinating seedlings [1]. Loganin, its methyl ester, serves as a precursor of the nontryptophan moiety of three different classes of indole alkaloids in the same plant [2, 3]. However, the occurrence of 7-deoxyloganin in V. rosea has also been reported and this glucoside was found to be hydroxylated to loganin in vivo. Since tracer studies in a related species, Menyanthes trifoliata, have revealed that the O-methyl group of loganin is derived from the methyl of methionine [4], it was of interest to determine which monoterpene, loganic acid or 7-deoxyloganic acid, is methylated prefentially in V. rosea.

* Part IV in a series on "Monoterpene Biosynthesis"; Part III: ref. [1].

2. Materials and methods

Tender leaves and shoots from 4–8 month old V. rosea plants were used for the preparation of cell-free extracts. Loganic acid was isolated from Swertia curoliniensis and purified as previously described [6].

Plant material was ground in liquid nitrogen and extracted with 3 volumes of 0.2 M phosphate buffer, pH 7.2 containing 0.01 M sodium metabisulfite and 1 mM dithiothreitol (DTT). To this Polyclar AT (tissue: Polyclar AT, 4: 1 w/w) was added and the extract squeezed through two layers of silk [7]. After centrifugation of the homogenate at 10,000 g for 20 min, the supernatant was fractionated on a column of Bio-gel P, eluting with 0.1 M phosphate buffer, pH 7.2 containing 0.01 M sodium metabisulfite and 1 mM dithiothreitol (DTT). To this Polyclar AT (tissue: Polyclar AT, 4: 1 w/w) was added and the extract squeezed through two layers of silk [7]. After centrifugation of the homogenate at 10,000 g for 20 min, the supernatant was fractionated on a column of Bio-gel P, eluting with 0.1 M phosphate buffer, pH 7.2, containing 1 mM DTT. To the first eluant after the void volume, 3 volumes of acetone were added at -15°C. The precipitate thus obtained was freed from acetone and dissolved in 0.1 M phosphate buffer, pH 7.2, containing 1 mM DTT. Denatured protein was removed by centrifugation and the clear supernatant was used for the enzymatic assays. Protein concentration was measured by the Lowry method [8].

Assays were conducted as mentioned in table 1 and the reaction was terminated by addition of 1 ml of methanol. Carrier loganin (0.5 mg) was added and the mixture centrifuged. The aqueous supernatant was subjected to preparative thin-layer chromatography [5], ion-exchange chromatography [5] and descending paper chromatography (Whatman no. 1 paper, developed with butanol--acetic acid--water, 15:1:4 v/v/v). In the latter loganin (Rf 0.56) was visualized with a vanillin reagent [9] and radioactive peaks were
Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Loganyrin formed (dpm/mg prot./hr)</th>
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<tbody>
<tr>
<td></td>
<td>Crude cell-free extract Acetone-treated enzyme</td>
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<tr>
<td>Complete system</td>
<td>1707</td>
</tr>
<tr>
<td>plus boiled enzyme</td>
<td>155</td>
</tr>
<tr>
<td>less DTT</td>
<td>620</td>
</tr>
<tr>
<td>DTT replaced by NADPH+FAD (2.5 μmoles each)</td>
<td>1122</td>
</tr>
</tbody>
</table>

The assay mixture contained potassium phosphate buffer, pH 7.1, 25 μmoles; loganic acid, 2.0 μmoles; DTT, 4.0 μmoles; MgCl₂, 1.0 μ mole; S-AM, 200 nmoles, 140,000 dpm and enzyme (crude cell-free extract, 450 μg, or acetone-treated enzyme, 300 μg) in total volume of 450 μl. The reaction mixture was incubated at 32–33°C for 3 hr.

Initially the identity of the reaction product as loganin-14C was further established by crystallization to constant specific activity after addition of carrier loganin (10 mg). In a representative experiment the specific activities of loganin in the third, fourth and fifth recrystallizations were 590, 567 and 570 dpm/mg respectively (2.22 × 10⁵ dpm/mmmole). The recrystallized loganin-14C was also acetylated and recrystallized to constant activity as loganin pentaacetate (356, 354 and 359 dpm/mg in the second, third and fourth recrystallizations respectively, 2.14 × 10⁶ dpm/mmmole).

3. Results

As seen in Table 1, cell-free extracts of V. rosea contain a methyl transferase capable of converting loganic acid into loganin in the presence of S-adenosyl-L-methionine (S-AM). The latter may be replaced by methionine and ATP although a smaller fraction of acid is methylated. Cell-free extracts prepared in the presence of DTT were nearly four times more active than extracts isolated in the presence of sodium metabisulfite alone. In the assay equimolar amounts of mono-sulfhydryl group reagents such as mercaptoethanol, reduced glutathione or cysteine were not capable of replacing DTT, whereas a combination of NADPH and FAD could partially substitute for the dithiol with the crude cell-free extract (Table 1). Under these same assay conditions no significant methylation of 7-deoxyloganin acid was observed.

Fig. 2(a) reveals the effect of pH on the enzymatic formation of loganin. The crude methyl transferase has a pH optimum between 6.9 and 7.4. Fig. 2(b) shows the time course of incorporation of methyl-14C of S-adenosylmethionine into loganin. Maximum incorporation occurs in about 3 hr.

4. Discussion

To our knowledge this is the first methyl transferase capable of converting the carboxyl group of a low molecular weight compound into a methyl ester in higher...
The methylation of the carboxyl groups of pectin by S-adenosyl-L-methionine has been shown to occur on the polysaccharide [10]. Recently synthesis of methyl esters of fatty acids by extracts of Mycobacterium phlei with S-adenosyl-L-methionine serving as methyl donor has been observed [11].

The evidence presented above suggests the importance of the presence of reduced sulfhydryl groups for enzymatic activity. This is supported by preliminary results indicating enzyme inhibition by p-chloromercuribenzoate, iodoacetamide and N-methylmaleimide. The specific requirement for DTT may be due to its strong reducing potential. DTT has been found to be the most suitable reducing agent for other methyl transferases [12]. Alternatively the enzyme may contain sulfhydryl groups which can form disulfide bridges and thus are more facilely reduced by dithiols. Reduced lipoic acid also restores methyl transferase activity. Preliminary studies indicate that the effect of FAD and NADPH decreases with increasing purification of the enzyme. This is not observed with DTT or lipoic acid. Further studies on the role of sulfhydryl groups are in progress.

In summary the ability of partially purified transferase to methylate loganic acid but not 7-deoxyloganic acid re-emphasizes the intermediacy of the former in indole alkaloid biosynthesis.

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References