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Studies on the late steps of (+) pisatin biosynthesis: Evidence for (−) enantiomeric intermediates

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Abstract

Pisatin, a 6a-hydroxyl-pterocarpan phytoalexin from pea (*Pisum sativum* L.), is relatively unique among naturally occurring pterocarpans by virtue of the (+) stereochemistry of its 6a–11a C–C bond. However, pisatin synthesizing pea tissue has an isoflavone reductase, first identified in alfalfa, which acts on the (−) antipode. In order to establish the natural biosynthetic pathway to (+) pisatin, and to evaluate the possible involvement of intermediates with a (−) chirality in its biosynthesis, we administered chiral, tritium-labeled, isoflavanones and pterocarpans to pisatin-synthesizing pea cotyledons and compared the efficiency of their incorporation. Pea incorporated the isoflavanone, (−) sophorol, more efficiently than either its (+) antipode, or the pterocarpans (+) or (−) maackiain. (−) Sophorol was also metabolized by protein extracts from pisatin-synthesizing pea seedlings in a NADPH-dependent manner. Three products were produced. One was the isoflavene (7,2′-dihydroxy-4′,5′-methylenedioxyisoflav-3-ene), and another had properties consistent with the isoflavanol (7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol), the expected product for an isoflavanone reductase. A cDNA encoding sophorol reductase was also isolated from a cDNA library made from pisatin-synthesizing pea. The cloned recombinant sophorol reductase preferred (−) sophorol over (+) sophorol as a substrate and produced 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol. Although no other intermediates in (+) pisatin biosynthesis were identified, the results lend additional support to the involvement of intermediates of (−) chirality in (+) pisatin synthesis.

Keywords: *Pisum sativum* L.; Leguminosae; Pea; Biosynthesis; Isoflavonoids; Pterocarpans

1. Introduction

The pterocarpan pisatin (**5**) (see Fig. 1) from garden pea was the first chemically identified phytoalexin (Cruickshank and Perrin, 1960) and is a product of the isoflavonoid pathway (Dixon, 1999). The basic pterocarpan carbon skeleton contains rings A–D as shown for pisatin (**5**) in Fig. 1A. In nature, pterocarpans exist in two stereoisomeric forms, one of which is exemplified by (+) pisatin (**5**) (Fig. 1A). Unlike pea, most legumes produce the (−) stereoisomer of pterocarpans (Dewick, 1988) and have the configuration at asymmetric carbons 6a and 11a shown for (−) maackiain (**3b**) (Fig. 1A). The late steps of pisatin (**5**) biosynthesis, particularly the steps that lead to the (+) enantiomer and to hydroxylation at the 6a carbon, are not understood. Most models for pterocarpan biosynthesis involve reduction of an isoflavone, catalyzed by isoflavone reductase (IFR), as an intermediate step in the biosynthesis of these compounds (Aoki et al., 2000; Dixon, 1999).

IFR was first detected in chickpea (Tiemann et al., 1987) and cDNAs encoding IFR have been cloned from chickpea, alfalfa, pea and *Lotus japonicus* (Paiva et al., 1991,

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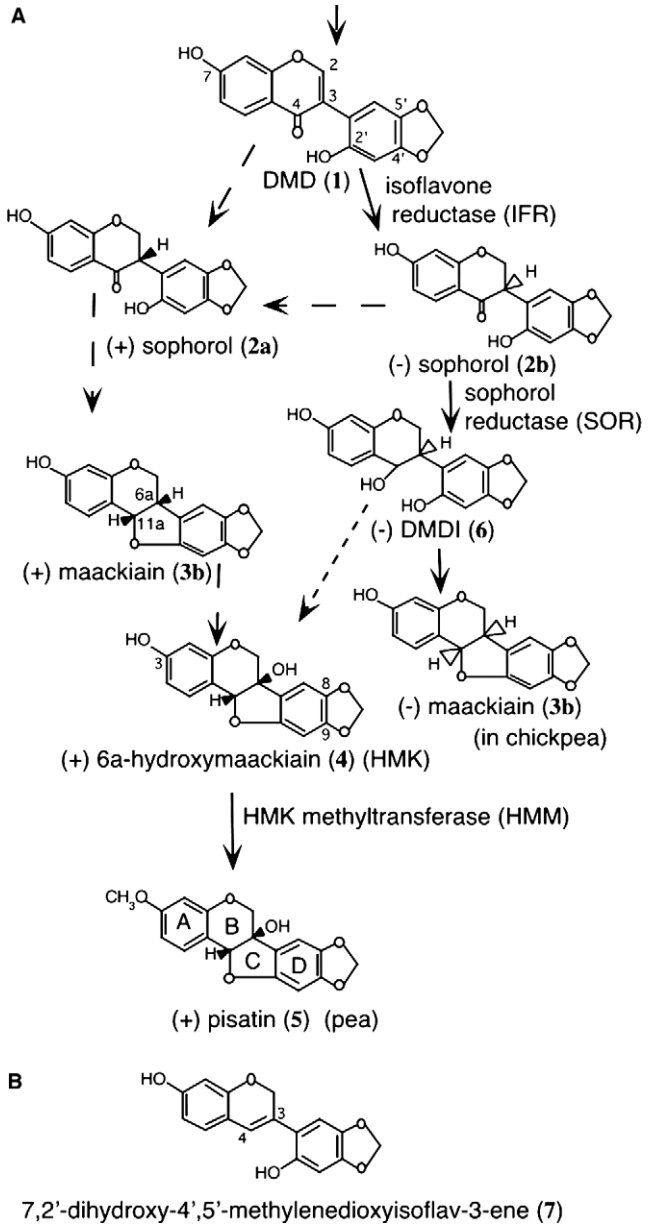

Fig. 1. (A) Proposed pathways for the biosynthesis of (+) pisatin (**5**) and (−) maackiain (**3b**). The pathway proposed by Banks and Dewick (1982) for (+) pisatin (**5**) is indicated by dashed arrows. Reactions for which the enzymatic activity has been confirmed are indicated by solid arrows. The occurrence of 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol (DMDI) (**6**) as an intermediate in the (+) pisatin (**5**) pathway is indicated by a dotted arrow. DMD = 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavone. (B) Structure of 7,2′-dihydroxy-4′,5′-methylenedioxyisoflav-3-ene (**7**).

1994; Shimada et al., 2000; Tiemann et al., 1991). IFR is of special significance, as the reduction of the 2,3 double bond is the first reaction in the pterocarpanoid pathway that produces an asymmetric product. Dewick and Ward (1977) proposed that this reaction could be the branchpoint leading to the two pterocarpan stereoisomers and that the IFR leading to (−) pterocarpans, converts optically inactive isoflavones to (−) isoflavanones and the IFR leading to (+) pterocarpans does the converse.

The IFR that has been isolated and characterized from alfalfa, a plant which makes only (−) pterocarpans, only converts optically inactive isoflavones into (−) isoflavanones (Paiva et al., 1991), supporting the prediction of Dewick and Ward that there is a specific reductase for the synthesis of (−) pterocarpans. However, the IFR enzymatic activity detected in pea tissue that synthesizes (+) pisatin (**5**) and the IFR encoded by an *Ifr* cDNA isolated from this tissue, catalyze the conversion of the achiral isoflavone 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavone (DMD) (**1**) to the (−) isoflavanone (−) "sophorol" (**2b**) (Fig. 1A) (Paiva et al., 1994). In alfalfa, the final two enzymes that form (−) medicarpin (3-hydroxy-9-methoxy pterocarpan) from the (−) isoflavanone (vestitone: 7,2′-dihydroxy-4′-methoxyisoflavanone) are isoflavanone reductase (vestitone reductase, VR), which acts on (−) vestitone to make (−) 7,2′-dihydroxy-4′-methoxyisoflavanol (DMI), and the isoflavanol dehydratase that dehydrates (−) DMI to make (−) medicarpin (Guo et al., 1994a,b) (structures not shown). A cDNA encoding VR from alfalfa has been cloned (Guo and Paiva, 1995).

Another uncertainty in the biosynthesis of (+) pisatin (**5**) involves the origin of the oxygen in the hydroxyl group at 6a. Radiolabeling studies in pea found that (+) maackiain (**3a**) is readily incorporated into (+) pisatin (**5**). ^{2}H NMR analysis of pisatin (**5**), which had incorporated (+) [11a-^{2}H, 6-^{2}H] maackiain (**3a/b**), revealed that both labeled protons were retained, suggesting that hydroxylation at the 6a carbon probably involved an O_2-dependent oxygenase and did not desaturate either the 11a–6a or 6–6a carbon bonds (Banks and Dewick, 1983a,b). However, subsequent labeling experiments with $^{18}O_2$ and $H_2{}^{18}O$ failed to support this model for the origin of the 6a-hydroxyl group in pisatin (**5**) and indicated that direct hydroxylation of maackiain (**3**) is not involved in (+) pisatin (**5**) biosynthesis (Matthews et al., 1987, 1989).

While the step(s) responsible for determining the stereochemistry of (+) pisatin (**5**) have yet to be described, all of the data indicate that the final step in pisatin (**5**) biosynthesis is methylation of (+) 6a-hydroxymaackiain (**4**) at the 3-0 position (Fig. 1A). A methyl transferase, (+) 6a-hydroxymaackiain 3-*O*-methyltransferase (HMM), that catalyzes this reaction has been purified from pea (Preisig et al., 1989). cDNA clones encoding HMM have been isolated and used to make antisense (anti *Hmm*) constructs of *Hmm*. When anti *Hmm* constructs are expressed in transgenic pea tissue (hairy roots), the tissue has a decreased ability to produce pisatin (**5**) (Wu et al., 1997; Wu and VanEtten, 2004).

The purpose of this present study was to evaluate further the participation of the (−) and (+) isoflavonoid intermediates in the synthesis of (+) pisatin (**5**). This was done by measuring the relative incorporation of sophorol (**2**) and maackiain (**3**) enantiomers into (+) pisatin (**5**) and by determining if an enzyme (sophorol reductase) and a gene analogous to (−) *Vr* from alfalfa are present in pea.