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Ipsdienol dehydrogenase (IDOLDH): A novel oxidoreductase important for *Ips pini* pheromone production

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ABSTRACT

Ipsdienone (2-methyl-6-methylene-2,7-octadien-4-one) is an important intermediate in the biosynthesis of pheromonal ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) and ipsenol (2-methyl-6-methylene-7-octen-4-ol) in male pine engraver beetles, *Ips pini* (Say). A novel ipsdienol dehydrogenase (IDOLDH) with a pheromone-biosynthetic gene expression pattern was cloned, expressed, functionally characterized, and its cellular localization analyzed. The cDNA has a 762 nt ORF encoding a 253 amino acid predicted translation product of 28 kDa and pI 5.8. The protein has conserved motifs of the Cp2 subfamily of "classical" short-chain dehydrogenases. Transcript levels were highest in pheromone producing tissue: the anterior midgut of fed males. The protein was detected only in male midguts and localized in the cytosolic fraction of midgut cells. Recombinant IDOLDH was produced in Sf9 cells using a baculovirus expression system. Enzyme assays of protein preparations showed IDOLDH used both NAD^+ and $NADP^+$ as coenzymes with specific activities in the nanomole range. Enzyme assays and GC/MS analysis showed that IDOLDH catalyzed the oxidation of racemic ipsdienol and (4*R*)-(−)-ipsdienol to form ipsdienone, while (4*S*)-(+)-ipsdienol was not a substrate. These data strongly implicate IDOLDH as an enzyme involved in terminal pheromone-biosynthetic steps, likely functioning to "tune" ipsdienol enantiomeric ratios.

1. Introduction

The major pheromone compounds of *Ips* spp. beetles are ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) and ipsenol (2-methyl-6-methylene-7-octen-4-ol) (Vité et al., 1972). Species specificity of aggregation signals is obtained by differences in the combination of compounds and/or their enantiomeric ratios (Vité et al., 1978). Additionally, differences in enantiomeric blends occur within populations of the same species. For example, populations of the pine engraver beetle *Ips pini* (Say) in California ("western *I. pini*") aggregate to a 97% (4*R*)-(−): 3% (4*S*)-(+)-ipsdienol pheromone blend (Seybold et al., 1995) while *I. pini* in New York ("eastern *I. pini*") respond to a 60% (−):40% (+)-ipsdienol blend (Lanier et al., 1980; Miller et al., 1989; Teale and Lanier, 1991). Both populations use (−)-ipsenol as an anti-aggregation semiochemical.

The overall metabolic pathway to ipsdienol branches from the normal mevalonate pathway at the step catalyzed by the dual-function enzyme, geranyl diphosphate/myrcene synthase (GPPS/MS) (Gilg et al., 2005, 2009). Myrcene hydroxylase (CYP9T1 and CYP9T2 from *Ips confusus* and western *I. pini*, respectively) then produces ipsdienol, however the mechanism(s) by which different enantiomeric ratios are produced are not clear (Sandstrom et al., 2006, 2008). CYP9T1 and CYP9T2 produce an intermediate enantiomeric blend of approximately 80% (−):20% (+)-ipsdienol, suggesting a more complex enantiomeric regulation system for pheromone biosynthesis. Ipsdienone and ipsdienol may be maintained in a constant ratio and are precursors for (−)-ipsenol in *Ips paraconfusus* (Fish et al., 1979, 1984). *Ips* spp. such as *I. paraconfusus* that use mostly (+)-ipsdienol in the pheromone blend may attain the correct enantiomeric ratio as (−)-ipsdienol is consumed to make (−)-ipsenol (Fish et al., 1984). Similarly, the fate of (+)-ipsdienol in species that do not use that enantiomer is unknown. It has been suggested that (+)-ipsdienol is enantioselectively converted to amitinol by allylic rearrangement (Kohnle et al., 1988). These observations suggest that additional oxidoreductases are required to make the final pheromone blend, including a dehydrogenase that oxidizes ipsdienol to ipsdienone, and at least one reductase to first reduce the diene from ipsdienone to form ipsenone, and then to reduce this product to ipsenol (Fig. 1). We wanted to identify the enzyme(s) that catalyze(s) the last steps of western *I. pini* pheromone biosynthesis in order to understand how *Ips* beetles make different enantiomeric blends of ipsdienol.

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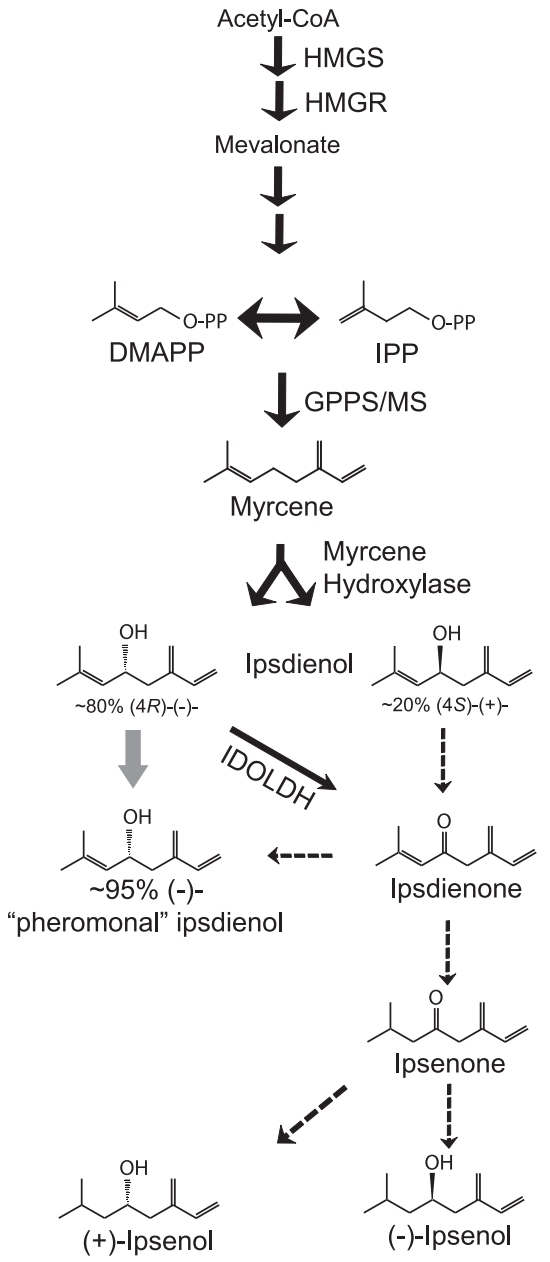

Fig. 1. *Ips* spp. pheromone component biosynthesis. Known reactions are shown by solid arrows, hypothesized reactions by dashed arrows. Western *I. pini* "pheromonal" ipsdienol likely results from stereo-specific ipsdienone reduction combining with (−)-ipsdienol produced from myrcene hydroxylase (gray arrow).

In this study we identify and functionally characterize ipsdienol dehydrogenase (IDOLDH), an oxidoreductase from western *I. pini*. The enzyme is produced in a pattern consistent with ipsdienol biosynthesis. Recombinant IDOLDH oxidized racemic and (−)-ipsdienol to ipsdienone, but showed no reaction with (+)-ipsdienol. These data strongly suggest that IDOLDH plays a role in pheromone biosynthesis, likely by contributing to the final enantiomeric blend of ipsdienol and/or creating ipsdienone necessary for ipsenol production.

2. Materials and methods

2.1. Insects and materials

I. pini-infected Jeffrey pine (*Pinus jeffreyi*) bolts were gathered from the Sierra Nevada in California and Nevada, USA and handled as described previously (Sandstrom et al., 2006). Fetal bovine serum (FBS) was from Atlas, Grace's 1× Insect Basal Medium and Hink's 1× TNM-FH Media (Supplemented Grace's Medium) were from Mediatech, Inc (Herndon, VA). Pluronic F-68, 4% Agarose Gel, and 2× Grace's Insect Media were from Gibco (Grand Island, NY). Sf9 cells were purchased from Invitrogen. Ipsdienone and ipsenone were kind gifts from D. Vanderwel (U. Winnipeg). Neat "racemic" ipsdienol (50% (+)-, 45% (−)-ipsdienol, 5% ipsdienone) was from Bedoukian Research, Inc. (Danbury, CT), and 80 mg/ml 2% (−):96% (+)-ipsdienol and 98% (−):1% (+)-ipsdienol in 1,3-butane diol were from PheroTech Inc. (Vancouver, Canada). Ip12D04F1 and qRT-PCR primers were from Integrated DNA Technologies (Coralville, IA) and cloning primers were from Sigma Genosys (St. Louis, MS).

2.2. cDNA isolation

The full-length sequence for the cDNA represented by *I. pini* EST IPG012D04 (GenBank ID# CB408666.1) in pBluescript SK$^-$ was determined by dideoxy-sequencing with an ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 on an ABI3730 DNA Analyzer at the Nevada Genomics Center (NGC). Sequence traces were analyzed using Vector NTIv.9 software (Informax, N. Bethesda, MD, USA). The cDNA and deduced amino acid sequence were renamed "ipsdienol dehydrogenase" (IDOLDH) after functional characterization.

2.3. Expression analysis

IDOLDH, IPG007C7 (GenBank ID: CB408556) and IPG024B01 (GenBank ID: CB407830) mRNA levels were determined by quantitative (Real Time) reverse-transcriptase (RT) PCR (qRT-PCR) using primers described in Keeling et al. (2006) to amplify template cDNA prepared from fed or unfed male and female tissues (Sandstrom et al., 2006). Relative mRNA levels were determined using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). *I. pini* cytoplasmic actin (IPG005E02; GenBank ID# CB408614.1), whose expression in *I. pini* is unchanged by feeding (Keeling et al., 2004), was used to normalize expression values. qRT-PCR reactions were done in 25 μl with optimal concentration of RT primers, template cDNA and SYBR® Green PCR Master Mix as per the supplier's recommendation (Applied Biosystems) and data was acquired using the ABI Prism 7000 sequence detection system. Dissociation curves for each amplicon were analyzed for non-specific amplification.

2.4. Antibody preparation

A 13 residue antigenic region of IDOLDH (SRNEYKNEFPHDQ) was identified with Vector NTIv.9 software and suggestions from GenScript Corp (Piscataway, NJ, USA). GenScript Corp was contracted to synthesize, purify, and produce rabbit-anti-IDOLDH polyclonal serum. The resultant polyclonal antibody was named anti-Ip12D04.

Similarly, a predicted antigenic region (KEDNKNSEAVREV) of *I. pini* geranyl diphosphate synthase/myrcene synthase (GPPS/MS (Gilg et al., 2005, 2009)) was identified and constructed with an added C-terminal cysteine by the Nevada Proteomic Center (NPC). Cocalico Biologicals, Inc. (Reamstown, PA, USA) was contracted to produce rabbit antisera to the peptide and conduct ELISA assays. The SulfoLink® Coupling Gel Column (Pierce, Rockford, IL, USA) was used to affinity purify the test bleeds as per the manufacturer's protocol. The antiserum collected was named anti-IpiGPPS.