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Novel tryptophan metabolic pathways in auxin biosynthesis in silkworm

Chiaki Yokoyama[a], Mami Takei[a], Yoshiaki Kouzuma[a], Shinji Nagata[b], Yoshihito Suzuki[a,*]

[a] Department of Food and Life Sciences, Ibaraki University, 3-21-1 Chuo, Ami-machi, Inashiki, Ibaraki 300-0393, Japan
[b] Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa-city, Chiba 277-8567, Japan

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ABSTRACT

In the course of our study of the biosynthetic pathway of auxin, a class of phytohormones, in insects, we proposed the biosynthetic pathway tryptophan (Trp) → indole-3-acetaldoxime (IAOx) → indole-3-acetadehyde (IAAld) → indole-3-acetic acid (IAA). In this study, we identified two branches in the metabolic pathways in the silkworm, possibly affecting the efficiency of IAA production: Trp → indole-3-pyruvic acid → indole-3-lactic acid and IAAld → indole-3-ethanol. We also determined the apparent conversion activities (2.05×10^{-7} U mL^{-1} for Trp → IAA, 1.30×10^{-5} U mL^{-1} for IAOx → IAA, and 3.91×10^{-1} U mL^{-1} for IAAld → IAA), which explain why IAOx and IAAld are barely detectable as either endogenous compounds or metabolites of their precursors. The failure to detect IAAld, even in the presence of an inhibitor of the conversion IAAld → IAA, is explained by a switch in the conversion from IAAld → IAA to IAAld → IEtOH.

1. Introduction

Phytohormonal studies of insect gall formation have strongly suggested that indole-3-acetic acid (IAA), an active form of auxin that is synthesized in galling insects, is involved in gall induction. Early studies showed that galling insects contain IAA, at levels that are sometimes much higher than those in the host plant tissues (Mapes and Davies, 2001; Tooker et al., 2011). We subsequently showed that the IAA concentration in a galling sawfly (*Pontania* sp.) larva is about 1000 ng g^{-1}, which is 100 times higher than that in the leaf tissue of its host willow plant. A conversion experiment using ^{13}C-labelled tryptophan (Trp) demonstrated that IAA is synthesized *de novo* in the sawfly from Trp (Yamaguchi et al., 2012). IAA signalling is also enhanced in the gall tissue compared with that in the control leaf tissue, supporting the involvement of insect-derived IAA in the induction and maintenance of gall tissues (Yamaguchi et al., 2012).

Although other galling insects also show biosynthetic activity from Trp to IAA (Tanaka et al., 2013; Takei et al., 2015), we have shown that non-galling insects, such as the fruit fly *Drosophila melanogaster*, the cotton aphid *Aphis gossypii*, the honey bee *Apis mellifera*, and the silkworm *Bombyx mori*, contain certain levels of IAA (8–120 ng g^{-1}) (Suzuki et al., 2014) and synthesize IAA *de novo* from Trp (Suzuki et al., 2014 for silkworm; unpublished data for the other insects). Because the IAA concentration is high in the galling sawfly, we hypothesized that the biosynthesis of IAA is intrinsic to insects, and that the sawfly has extended this ability to facilitate gall formation. Based on metabolic studies using a crude silk-gland extract from the silkworm and regurgitant fluid from *Pontania*, we proposed the pathway Trp → indole-3-acetaldoxime (IAOx) → indole-3-acetadehyde (IAAld) → IAA, which commonly operates in both non-galling silkworm larvae and the galling sawfly. Detection of the pathway was largely based on the finding that the conversion rates from each precursor to IAA were in the following order: [Trp → IAA] < [IAOx → IAA] < [IAAld → IAA]. For example, the amounts of IAA produced from 1 μg of Trp, IAOx, or IAAld during overnight incubation with the crude silk-gland extract were ~5 ng, ~90 ng, and ~450 ng, respectively (Suzuki et al., 2014). The linear relationship of the three steps was further supported by the finding of an inhibitor of the conversion from IAAld to IAA, designated 'IAA-biosynthetic-inhibitor-1' (IBI1), which inhibits IAA production from all three precursors. Screening of chemical libraries for compounds to inhibit the conversion from IAOx to IAA identified two inhibitors, IBI1 and IBI2, which were later shown to inhibit the single conversion step from IAAld to IAA (Suzuki et al., 2014). IBI1 was a strong inhibitor of the conversion reaction, in both the silk-gland extract and the sawfly regurgitant fluid, whereas IBI2 was a strong inhibitor of the conversion reaction in the sawfly regurgitant fluid, and a weak inhibitor in the silk-gland extract. These results support the proposed biosynthesis pathway, and indicate that the enzymes involved in the conversion of IAAld to IAA in the silkworm and sawfly have a common evolutionary origin. Despite the evidence for the proposed pathway described above, this

Abbreviations: ABA, abscisic acid; HPLC, high-performance liquid chromatography; IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; IAOx, indole-3-acetaldoxime; IEtOH, indole-3-ethanol; ILA, indole-3-lactic acid; IPyA, indole-3-pyruvic acid; LC–MS/MS, liquid chromatography–tandem mass spectrometry; Trp, tryptophan

* Corresponding author at: Department of Bioresource Science, Ibaraki University, 3-21-1 Chuo, Ami-machi, Inashiki-gun, Ibaraki 300-0393, Japan.
E-mail address: yoshihito.suzuki.chemeco@vc.ibaraki.ac.jp (Y. Suzuki).

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pathway has not been confirmed in step-by-step analyses. IAOx was barely detectable as either an endogenous compound or a metabolite of Trp. One interpretation is that the enzyme activity responsible for metabolizing IAOx was stronger than that producing IAOx. IAAld was also difficult to detect as a metabolite of either Trp or IAOx. In our previous study, IAAld was analysed with liquid chromatography–tandem mass spectrometry (LC–MS/MS) after derivatization to 2,4-dinitrophenylhydrazone (DNPH). Later, because the sensitivity of LC–MS/MS in detecting the DNPH derivative was low, IAAld was analysed as a thiazolidine derivative, which allowed us to quantify IAAld more sensitively and accurately. However, negligible IAAld was still detected as a metabolite of Trp or IAOx. This situation was not even improved by using the IBI1 inhibitor, which should have inhibited the further metabolism of IAAld to IAA. This situation was interpreted as arising from the presence of other metabolites of IAAld that do not lead to IAA production.

Under the hypothesis that insects share an IAA biosynthesis system with a common evolutionary origin, we further characterized IAA biosynthesis using silk-gland extract in this study. We exploited the advantage of this material, which is easily obtained in large amounts at any time of year. We examined the metabolites of Trp, IAOx, and IAAld formed by their incubation with a crude silk-gland extract to obtain information on the conversion steps that could affect IAA production and yield. We also analysed the apparent activity required for the conversion steps from each precursor to IAA, to understand why these two intermediates are so difficult to detect.

2. Materials and methods

2.1. Chemicals

Stable isotope-labelled compounds [$^{13}C_6$]IAA and [$^{13}C_{11}$,$^{15}N_2$]l-Trp were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). [$^{13}C_6$]IAOx was synthesized from [$^{13}C_6$]IAA according to a published method (Novák et al., 2012). [$^{13}C_2$]ABA was a kind gift from Dr. Asami (The University of Tokyo, Asami et al., 1999). IBI1 is 2-{[3-(3-nitrophenyl)(1, 2, 4-oxadiazol-5-yl)]methylthio}-1-benzyl-2-imidazoline, a component of the Maybridge chemical library (code BTB02880; Thermo Fisher Scientific, MA, USA).

2.2. Preparation of crude silk-gland enzyme extract

Fifth-instar larvae of the silkworm *Bombyx mori* (the hybrid strain Kinshu × Showa) were purchased from Kogensha Co. Ltd (Matsumoto, Japan) and reared on an artificial diet (Nihon-nosanko, Yokohama, Japan) for a few days until just before pupation. The mature silk glands were isolated from the larvae and homogenized in 0.1 M citric acid and 0.2 M sodium phosphate buffer (pH 8.0, 1.2 mL per 1.0 g of silk gland). The homogenate was filtered by squeezing it through two-layered gauze and the filtrate was used in the conversion experiments.

2.3. Identification of indole-3-lactic acid (ILA) as a metabolite of Trp

Tryptophan (200 mg) was incubated at 25 °C for 1 week with 25 mL of the crude silk-gland extract in the presence of 0.6 mM IBI1 in a total volume of 50 mL. The reaction mixture was partitioned three times with an approximately one-third volume of ethyl acetate, and the combined ethyl acetate fraction was dried over sodium sulfate and concentrated under reduced pressure. The concentrate was separated on an InertSustain C-18 column (14.0 mm i.d. × 150 mm, 5 μm; GL Sciences) using linear gradient elution from 0.1% (v/v) acetic acid in water to methanol within 15 min and isocratic elution with methanol for 15 min, at 3 mL min^{-1}. The elution was monitored with a fluorescence detector (emission [em] 350 nm, excitation [ex] 280 nm). A fraction containing a fluorescent peak eluted at 16.6–17.6 min and was further purified with an Inertsil ODS-EP column (6.0 mm i.d. × 250 mm, 5 μm; GL Sciences) with linear gradient elution from 0.1% (v/v) acetic acid in water to methanol within 15 min and isocratic elution with methanol, for 15 min at 1 mL min^{-1}. ILA (2.3 mg) was obtained as a single peak that eluted at 21 min. ^{1}H-Nuclear magnetic resonance (NMR; 400 MHz) and ^{13}C-NMR (100 MHz) were recorded on an Avance III FT-NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 5 mm broadband fluorine observation probe. The chemical shifts (δ) were referenced to solvent signals ($\underline{C}HD_2OD$, δ_H3.329; $\underline{C}D_3OD$ δ_C 49.03). ^{1}H-NMR (CD_3OD) δ_H 3.06 (1H, dd, H-10a, $^3J_{10a,11}$ = 7.5, $^3J_{10a,10b}$ = 14.7), 3.26 (1H, dd, H-10b, $^3J_{10b,11}$ = 4.3, $^3J_{10a,110b}$ = 14.7), 4.36 (1H, dd, H-11, $^3J_{10b,11}$ = 4.3, $^3J_{10a,11}$ = 7.5), 6.99 (1H, dd, H-6, $^3J_{6,7}$ = 8.1, $^3J_{5,6}$ = 8.0), 7.06 (1H, dd, H-5, $^3J_{5,6}$ = 8.0, $^3J_{4,5}$ = 7.9), 7.13 (1H, s, H-2), 7.31 (1H, d, H-7, $^3J_{6,7}$ = 8.1), 7.60 (1H, d, H-4, $^3J_{4,5}$ = 7.9); ^{13}C-NMR (CD_3OD) δ_C 31.7 (C-10), 72.8 (C-11), 111.7 (C-3), 112.1 (C-7), 119.5 (C-4), 119.6 (C-6), 122.1 (C-5), 124.5 (C-2), 129.2 (C-9), 138.1 (C-8), 178.5 (C-12).

2.4. Identification of indole-3-ethanol (IEtOH) as a metabolite of IAAld

IAAld (6 mg) was incubated at 25 °C for 19 h with 25 mL of the crude silk-gland extract in the presence of 0.6 mM IBI1 in a total volume of 51 mL. The reaction mixture was partitioned three times with an approximately one-third volume of ethyl acetate, and the combined ethyl acetate fraction was dried over sodium sulfate and concentrated under reduced pressure. The concentrate was separated on an Inert Sustain C-18 column (14.0 mm i.d. × 150 mm, 5 μm; GL Sciences). Elution was performed by varying the relative concentrations of solvents A (0.1% [v/v] acetic acid in water) and B (methanol): linear gradient elution from 0% B to 50% B in 12 min, isocratic elution with 50% B for 8 min, linear gradient elution from 50% B to 100% B in 10 min, and isocratic elution with 100% B for 10 min, at a flow rate of 3 mL min^{-1}. The elution was monitored with a fluorescence detector (em 350 nm, ex 280 nm). A fraction containing a fluorescent peak was eluted at 23.73–26.20 min and was further purified with an Inertsil ODS-EP column (6.0 mm i.d. × 250 mm, 5 μm; GL Sciences) with isocratic elution with 40% B at a flow rate of 1 mL min^{-1}. IEtOH (0.6 mg) was obtained as a single peak that eluted at 34 min. The procedures for the ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) measurements were identical to those for ILA. ^{1}H-NMR (CD_3OD, 400 MHz) δ_H 2.97 (2H, dt, $^3J_{10,11}$ = 7.4, $^4J_{10,2}$ = 0.7), 3.80 (2H, t, H-11, $^3J_{10,11}$ = 7.4), 6.99 (1H, ddd, H-5, $^3J_{5,6}$ = 8.1, $^3J_{4,5}$ = 7.9, $^4J_{5,7}$ = 1.0), 7.06 (1H, s, H-2), 7.07 (1H, ddd, H-6, $^3J_{5,6}$ = 8.1, $^3J_{6,7}$ = 7.9, $^4J_{4,6}$ = 1.0), 7.32 (1H, ddd, H-7, $^3J_{6,7}$ = 8.1, $^4J_{5,7}$ = 1.0, $^4J_{4,7}$ = 0.9), 7.52 (1H, ddd, H-4, $^3J_{4,5}$ = 7.9, $^4J_{4,6}$ = 1.0, $^4J_{4,7}$ = 0.9); ^{13}C-NMR (CD_3OD, 400 MHz) δ_C 29.8 (C-10), 63.7 (C-11), 112.2 (C-4), 112.7 (C-3), 119.2 (C-7), 119.5 (C-5), 122.2 (C-6), 123.5 (C-2), 128.9 (C-9).

2.5. Conversion experiments

The enzyme reaction was performed by mixing 25 μL of the silk-gland extract with a substrate (10 μg of [$^{13}C_{11}$,$^{15}N_2$]Trp, 0.5 μg of [$^{13}C_6$]IAOx, 0.5 μg of IAAld, or 1.0 μg of indole-3-pyruvic acid [IPyA]) in the presence or absence of IBI1 (0.6 mM) in a total volume of 50 μL. To measure the enzyme activity for the conversion from IAAld to IAA, the original crude extract solution was diluted 500-fold. The undiluted crude extract solution was used for the other reactions. The incubation time is shown in each figure. IPyA and IAAld were converted to their thiazolidine derivatives (IPyA-TAZ and IAAld-TAZ, respectively), according to Novák et al. (2012). Briefly, the reaction mixture was mixed with 200 μL of 0.25 M aqueous cysteamine (pH adjusted to 8.0 with aqueous NH_3) and incubated for 1 h at the ambient temperature. IAA, ILA, IPyA-TAZ, IEtOH, and abscisic acid (ABA) were extracted with ethyl acetate from the reaction mixture after acidification with 1 M HCl (ca. pH 3), and then subjected to LC–MS/MS analysis. IAAld-TAZ was purified from the reaction mixture with an Oasis HLB column (1 mL/30 mg; Waters). The reaction mixture was applied to the column, which