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In vitro assay for sex pheromone biosynthesis by the female yellow mealworm beetle and identification of a regulated step

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Abstract

Several pheromones are known to be involved in mediating the mating behavior of the yellow mealworm beetle, *Tenebrio molitor* (Coleoptera: Tenebrionidae). The only sex pheromone that has been identified to date in this species is the female-produced male attractant, 4-methylnonanol (MNol). The objective of this study was to develop an in vitro assay for the last biosynthetic step involved in MNol biosynthesis, the reduction of 4-methylnonanoic acid (MNA) to the corresponding pheromone product. In vitro assays were conducted by adding appropriate cofactors and radioactively labelled precursor. Radioactive pheromone product was separated from the radioactive precursor by column chromatography, and quantified through scintillation counting. The conversion of MNA to MNol was observed, and was comparable to that observed in vivo using the same radiolabelled substrate. In both the in vivo and in vitro assays, the ^{3}H-MNA was also converted to a nonpolar product, tentatively identified as a triacylglyerol (TAG) derivative. The reduction of ^{3}H-MNA to pheromone proceeded through the coenzyme A intermediate. Mature females reduced significantly more precursor to pheromone than the immature females and the males, both in vivo and in vitro, indicating that the reduction of MNA to MNol is a regulated step involved in pheromone production. Using the in vitro assay reported herein, studies are ongoing in our lab to purify and characterize the enzyme catalyzing the reduction.
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Keywords: *Tenebrio molitor*; Beetle; Sex pheromone; Biosynthesis; Regulation; Acyl-CoA reductase

1. Introduction

The biosynthetic pathways used by beetles to produce their pheromones, and the mechanisms by which these pathways are regulated, have been the targets of consistent interest. The biochemistry of aggregation pheromone production has been extensively examined in the beetles (reviewed in Seybold and Vanderwel, 2003), but the biochemistry involved in the production of other types of beetle pheromones has been largely ignored. In order to gain a more comprehensive understanding of coleopteran pheromone biochemistry, we initiated an investigation of *sex* pheromone biosynthesis in the yellow mealworm, *Tenebrio molitor*. Several different pheromones are known to mediate mating in *T. molitor* (reviewed in Plarre and Vanderwel, 1999), but only one has been identified to date: the female-produced sex pheromone, (4*R*)-(+)-4-methyl-1-nonanol (MNol) (Tanaka et al., 1986, 1989). Newly eclosed and immature adult females produce relatively little sex pheromone, but production increases as the females mature (Happ and Wheeler, 1969) due to stimulation by Juvenile Hormone (JH) III (Menon, 1970). The biosynthetic pathway to MNol is a modification of normal fatty acid biosynthesis (Islam et al., 1999). The objective of this study was to develop an in vitro assay for sex pheromone production in female *T. molitor*, focusing on the last step of the process: the reduction of 4-methylnonanoic acid (MNA) to the pheromone (Fig. 1). The reduction of a fatty acid generally proceeds through an acyl-Coenzyme A (acyl-CoA) intermediate, and also involves reducing cofactors such as reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Riendeau and Meighen, 1985).

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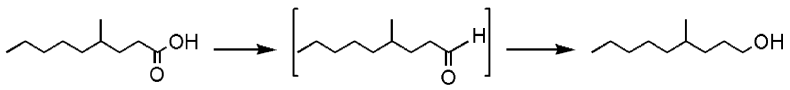

4-methylnonanoic acid (MNA) 4-methylnonanal (MNal) 4-methylnonanol (MNol)

Fig. 1. Final step of 4-methylnonanol (MNol) biosynthesis in female *T. molitor*, involving the reduction of 4-methylnonanoic acid (MNA) to MNol through the aldehyde intermediate, 4-methylnonanal (4-MNal).

Our approach was to determine if homogenates of the beetles could convert the precursor in radiolabelled form, [3,4-^{3}H]MNA (^{3}H-MNA), to radiolabelled pheromone, in the presence of an appropriate buffer and cofactors. The acid and corresponding alcohol products were readily separable by column chromatography, and quantifiable by scintillation counting. The in vitro results were compared to the products formed in vivo from ^{3}H-MNA injected into live beetles. This assay will facilitate the purification and characterization of what we now know to be a key regulatory enzyme involved in sex pheromone biosynthesis.

2. Materials and methods

2.1. Rearing of insects

T. molitor larvae were purchased from Northwest Scientific Supply Ltd. (Victoria, British Columbia, Canada). The insects were maintained in an incubator at 25 °C (±0.5) with a 12 h light/12 h dark reverse photoperiod and a humidity of 20–30%. Moist paper towels were spread over the colonies and the larvae were occasionally fed potatoes in order to provide liquid moisture to the insects. The larvae were fed a diet of wheat bran, rolled oats, and wheat germ (4:2:1 by volume). Pupae were removed from the colonies and separated by sex by examining the developing genitalia behind the 7th visible abdominal sternite (Bhattacharya et al., 1970). Males and females were maintained separately, in order to ensure virginity. Mature virgin females 4–7 days post-emergence were used for experiments.

2.2. Chemicals

Adenosine 5′-triphosphate disodium salt (ATP, 98% purity), glycerol (99% purity), nicotinamide adenine dinucleotide disodium salt (reduced form, NADH, 98% purity), NADPH tetrasodium salt (reduced form, NADPH, 95% purity), coenzyme A lithium salt (CoA, 94% purity), mammalian protease inhibitor cocktail, *N*-octyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulphonate (Zwittergent® 3-8, 97% purity), pancreatic porcine lipase, and Florisil® activated magnesium silicate (100–200 mesh) were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Dithiothreitol (oxidized form), and potassium chloride (99% purity) were obtained from Malinckrodt Inc. ScintiSafe™ Econo 2 scintillation fluid and ScintiSafe™ Plus 50% scintillation fluid were obtained from Fischer Scientific (Fairlawn, NJ, USA).

The radioactive precursor, ^{3}H-MNA, was obtained through the custom tritiation (ChemSyn, Lenexa, Kansas, USA) of the related alkene, methyl 4-methyl-3-nonenoate, synthesized in our laboratory (unpublished). The tritiated ester product obtained from ChemSyn was diluted with unlabelled methyl 4-methylnonanoate, hydrolyzed with 0.5 M KOH/ethanol, and purified by column chromatography and high performance liquid chromatography (HPLC). The radioactive precursor used for experiments was >95% chemically and radiochemically pure, with a specific activity of about 430 mCi/mmol, and stored in ethanol (1×10^6 DPM/μL). Radioactive ^{3}H-MNol standard was obtained through the lithium aluminum hydride reduction of the corresponding ester.

2.3. In vivo assay and extraction method

Beetles were injected with 0.5 μL ^{3}H-MNA in ethanol (500,000 DPM) between the thorax and the abdomen, using a Hamilton 5 μL syringe (needle gauge 33.5, point style 4). The beetles were placed on food in an incubator in the dark at 25 °C. After 15 min the beetles were extracted individually by an adaptation of the method of Hara and Radin (1978). Each beetle was crushed in 2 mL hexane/isopropanol (3/2, v/v), and the extract removed. The procedure was repeated two additional times with 1 mL of solvent. The extracts were combined and extracted with 10% aqueous $MgSO_4$ (5 mL). When necessary a bench-top centrifuge was used to separate the layers. The upper hexane layer was removed. The lower layer (containing water and isopropanol) was extracted with hexane (3×1 mL) to remove any organics still present. The hexane layers were combined and extracted with 10% aqueous $MgSO_4$ (3×2 mL) to remove all traces of isopropanol. The organic extract was dried over granular crystalline Na_2SO_4 and subjected to column chromatography. Unless otherwise stated, each treatment was replicated five times.

2.4. In vitro assay and extraction method

Unless otherwise specified, the assay solution contained 2 mM ATP, 60 mM CoA, 1 mM NADH, 1 mM NADPH, 5×10^5 DPM of ^{3}H-MNA, and 50 mM Zwittergent™3-8 in 50 mM phosphate buffer (pH 7.4, containing 1 mM dithiothreitol, 50 mM KCl, 2 mM $MgCl_2$, and10% v/v glycerol), in a total volume of 1 mL. Each reaction was initiated when one whole beetle was homogenized in the assay solution with a flattened glass rod (except for the "no protein controls", to which no beetles were added). The

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