Degradation of an alkaloid pheromone from the pale-brown chafer, *Phyllopertha diversa* (Coleoptera: Scarabaeidae), by an insect olfactory cytochrome P450

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Abstract The pale-brown chafer, *Phyllopertha diversa*, utilizes an unusual alkaloid, 1,3-dimethyl-2,4-(1*H*,3*H*)-quinazolinedione, as its sex pheromone. This compound is rapidly degraded in vitro by the antennal protein extracts from this scarab beetle. Demethylation at the N-1 position and hydroxylation of the aromatic ring have been identified as the major catabolic pathways. The enzyme responsible for the pheromone degradation is membrane-bound, requires NAD(P)H for activity and is sensitive to cytochrome P450 inhibitors, such as proadifen and metyrapone. The ability to metabolize this unusual pheromone was not detected in 12 species tested, indicating that the P450 system, specific to male *P. diversa* antennae, has evolved as a mechanism for olfactory signal inactivation.

Key words: Pheromone-degrading enzyme; Olfaction; Scarab beetle; Pheromone inactivation

1. Introduction

Detection of odorants in insects takes place in their olfactory sensilla. Monitoring the changes of chemical signals in the environment requires that the molecules that have already entered the sensilla and stimulated olfactory neurons are rapidly inactivated. Enzymatic transformation of pheromones to inactive forms has early been recognized as a likely mechanism for maintaining sensitivity to small changes in the concentration of semiochemicals [1]. Since the Lepidoptera have become a model system for studying the olfactory perception, pheromone degradation has been best-characterized in this order of insects (reviewed in [2–4]). However, the repertoire of lepidopteran pheromones is quite limited and most species utilize straight chain alcohols, aldehydes and esters. Therefore, studies of the degradation pathways have centered on enzymes capable of transforming these functional groups. These efforts have led to the identification of esterases, aldehyde oxidases and dehydrogenases in a number of species (for review see [2]), an alcohol oxidase and dehydrogenase in *Bombyx mori* [5] and an epoxide hydrolase in *Lymantria dispar* [6,7].

Recently, we have isolated an unusual pheromone, 1,3-dimethyl-2,4-(1*H*,3*H*)-quinazolinedione, from a scarab beetle *Phyllopertha diversa* [8]. The structure of this molecule resembles that of caffeine-type alkaloids. It was synthesized four decades ago and shown to have anti-inflammatory and analgesic effects in mammals [9,10]. None of the insect pheromone-degrading systems characterized so far seemed capable of metabolizing this peculiar molecule. We have investigated its transformation by antennal enzymes and found that it is mediated by a specific, olfactory cytochrome P450.

2. Material and methods

2.1. Chemicals

NADH, NADPH, H_2O_2, tris(hydroxymethyl)aminomethane and all solvents were from Wako. Metyrapone, methimazol and proadifen (SKF525A) were from Sigma. *t*-Butyl hydroperoxide was from Nacalai Tesque. The tritiated pheromone was prepared commercially (Amersham) from benzoyleneurea and tritiated methyl iodide, as described previously for the unlabelled compound [8] at a specific activity of 162 Ci/mmol.

2.2. Enzymatic assays with the tritiated pheromone

Insect body parts were collected and homogenized as described previously [11]. The radioactive pheromone (1 μCi, 6 pmol) was added to glass tubes in dichloromethane and the solvent was evaporated under a stream of nitrogen gas. Extracts of antennal or leg proteins (20 μl in 20 mM Tris-HCl buffer, pH 7.5) were transferred to the tubes containing the substrate, vortexed briefly, incubated for 1 h at room temperature and applied directly to a thin layer chromatography (TLC) plate (Whatman LK6D). The plate was developed in diethyl ether:hexane 9:1, sprayed with a fluorophore (EN^{3}HANCE, DuPont NEN) and exposed to X-ray film.

2.3. Separation of subcellular fractions

The 12 000×*g* supernatant from the antennal homogenate was centrifuged at 105 000×*g* in a Beckman XL-90 ultracentrifuge (SW-60 Ti rotor, 32 000 rpm) for 1 h. The supernatant was transferred to a fresh tube and the pellet was resuspended in 2 mM Tris-HCl, pH 7.5. Both fractions were re-centrifuged at the same speed for 45 min. The soluble fraction was lyophilized and reconstituted in water. The membrane fraction was resuspended in 20 mM Tris-HCl, pH 7.5. Enzymatic activity of both fractions was tested with 1 μCi of the tritiated alkaloid as described above (radio-TLC). The membrane fraction was tested without cofactors or in the presence of 1 mM NADPH, NADH, *t*-butyl hydroperoxide or hydrogen peroxide.

2.4. Enzyme inhibition

The membrane fraction was pre-incubated with inhibitors for 30 min at room temperature. NADPH (1 mM) was added either before or after the pre-incubation period. The following compounds were used at a 0.5 mM concentration: proadifen, metyrapone and methimazole. Samples pre-incubated with the inhibitors were transferred to glass tubes containing 1 μCi of the radioactive pheromone, incubated for 1 h at room temperature and analyzed by radio-TLC.

2.5. Identification of the degradation products

Extracts of male antennal proteins (20 ml, 67 antenna equivalents) were incubated with 10 μg of cold 1,3-dimethyl-2,4-(1*H*,3*H*)-quinazolinedione or caffeine for 2 h at 30°C. The reaction mixture was separated on a Hypersil OSD (125×4 mm) column using the Hewlett-Packard series 1100 high performance liquid chromatography (HPLC) system. Elution was performed with acetonitrile in water: 5% for

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5 min, 5–25% gradient in 20 min, followed by a column wash with 100% CH_3CN. The substrate incubated with the buffer or the protein extract without the substrate were taken as controls. Fractions from the HPLC were lyophilized, dissolved in a small volume of dichloromethane and analyzed by gas chromatography-mass spectrometry (GC-MS). GC-MS was carried out on an HP 5890 II Plus gas chromatograph linked to a mass spectrometer (MSD 5973, Hewlett-Packard). A HP-5MS capillary column (30 m×0.25 mm×0.25 μm) was operated at 110°C for 1 min, followed by a gradient to 270°C at 10°C/min and operated at this temperature for 10 min. Alternatively, the reaction mixture was separated on an Inertsil OSD-3 column using the Hewlett-Packard series 1100 LC/MSD system (HPLC directly coupled to a mass spectrometer). Fractions containing the hydroxylated product were also analyzed by GC (HP6890, Hewlett-Packard) coupled to a vapor phase Fourier transform infrared (FTIR) (FTS-40A, Bio-Rad). The light pipe was operated at 240°C and the transfer line at 250°C.

3. Results

Separation of the radioactive metabolites by TLC indicated the presence of one major and at least two minor degradation products (Fig. 1). Analysis by HPLC confirmed the presence of three compounds (Fig. 2). The most abundant metabolite was identified as 3-methyl-2,4-(1*H*,3*H*)-quinazolinedione, the pheromone demethylated at position N-1 (Fig. 3C). Its retention time in GC and its mass spectrum matched those of the standard compound. The second product could not be identified unambiguously, but its mass spectrum strongly suggested incorporation of an oxygen atom. The molecular ion peak for this compound was consistently 206 in both analytical systems (GC-MS and LC-MS). The fragmentation pattern, particularly the shift of the 104 and 105 peaks by 16 U, strongly indicates oxidation of the aromatic ring (Fig. 3B). The mass spectrum of the most polar product could not be obtained in the GC-MS system, due to its low volatility. However, its molecular mass obtained in the LC-MS system was 192, corresponding to the double metabolite, i.e. the demethylated and oxidized pheromone molecule. In addition to the mass spectral data, bathochromic shift in the UV spectrum from 312 to 340 nm also suggested oxidation of the aromatic ring both in compounds B and D. The presence of a hydroxy group was also indicated by the FTIR spectrum of compound

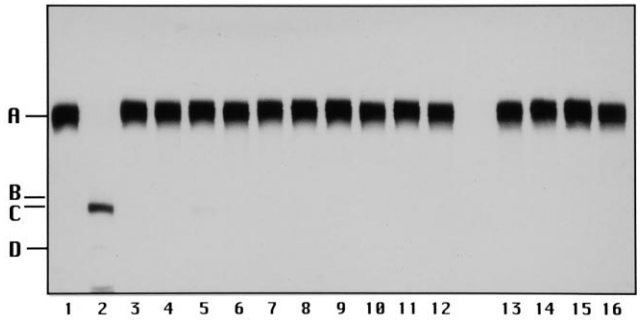

Fig. 1. Degradation of the tritiated pheromone by various insect species. The radio-TLC assay was performed with a buffer or protein extracts from the following sources: 1: 10 mM Tris-HCl, pH 7.5, 2: *P. diversa* male antennae (33 equivalents), 3: *P. diversa* female antennae (33 equivalents), 4: *P. diversa* male legs (three equivalents), antennae from the following species of scarab beetles (33 equivalents each): 5: *P. japonica*, 6: *Anomala osakana*, 7: *Anomala octiescostata*, 8: *Anomala cuprea*, 9: *Anomala schoenfeldti*, 10: *Exomala orientalis*, 11: *Heptophylla picea*, 12: *Holotrichia paralella* and the following species of moths: 13: *Antheraea polyphemus* (one equivalent), 14: *Manduca sexta* (one equivalent), 15: *B. mori* (three equivalents), 16: *Samia cynthia ricini* (1.5 equivalents). The number of body part equivalents given in parentheses gives an approximately equal amount of total protein, as determined by gel electrophoresis.

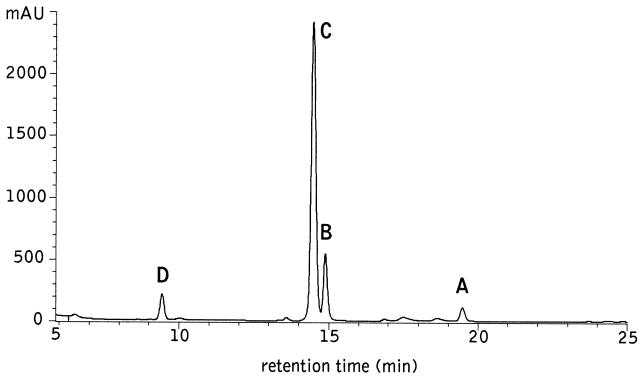

Fig. 2. Separation of the degradation products of the cold pheromone by HPLC (Hypersil OSD column). Elution was monitored at 210 nm.

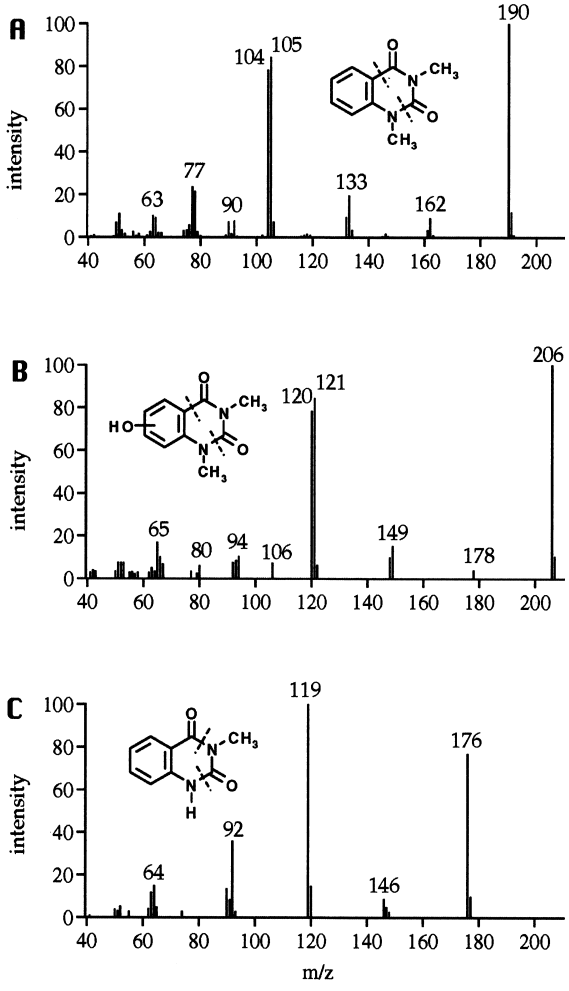

Fig. 3. Mass spectra of compounds A (the pheromone), B and C (two major metabolites) separated by HPLC (Fig. 2), obtained in the GC-MS system (MSD 5973). Structures of the molecules and their major fragmentation patterns (dashed lines) are presented as insets.

B obtained in the gas phase (data not shown). An unchanged absorbance of the carbonyl group suggested that the oxidation did not occur in the *ortho* position to this group.

The degradation pathway consistent with these results is presented in Fig. 4. These reactions (*N*-demethylation and oxidation of the aromatic ring) could be attributed to at least three classes of oxidases: cytochromes P450, flavin-containing monooxygenases and peroxidases [12,13]. To characterize the enzyme(s) more closely, we determined its subcellular localization, cofactor requirements and sensitivity to inhibitors.

The enzyme degrading the *P. diversa* pheromone was primarily membrane-bound (105 000 × *g* pellet), although some activity (15–20%) remained in the soluble fraction. Such a phenomenon has been frequently observed for cytochrome P450 enzymes during separation by differential ultracentrifugation [14]. The membrane fraction was inactive in the absence of cofactors but the activity was fully restored by the addition of NADPH. NADH also supported the reaction, but to a lesser degree. Although NADPH seems to be the primary source of reducing equivalents for cytochromes P450 (as a substrate for NADPH-cytochrome P450 oxidoreductase), it can be replaced by NADH in a number of cases, particularly when crude microsomes are tested [14]. *t*-Butyl hydroperoxide and hydrogen peroxide were not able to support the reaction, indicating that peroxidases do not participate in this process.

Degradation of the pheromone was inhibited by proadifen, a general, widely used inhibitor for cytochromes P450 [14]. Metyrapone, an inhibitor used to inhibit the mammalian olfactory cytochrome P450 [15], was even more potent, providing complete inhibition at a 0.5 mM concentration, when pre-incubation was carried out in the absence of NADPH. Both inhibitors were equally potent (complete inhibition at 0.5 mM) when pre-incubation was carried out in the presence of NADPH (data not shown). Methimazole, which may inhibit a number of enzymes, but is considered a specific, competitive inhibitor for flavin-containing monooxygenases [16], was completely ineffective (Fig. 5).

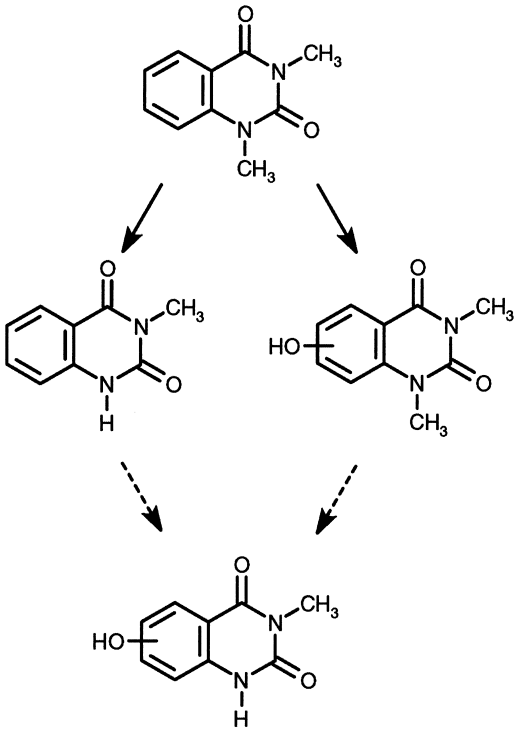

Fig. 4. Proposed degradation pathway of 1,3-dimethyl-2,4-(1*H*,3*H*)-quinazolinedione in the male antennae of *P. diversa*.

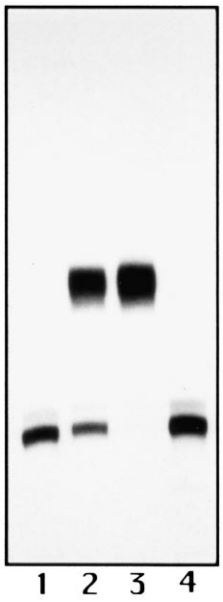

Fig. 5. Influence of inhibitors on the pheromone degradation by antennal microsomes (20 antenna equivalents per reaction) in the radio-TLC assay: 1: no inhibitors, 2: proadifen, 3: metyrapone, 4: methimazole. NADPH (1 mM) and the substrate were added after pre-incubation with the inhibitors for 30 min.

To assess the specificity of the enzyme, we have tested the ability of the antennal enzymatic system to metabolize caffeine, a compound closely resembling the structure of the pheromone from *P. diversa*, which has become a model molecule in studying a number of monooxygenase systems [17,18]. Although caffeine was degraded, primarily to theophylline, the rate was at least an order of magnitude slower than for the pheromone (data not shown), indicating that the enzyme has developed a considerable substrate-specificity.

We tested the ability to degrade 1,3-dimethyl-2,4-(1*H*,3*H*)-quinazolinedione in antennal protein extracts from a number of insect species, closely related scarab beetles from subfamilies Rutelinae and Melolonthinae, and also some model lepidopteran species. We detected traces of similar enzymatic activity only in one case, the Japanese beetle (*Popillia japonica*). All other species were not able to metabolize the alkaloid (Fig. 1).

4. Discussion

Degradation products, association with membranes, requirement for NADPH and sensitivity to inhibitors point towards involvement of a cytochrome P450 system in the degradation of the pheromone from *P. diversa*. Membrane localization of the enzyme requires that it is exposed to the sensillar lumen, where it could get in immediate contact with the pheromone and ensure its rapid inactivation. Although most cytochromes P450 are associated with the endoplasmic reticulum, several reports described detection of these enzymes on the surface of the plasma membrane. Recently, Pompon and co-workers have also demonstrated that a yeast cyto-

chrome P450 (CYP2D6) expressed in this location is perfectly functional [19]. The precise localization of the antennal enzyme from *P. diversa* remains to be established, but obviously, it would perform its function most efficiently if expressed on the surface of olfactory neurons. It would be a system analogous to the degradation of acetylcholine, which is inactivated by enzymes located in post-synaptic membranes.

In vertebrates, a high activity of cytochrome P450 and other monooxygenases in the olfactory system has been detected and their participation in odorant clearance has been postulated a long time ago (reviewed in [20]). Several isoforms have been identified in the olfactory epithelium and a specific, olfactory-type was cloned from rat [21]. In insects, a number of cytochromes P450 have been characterized, because of their involvement in pesticide metabolism and resistance [22,23]. Their role in inactivation of plant toxins has also drawn considerable attention [22–24]. Although structures of many pheromones bring to mind the possible role of P450 monooxygenases in their degradation [23], activity of these enzymes in the insect olfactory tissues has been demonstrated only in one case, in the house fly, where Z-9-tricosene is oxidized to the epoxide and ketone [25]. Recently, high expression of NADPH-cytochrome P450 oxidoreductase has also been detected in the antennae of *Drosophila melanogaster* and participation of a P450 system in odorant clearance has been postulated in this species [26]. We have now provided evidence that a cytochrome P450 system is involved in the degradation of the alkaloid pheromone from *P. diversa*.

The role of pheromone-degrading enzymes in the termination of olfactory stimuli has been a matter of considerable debate [2,3,27]. In vitro experiments with the esterase purified from the antennae of *Antheraea polyphemu* demonstrated that the rate of enzymatic inactivation was sufficient to account for the rapid decline of receptor potential [28]. On the other hand, measurements of the pheromone degradation in vivo indicated that the breakdown was too slow [29,30]. A model postulating that pheromone binding proteins participate in pheromone inactivation has even been proposed [31,32]. Our data, however, provide support for the hypothesis that the antennal enzymes play a primary role in the inactivation of olfactory signals. The P450 system in *P. diversa* antennae has apparently been evolved specifically to degrade the pheromone of this species. No such enzymatic activity has been detected in essentially all other species which we tested. On the other hand, we have observed a strong esterase activity and detected antennae-specific esterases in a number of scarab beetles, which do not utilize esters or lactones as sex pheromones (Wojtasek and Leal, unpublished data). This is not surprising, since these compounds (and similarly aldehydes or alcohols) share a functional group with a number of general odorants, such as plant volatiles. Therefore appropriate enzymatic systems involved in their degradation have evolved in a large number of species. However, very specific enzymatic systems evolved to degrade unusual molecules, such as the alkaloid pheromone from *P. diversa* or the epoxide described previously in *L. dispar* [6,7], point out that the antennae-specific enzymes have evolved to participate in deactivation of semiochemicals. It is conceivable that in general, the evolution of chemical signals from the environment is commonly coupled to specific degrading enzymes as it has been well-established in the nervous system for the degradation of acetylcholine.

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