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Substrate specificity of membrane-bound alcohol oxidase from the tobacco hornworm moth (*Manduca sexta*) female pheromone glands

Anna Luxová [a], Aleš Svatoš [a,b,*]

[a] *Institute of Organic Chemistry and Biochemistry (IOCB), Academy of Sciences of the Czech Republic, Flemingovo náměstí 2, Prague 6-Dejvice, CZ-166 10, Czech Republic*
[b] *Max-Planck-Institute for Chemical Ecology, Hans-Knöll-Str. 8, D-07745 Jena, Germany*

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Abstract

A putative alcohol oxidase (AO) from abdominal tips (ATs) of *Manduca sexta* virgin females was studied in a biphasic system hexane/aqueous phosphate buffer. The pH optimum closest to neutral range (6.8) and the temperature optimum closest to room temperature ($25 \pm 3\,^{\circ}C$) were measured for the highest AO activity. AOs that are in intact membranes have long lifespans and may oxidize repeatedly. A high selectivity for primary alcohols of benzylic, saturated, and allylic type was observed. Neither the secondary alcohols nor the primary alcohols with bulky alkyl groups on C_2-carbon are oxidized. This pronounced substrate specificity can be used for specific oxidation of alcohols in mixtures.
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Keywords: Alcohol oxidase; Insects; Aldehydes; Biphasic system

1. Introduction

Bacteria and yeast have been shown to be valuable sources of diverse enzymes useable in enzyme-mediated chemical transformations [1,2]. Insects, however, have not been used as enzyme sources, even though they represent more than 50% of all described species [3]. Unsurprisingly this overlooked resource of vast biochemical diversity houses a valuable supply of enzymes with unusual substrate specificity. Some of those enzymes, e.g., desaturases [4], oxidases [5], reductases [6], are important for the production of sex pheromones in female moths [7], typically long-chain fatty-acid-derived compounds for attracting conspecific males to mate.

Oxidation of a sex-pheromone-related alcohol is the terminal step in aldehyde sex pheromone biosynthesis. Putative alcohol oxidases (AOs) were found in the sex pheromone gland epidermis on the tips of the abdomen of *Heliothis visescens* [8] and *Manduca sexta* (Lepidoptera, Sphingidae) females [9].

Here, we report substrate specificity in the membrane-bound alcohol oxidase (AO).

2. Materials and methods

2.1. Insects

Pupae of *M. sexta* were obtained from the Institute of Organic Chemistry and Biochemistry (Prague, Czech Republic). They were sexed and female pupae were placed in cages at 25 °C, 45% relative humidity, under L/D 16:8 photoperiod. Emerging adults were transferred into separate cages. For experiments virgin 2- or 3- day-old females were used.

2.2. Gland dissections

Abdominal tip preparations (ATs) obtained as described [10] were used for experiments. In selected cases, the pheromone gland epidermis (PGE) was removed from terminal papillae anales (PA) and the preparations were used separately. The preparations were kept in hexane at −20 °C prior to further use. The dissected females were frozen at −20 °C and discarded after one week.

2.3. Chemicals and preparation of solutions

Alcohols, manganese dioxide, chromium trioxide, and pyridiumdichlorochromate (PCC) were purchased from Aldrich (Mil-

* Corresponding author. Tel.: +493641571700; fax: +493641571701.
E-mail address: svatos@ice.mpg.de (A. Svatoš).

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waukee, WI, USA) and used without further purification. Hexane and dimethylsulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). Solvents were HPLC-pure. The alcohols used were dissolved in hexane-containing (1 mg mL^{-1}) *n*-hexadecane as an internal standard (3.1 mg mL^{-1}). Solution A was prepared from benzylalcohol in hexane-containing (1 mg mL^{-1}) *n*-hexadecane.

The standards of the corresponding aldehydes were prepared by oxidation with PCC or MnO_2 (benzylic type); their chemical identity was confirmed by spectral methods. The calibration was performed using those aldehydes dissolved in the hexane-containing *n*-hexadecane (see above). Other solvents and concentrations used are described later. Phosphate buffer (PB) (pH 6.8) was prepared by mixing 0.2 M KH_2PO_4 and 0.2 M Na_2HPO_4 (7:18); PB of different pH were prepared by mixing the solutions in another proportions and pH values checked on a pH meter (inoLab pH Level 2, WTW, Weilheim, Germany).

2.4. AT homogenation

Several AT tissues were placed in an Eppendorf vial (0.5 mL) and 5 μL of phosphate buffer PB per gland were added. The vial was cooled in liquid nitrogen, and tissues were crushed with a Teflon hand homogenizer. This material was divided into several parts and added to reaction vessels.

2.5. Instrumentation

Gas chromatographic (GC) analyses were performed on an HP-6890 GC (Hewlett-Packard) equipped with an HP-5 capillary fused-silica column (5% phenylmethylsiloxane), 30 m × 0.32 mm with a film thickness of 0.25 μm. Samples were injected using a split inlet (split ratio 50:1). Helium was used as a carrier gas at constant pressure (66 kPa, flow of He at 50 °C was 2.0 mL min^{-1}). An FID detector was used. The temperature program started at 50 °C (2 min), then increased 10 °C/min to 280 °C (10 min). The temperature of the split injector was 250 °C.

2.6. Dependence of enzyme activity on temperature

Twenty-eight ATs were homogenized and divided among the twelve glass ampules (1 mL) and stored at −20 °C. Approximately, 3.5 mg of the AT homogenate represent the equivalent of one gland. Before a reaction, each AT equivalent was dosed with solution A (50 μL) and PB (5 μL), and ampules were thawed out. For each tested temperature (16, 38, 45, 55, and 78 °C), a pair of ampules was put into a thermostat adjusted to the desired temperature. After incubating 3 h, the contents were centrifuged and the upper hexane layer immediately analyzed by GC. The values for 0 °C were obtained from incubation performed in an ice bath.

In a similar experiment, pairs of glass-ampule-sealed AT homogenates (ca 3.5 mg each) were simultaneously heated to 38, 45, 60, and 78 °C for 3 h. Then the ampules were opened, incubated with solution A (50 μL) and PB (5 μL), and shaken for 12 h. The oxidation of substrate to aldehyde was determined by GC.

2.7. Dependence of enzyme activity on pH

Ten ATs were homogenized without adding PB and divided among five ampules. Each AT equivalent (3.5 mg) was dosed with solution A (50 μL) and a PB (10 μL) with desired pH values (5.7, 5.8, 6.2, 6.7, 6.9, 7.6, and 8.0), and ampules were thawed out. The reaction mixture was analyzed by GC after 24 h.

2.8. Subcellular localization

Ten ATs were homogenized and divided among two Eppendorf vials. More PB (10 μL) was added to one AT equivalent (3.5 mg). The materials were sonicated for 5 min and then centrifuged (1600 rad s^{-1}) for 20 min at 4 °C. The supernatant was transferred into clean Eppendorf tube and identical amounts of the hexane solution of benzyl alcohol (50 μL to 3.5 mg equiv^{-1}) were added to the solid phase and to the supernatant. The GC data were collected after 24 h.

2.9. Substrate specificity

2.9.1. Linear primary alcohol homolog series

2.9.1.1. In vitro oxidation. Individual solutions (0.1 M, 1 mL) of linear-chain alcohols with the following number of carbons, C_6, C_8, C_{10}, C_{11}, C_{12}, C_{14}, C_{16}, C_{17}, C_{18}, and C_{20}, respectively, were prepared. An equimolar ratio solution was prepared by mixing 0.1 mL of each individual alcohol hexane solution (0.1 M) and filled with *n*-hexane-containing (3 mL) *n*-hexadecane (1 mg mL^{-1}) as an internal standard.

A glass ampule (1 mL) was filled with this solution (50 μL), with PB (5 μL), and with ATs. The ampule was sealed and after 3 days of being shaken at room temperature, the reaction mixture was analyzed by GC.

The intact epidermis of the ATs of females was used for oxidation. In vitro oxidations with individual glands were conducted by incubating the hexane solutions of appropriate substrates (50 μL) and PB (5 μL) for a defined time (Fig. 3A). Alternatively, incubation with substrate solutions was carried out using AT homogenates by adding hexane solutions of substrates and PB in amounts equivalent to the homogenate weight; in this way order the proportion of 50 μL hexane solution of substrate to 5 μL of PB per 3.5 mg homogenate was maintained (Fig. 3B).

2.9.1.2. In vivo oxidation. Equimolar amounts (20 mmol) of the linear-chain alcohols were weighed in 25 mL volumetric flasks, hexadecane (25.3 mg) was added, and the mixture was diluted with DMSO to a final volume of 25 mL. *M. sexta* females were introduced into an anesthetization chamber and their extruded pheromone gland was treated twice at intervals of 20 min with the above-prepared in DMSO solution (2 × 1 μL) [10]. After the solution of a metabolic probe was absorbed into the gland (45 min), clips were removed, and the moths were returned to their cage. After 6 h, the pheromone glands were excised and

extracted with hexane (50 μL). The extracts were analyzed by GC.

2.9.2. Chemical oxidation

An equimolar solution of linear alcohols (C_8–C_{18}, mixture 6.6 mg mL^{-1} hexane solutions of individual alcohol) in hexane was chemically oxidized with chromium trioxide. Chromium trioxide (0.66 mg) was added to 100 μL of this mixture. After being stirred for 18 h at room temperature, the mixture was passed through a silica gel column. The purified filtrate was analyzed by GC.

2.10. Oxidation of substrate analogs

2.10.1. In vitro

The equivimolar amounts (30 mmol) of the alcohols (benzylalcohol, *rac*-octan-2-ol, (*S*)-(+)-1-phenylethanol, (*Z*)-2-hex-2-en-1-ol, cyclohexylmethanol and hexadecan-1-ol) were dissolved in hexane-containing *n*-hexadecane (1 mg mL^{-1}, 2.5 mL). The above-prepared solution (50 μL) and PB (5 μL) were applied to individual ATs and after 3 days being shaken, the GC data were measured.

In repeated oxidations using intact ATs, the AT preparation was removed after 3 days of reaction and washed in hexane (50 μL), after which a new batch of alcohol solution and PB was added. This was repeated 5 times and the organic phase from each oxidation was individually measured by GC.

2.11. Oxidations of enantiomeric of 1-phenylethanols

Eight ATs were homogenized and the material was divided among six glass vials. The equivalent of one gland was added to the solution of (*S*)-(+)-1-phenylethanol or (*R*)-(−)-1-phenylethanol (1 mg mL^{-1}, 50 μL) and PB (5 μL) in two glass vials. After three days of shaking, the organic phase was separated and measured by GC.

2.12. Isomerization of the double bond in position 2 of primary alcohols

A solution of (*Z*)-hex-2-en-1-ol (50 μL, 1 mg mL^{-1}) and PB (5 μL) was added to the individual ATs. The same mixture without ATs was used as a control. After 3 days, the GC data were collected.

3. Results and discussions

3.1. Insect rearing, pheromone gland dissections, and homogenations

An insect-rearing facility was established to provide a reliable source of insect material. This colony has been maintained at IOCB for several years, and this homogenous insect material from it has been used for several experiments [10,11]. Females utilized in this experiment were anesthetized before the pheromone gland was excised and fat tissue was carefully removed [10]. In preliminary experiments, the whole AT was used for oxidations, but the reproducibility of the oxidations was poor, i.e., the activity of ATs from individual females was variable. Originally the experiments were performed in aqueous phosphate buffer; however, the oxidative activity of AT preparation has been recognized to increase in bi-phase system hexane—aqueous phosphate buffer (10/1) [10]. The ATs could be stored in the same medium at −20 °C for several months. More reproducible results were obtained after homogenizing a large number of ATs and using homogenate aliquots that correspond to one AT in the bi-phase system as above.

3.2. Temperature and pH optimum

Temperature (*T*) and pH optimums for AT homogenate preparations were determined using benzyl alcohol (shown in preliminary experiments to be an effective substrate). The *T* optimum is 25 ± 3 °C (Fig. 1A). In another experiment we tested *T* tolerance of the homogenate for the same reaction. Enzymatic activity ceased dramatically when the homogenate was heated at 78 °C for 3 h (Fig. 1B), which is to be expected for enzymatic oxidation. The isolated ATs can be stored for several months at −20 °C in hexane, indicating their high stability. However, after homogenization, stability is markedly impaired presumably due

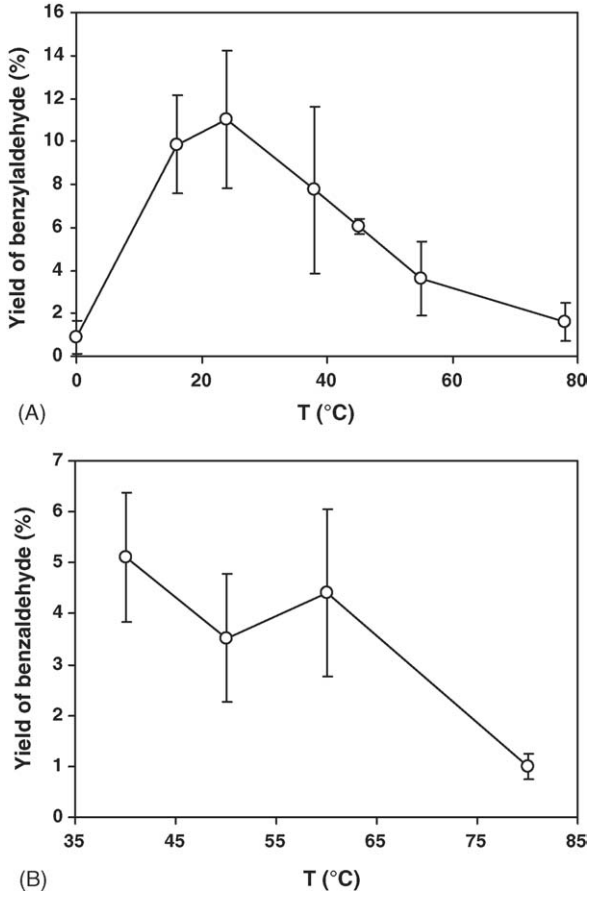


Fig. 1. (A) Temperature dependence of yield of benzaldehyde from incubation of benzyl alcohol with AT homogenate. (B) Temperature tolerance of oxidative enzymes in AT homogenate determined as yield of benzaldehyde ($n = 3$).

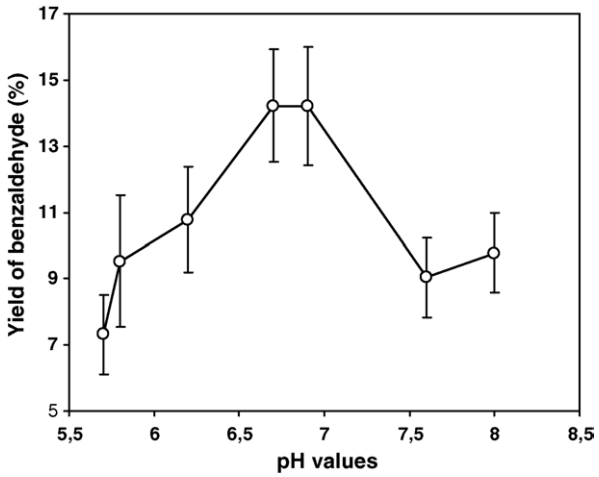


Fig. 2. Dependence of enzyme activity of AT homogenate on pH ($n = 3$).

to membrane fragmentation, and thus the homogenate should be prepared freshly before each use. The measured pH range optimum (Fig. 2) is pronounced and it is centered on 6.8. At values lower than pH 6.5, the oxidative activity diminished, and at the higher pH range as well (more than 7.5). The optimum pH range was reflected in further incubation experiments.

3.3. Subcellular localization

As already suggested, the AO is probably localized in a lipid membrane. Consecutive homogenization of the ATs, centrifugation of the pellets from the supernatants at 1600 rad s^{-1}, and the subsequent determination of the oxidative ability of isolated pellets or supernatant using benzylalcohol clearly show that the enzymatic activity remains associated with the membrane fractions. In the experiments with AT homogenate pellet the yield of the benzylaldehyde was $4.3 \pm 0.1\%$. In the corresponding supernatant only $0.1 \pm 0.01\%$ yield was recorded ($n = 3$) after 24 h incubations.

3.4. Substrate specificity

3.4.1. Linear alcohols

Linear long-chain alcohols are native substrates for the pheromone gland AO [8,9]. Substrate specificity was determined on intact anesthetized females, ATs, and homogenized ATs. Although for individual ATs noticeable insect-to-insect variation was observed (Fig. 3A), the substrate profiles show a similar trend. Surprisingly, the "native" chain length C16 is not oxidized with the highest conversion, but it lies on the bottom of a shallow minimum ranging from C_{11} to C_{17}. Longer (C_{17}–C_{20}) or shorter (C_6–C_{10}) alcohols are oxidized with noticeably higher yields. This trend is much more pronounced when AT homogenate is used, due to the lower variability of pooled ATs (Fig. 3B). To eliminate possible artifacts under in vitro conditions, the same mixture of alcohols dissolved in DMSO was applied on abdominal parts of virgin females. Due to the high volatility of the short-chain aldehydes (C_6–C_{10}), we were not able to determine their conversion, but the obtained results from longer chain lengths corroborate the above-mentioned substrate specificity profile. Control chemical oxidation experiments showed no chain-length discrimination (the yield for all substrate lengths [C_8–C_{18}] was in the range 33–40, not shown).

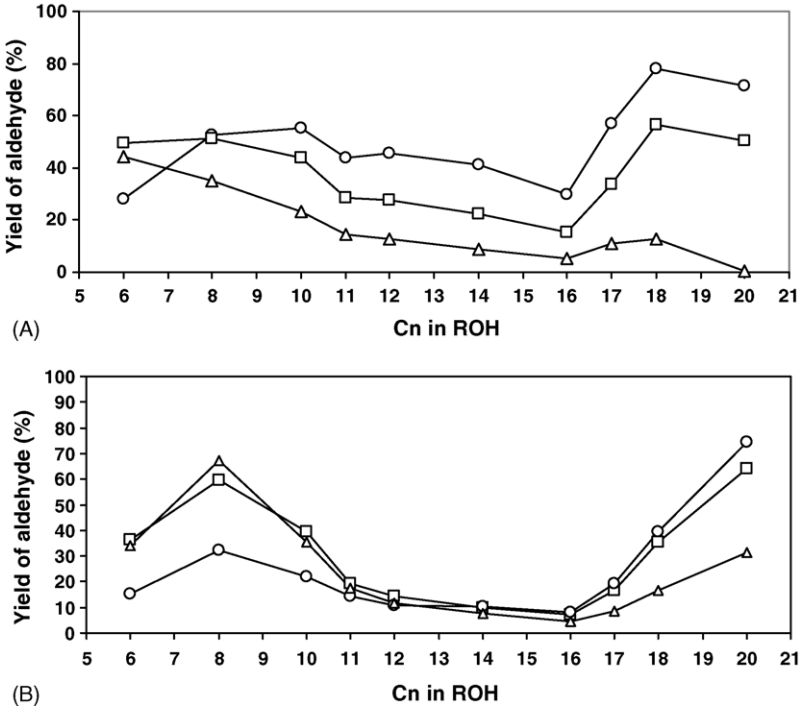
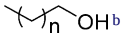
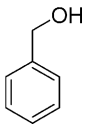
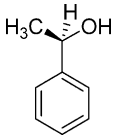
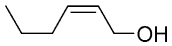
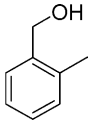
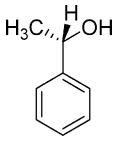
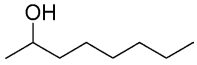
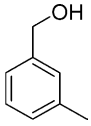
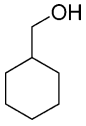
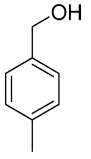


Fig. 3. Relative oxidation rates of a series of aliphatic primary alcohols with three individual ATs (A) or pooled AT homogenates (B) ($n = 3$).

Table 1
Relative yields[a] of carbonyl compounds for selected alcohols with ATs isolated from virgin *Manduca sexta* females

Aliphatic substrates	Relative yield of product	Aromatic substrates	Relative yield of product	Aromatic substrates	Relative yield of product
 [b]					
					
$n = 4$	91		185		2
$n = 12$	115				
$n = 14$	100				
$n = 18$	250				
	18		151		1.2
	0.8		115		
	2.2		166		

[a] Based on yield of hexadecan-1-ol.
[b] See Fig. 3 for the whole profile.

This unexpected substrate specificity, which operates both in vivo and in vitro, might be significant for insects. We speculate that the low conversion rates for physiological-chain-length alcohols may show the ability of an insect to regulate its sex pheromone production; a slow rate may prolong the effect of the pheromone.

3.4.2. Structurally diverse alcohols

A diverse series of primary and secondary alcohols of benzylic, allylic, and aliphatic types were tested as AO substrates, both individually and in mixtures using AT homogenate (Table 1, Fig. 3). Benzyl alcohol is as good a substrate as the native hexadecan-1-ol, followed by hex-2-en-1-ol isomers. As the oxidation using AT was performed under neutral conditions, low or no isomerization of the double bond in formed α,β-unsaturated aldehyde was expected. However, when (*Z*)-hex-2-en-1-ol was oxidized, an *E*/*Z* mixture of hex-2-enals (9/1) was obtained.

In strong contrast to benzyl alcohol, cyclohexylmethanol is a very poor substrate and the *rac*-octan-2-ol is likely not oxidized by AO. Instead, slow, spontaneous oxidations were observed in experiments without AT homogenate. Striking differences between benzyl alcohol and cyclohexylmethanol potentially show the importance of the steric hindrance in the active center of the AO. The steric effects were further screened using positional isomers of methylbenzyl alcohols (Table 1). Substantial reduction of oxidation was observed only for meta-isomer, the other two isomers are readily oxidized. The steric hindrance appears regio-sensitive.

Several experiments have shown that membranes in ATs can be used repeatedly, up to 3 times. One oxidation that lasted for 3 days showed the enzyme was still active after 9 days of incubation. In contrast, using AT homogenate showed that disrupted membranes largely impaired the oxidative power of the enzyme, making repeated oxidation with homogenate impossible.

3.4.3. Optically active substrates

We have shown that the oxidation of prochiral hydrogen atoms of the $–CH_2OH$ group proceeds with exclusive *Re* specificity [10]. To further evaluate the oxidase enantiospecificity, chiral *sec*-alcohols were tested using (*S*)-(+)-1-phenylethanol and (*R*)-(−)-1-phenylethanol. Conversions rates for both enantiomers were very low (2%) and no significant difference between them was observed. In control experiments without the AT, conversion was also about 1%. The observed strong preference of the AO for primary alcohols limits synthetic utility of the studied insect oxidase as no enantiospecific resolution of *sec*-alcohols [12] is feasible.

4. Conclusions

The putative AO from ATs of *M. sexta* females has a pH optimum almost within the range of neutral pH, and the temperature optimum for oxidations is close to room temperature and ther-

mally stable up to 60 °C. If it is in intact membrane, it will last for a long time and repeated oxidation with the ATs will be possible. A strong preference for primary alcohols of benzylic, saturated, and allylic types was observed. Neither the secondary alcohols nor primary alcohols with voluminous alkyl parts (CHMe) are oxidized. This pronounced substrate specificity can be used to oxidize alcohols in mixtures and poly-hydroxylated substrates with primary/secondary hydroxyl group. The overall substrate specificity closely resembles yeast alcohol dehydrogenase [13,14], although with a specificity shifted for long-chain linear alcohols as reported for fatty alcohol oxidase from *Candida apicola* [15]. The enzyme has not been successfully isolated, but the isolation of DNA that codes for this enzyme using the RT-PCR method is being worked on in our laboratory. If recombinant MsAO is successfully produced, we will perform a more detailed study of the substrate specificity using purified enzyme.

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