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Journal of Molecular Catalysis B: Enzymatic 38 (2006) 37–42
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

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

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Received 8 December 2004; received in revised form 22 October 2005; accepted 28 October 2005
Available online 5 December 2005

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.

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-

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doi:10.1016/j.molcatb.2005.10.006

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