Comparative Biochemistry and Physiology, Part C 205 (2018) 8–14

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Different response of acetylcholinesterases in salt- and detergent-soluble fractions of honeybee haemolymph, head and thorax after exposure to diazinon

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ARTICLE INFO

Keywords:
Acetylcholinesterase
Honeybee
Diazinon
Soluble
Membrane
Haemolymph

ABSTRACT

Organophosphate pesticide diazinon is a specific inhibitor of acetylcholinesterase (AChE), which is a common neurotoxicity biomarker in environmental studies. In honeybees, AChE exists in two forms having different physiological roles, one existing as a soluble form and the other as membrane-bound. In most studies AChE activity has been analysed without paying considerable attention to different forms of AChE. In this study, we exposed honeybees *Apis mellifera carnica* for 10 days to diazinon *via* oral exposure and analysed the total AChE activities in salt soluble (SS) and detergent soluble (DS) fractions. We assumed that SS fraction would preferentially contain the soluble AChE, but the DS fraction would contain only membrane AChE. On the contrary, our results showed that SS and DS fractions both contain soluble and membrane AChE and the latter has considerably higher activity. Despite this we obtained a differential response of AChE activity in SS and DS fractions when exposed to diazinon. The head/thorax AChE activity in DS fraction decreased, while the head/thorax AChE activity in SS fraction increased at sublethal concentrations. The AChE activity in honeybee hemolymph shown here for the first time is a salt soluble enzyme. Its activity remained unaltered after diazinon treatment. In conclusion, we provide evidence that varying results regarding AChE activity alterations upon stressor exposure are obtained when extracted through different procedures. In further environmental studies with honeybees this differential response of AChE activity should be given considerable attention because this affects the outcome of ecotoxicity study.

1. Introduction

The extensive use of pesticides has numerous negative effects including honeybee (*Apis mellifera* L.) colony loss (Johnson et al., 2010). Among the most commonly used pesticides are organophosphates which are known to inhibit acetylcholinesterase enzyme (AChE) activity (Thompson, 1999). Acetylcholinesterase is an ubiquitous enzyme found in almost all taxa, from uni- to multi-cellular organisms (Karczmar, 2010). The most well-known role of AChE is at cholinergic synapses where it terminates the synaptic signal transmission *via* rapid hydrolysis of the neurotransmitter acetylcholine (Ach; Kwong, 2002; Oruç and Usta, 2007; Kim et al., 2012). A number of non-neuronal roles of AChE have been reported as well. In vertebrates, AChE is involved in proliferation, differentiation, locomotion, migration, cell-cell contact, ciliary activity, immune function, and in the organization of the cytoskeleton (Karczmar, 2010). In invertebrates, reports show that AChE plays a role in fertilisation, embryogenesis (Cariello et al., 1986; Lu et al., 2012), tissue regeneration (Lenique and Feral, 1976; Fossati et al., 2015), honeybee brood rearing (Kim et al., 2017), and xenobiotic defense (Kang et al., 2011; Kim et al., 2012).

The physico-chemical properties and molecular structure of AChE isolated from honeybee heads has been thoroughly characterized (Belzunces et al., 1988a; Belzunces and Debras, 1997; Badiou et al., 2007; Kim et al., 2017). Head AChE occurs in two main forms, a major membrane form representing 94–97% of the total head AChE activity and a minor soluble form responsible for the remaining 4.5 ± 1.5% activity (Belzunces et al., 1988b). Both forms differ in their electrophoretic mobility, kinetic parameters, thermal stability and temperature dependence (Belzunces and Debras, 1997). The major difference between these two forms is the presence of the GPI- anchor sequence at the hydrophobic C-terminal region of membrane-AChE (Kim et al., 2017). It was shown that soluble-AChE is not the mere cleavage product

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https://doi.org/10.1016/j.cbpc.2017.12.004
Received 26 October 2017; Received in revised form 8 December 2017; Accepted 14 December 2017
Available online 16 December 2017
1532-0456/

of membrane-AChE by endogenous phospholipases or proteases indicating their regulated expression and possibly varying physiological functions (Belzunces et al., 1988a). Two separate gene locuses, *ace1* and *ace2*, exist for soluble- and membrane- AChE in *A. mellifera*, respectively (Kim and Lee, 2013). The membrane honeybee AChE form has an approximately 2500-fold higher catalytic efficiency towards acetylthiocholine than the soluble form which suggests that membrane-AChE is most probably involved in synaptic transmission (Kim et al., 2012). A tissue-specific expression profile distribution of AChE in honeybees showed that the membrane form is found primarily in tissues associated with the central nervous system (head and thoracic ganglia), while the soluble form is abundant in not only the central nervous system but also in the thorax, abdomen and legs (Kim et al., 2012).

The physiological roles of soluble-AChE in honeybees have been investigated less thoroughly. Kim et al. (2012) have shown that *in vitro* pre-incubation of OP and carbamate insecticides with a mixture of membrane- and soluble-AChEs significantly reduced the inhibition of membrane-AChE. This was interpreted as a protective role of soluble-AChE against xenobiotics. Also, Lee et al. (2015) reported that a chemical stressor induced synthesis of soluble-AChE in a fruit fly. Recently, it was found that soluble honeybee AChE expression intensity is inversely correlated to brood rearing: the soluble-AChE expression level increased in the honeybee head and abdomen when the brood-rearing activity was significantly suppressed (Kim et al., 2017). These authors suggested that soluble-AChE was likely to be involved in signal pathways related to brood-rearing activities, or alternatively the soluble-AChE expression level could be a simple downstream consequence of the brood-rearing status. In parallel, it has been reported that the hypopharyngeal gland of honeybees secretes ACh in royal jelly which is fed to larvae (Colhoun and Smith, 1960; Wessler et al., 2016). When acetylcholinesterase was added to the royal jelly, ACh levels were decreased leading to disturbed development of the brood (Wessler et al., 2016). Considering the finding of Wessler et al. (2016) and Kim et al. (2017) it may be that the high AChE levels in the non-brood rearing period are tightly connected to low levels of ACh, but this needs to be explored further.

Acetylcholinesterase activity is among one of the most frequently used neurotoxicity biomarkers in environmental studies. In most of these the homogenates without separating the salt soluble and detergent soluble fractions were used to assess AChE activity (Najimi et al., 1997; Olsen et al., 2001; Varó et al., 2002; Lionetto et al., 2003; Frasco et al., 2005; Duquesne, 2006). In honeybees, only a few studies paid attention to both types of AChEs and their different physiological roles (Belzunces and Debras, 1997). The AChE activity was most commonly measured in the brain (Milivojević et al., 2015) or head capsules of honeybees (Badiou et al., 2007; Carvalho et al., 2013; Wessler et al., 2016), but only very limited data is available for other body regions (Rabea et al., 2010). According to the various physiological roles and distinct biochemical and kinetic properties of both membrane- and soluble-AChE, it could be expected that pollutants affect these two AChE forms differently (Belzunces and Debras, 1997; Kim et al., 2012). However, it is difficult to distinguish between soluble and membrane AChEs activities in the homogenate. Previously, the activities of soluble and membrane AChE were analysed by Badiou et al. (2008) where the relative activities after treating honeybees with deltamethrin were measured by scanning of non-denaturing electrophoretic gels after AChE staining. In the work presented here, we assessed the protocol to isolate AChE in two homogenate fractions: the salt soluble (SS) and detergent soluble (DS) fractions according to Das et al. (2001). We presumed that the DS fraction comprises predominately membrane AChE, because we extracted proteins by adding Triton X-100 from pellets only which contain membranes. The SS phase was removed prior to extraction without adding the Triton X-100 and we assumed that this fraction contains predominately the soluble AChE. Polyacrylamide gel electrophoresis followed by AChE staining was utilized to evaluate the presence of soluble and membrane AChEs in SS and DS tissue fractions and haemolymph of untreated honeybees.

The aim of our work was to investigate the alterations of AChE activity in SS and DS fractions of honeybee *Apis mellifera carnica* head, thorax and haemolymph, when exposed to organophosphate diazinon *via* a 10-days sucrose feeding experiment. Diazinon was selected as a test substance because it is a well-known irreversible AChE specific inhibitor (Čolović et al., 2011). Although the use of this pesticide active ingredient is not approved in the EU and USA (EU Pesticides Database, 2017), it is still in use in some countries (Pest Management Regulatory Agency, 2013). We discuss the alterations of AChE activities in different honeybee tissue fractions and haemolymph. Specifically, we focus on the importance of protein extraction procedures when using this enzyme as a biomarker in environmental studies.

2. Materials and methods

2.1. Materials

Bideionized water (MQ) with a resistivity of 18.2 MΩ·cm (Milli-Q Advantage A10, MILLIPORE) was used for standards and sample solutions preparation. Diazinon PESTANAL® standard (98.4% HPLC), absolute ethanol, acetonitrile, sucrose, non-ionic detergent Triton X-100; dibasic and monobasic potassium phosphate, sodium hydrogencarbonate ($NaHCO_3$); 5,5′ dithiobis-2-nitrobenzoic acid (DTNB); acetylthiocholine chloride (ACh-Cl), $CuSO_4$, trisodium citrate, and bovine serum albumin analytical standard (BSA) were all of analytical grade and purchased from Sigma-Aldrich Co. BCA Protein Assay Reagent A and BCA Protein Assay Reagent B were purchased from Pierce (Thermo Fisher Scientific, USA). Tris and glycine were from Serva; HCl, acetic acid, and glycerol from Carlo Erba; acetate and bromphenol blue from Kemika; and potassium ferricyanide from Fluka. The 96-well flat base, transparent, polystyrene plates were used for enzyme assays (Sarstedt, Germany). All buffer solutions were filter-sterilized through a 0.22 μm filter (MILLIPORE).

2.2. Collection and preparation of the honeybees

The western honeybee subspecies used in the study was *Apis mellifera carnica*, Pollman 1879 (Insecta, Hymenoptera: Apidae). Honeybees originated from the hive on the estate at the Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, Ljubljana, Slovenia, and were maintained according to good honeybee practice. The bees were obtained from the same adequately fed, healthy, queen-right colony with known history and physiological status, and were not treated with any chemical substances at least 4 weeks prior to experiments.

The summer adult worker honeybees were collected from honeycomb frames inside the hive using an aspirator on the morning of the experiment. The collected honeybees were randomly allocated to test cages to form groups of 20–23 bees, and cages were then randomly designated a treatment. Bees were provided *ad libitum* with dechlorinated water and 1.5 M sucrose solution during the collection. After collection, the cages with bees were transferred to an incubator (34 °C, 60% RH), provided *ad libitum* with dechlorinated water only, and left to starve for 2 h so that they were all equal in terms of their gut contents at the start of the test.

2.3. Test cages and feeders

Rectangular wooden cages (10 × 6 × 7 cm; length × width × - height) were used. The frame was made of untreated spruce wood, steel wire mesh, and sliding transparent glass. The cage roof had 2 openings for feeders with food and water, and floor was covered with disposable cork to prevent contamination of the wood. The cages were well-ventilated and easy to clean. Graduated 5 mL sterile syringes for single use with open ends were used as feeders (polypropylene + polyethylene;