Pesticide Biochemistry and Physiology 103 (2012) 94–100

Contents lists available at SciVerse ScienceDirect

Pesticide Biochemistry and Physiology

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

Cloning of the acetylcholinesterase 1 gene and identification of point mutations putatively associated with carbofuran resistance in *Nilaparvata lugens*

Deok Ho Kwon [a], Deok Jea Cha [a], Young Ho Kim [b], Si Woo Lee [c], Si Hyeock Lee [a,b,*]

[a] Department of Agriculture and Life Science, Seoul National University, Seoul 151-742, Republic of Korea
[b] Research Institute for Agriculture and Life Science, Seoul National University, Seoul 151-742, Republic of Korea
[c] National Academy of Agricultural Science, Rural Development Administration, Suweon 441-707, Republic of Korea

ARTICLE INFO

Article history:
Received 15 November 2011
Accepted 16 April 2012
Available online 3 May 2012

Keywords:
Nilaparvata lugens
Carbofuran
Resistance
Acetylcholinesterase
Point mutation

ABSTRACT

Molecular mechanisms of carbofuran resistance in the brown planthopper, *Nilaparvata lugens* Stål, were investigated. A carbofuran-resistant strain (CAS) showed approximately 45.5- and 15.1-fold resistance compared with a susceptible strain (SUS) and a non-selected field strain (FM), respectively. Activities of the esterase and mixed-function oxidase were approximately 2.8- and 1.6-fold higher, respectively, in the CAS strain than in the SUS strain, suggesting that these enzymes play a minor role in carbofuran resistance. Interestingly, the insensitivity of acetylcholinesterase (AChE) to carbofuran was approximately 5.5- and 3.7-fold higher in the CAS strain compared to the SUS and FM strains, respectively, indicating that AChE insensitivity is associated with carbofuran resistance. Western blot analysis identified two kinds of AChEs, of which the type-1 AChE (encoded from *Nlace1*, which is paralogous to the *Drosophila* AChE gene) was determined to be the major catalytic AChE in *N. lugens*. The open reading frame of *Nlace1* is composed of 1989 bp (approximately 74 kD) and revealed 52.5% and 24.3% amino acid sequence identities to those of *Nephotettix cincticeps* and *Drosophila melanogaster*, respectively. Screening of point mutations identified four amino acid substitutions (G119A, F/Y330S, F331H and H332L) in the CAS strain that likely contribute to AChE insensitivity. The frequencies of these mutations were well correlated with resistance levels, confirming that they are associated with reduced sensitivity to carbofuran in *N. lugens*. These point mutations can be useful as genetic markers for monitoring resistance levels in field populations of *N. lugens*.

1. Introduction

The brown planthopper, *Nilaparvata lugens* Stål, is one of the serious pests of rice in Southeast Asia [1]. It causes yield loss of rice directly by sucking saps from the stem and indirectly by transmitting the grassy stunt virus disease. In Korea, *N. lugens* has been known as a major migratory insect species that invaded from China [2]. The control of *N. lugens* has mainly depended on organophosphate (OP), carbamate (CB), pyrethroid, nereistoxin and neonicotinoid insecticides. Among them, CB and OP insecticides have been used for the past 50 years to control *N. lugens*. The extensive use of CB and OP insecticides has led to resistance development in the *N. lugens* populations in Japan, Taiwan, Solomon Islands, Philippines, Malaysia and Korea [2–4].

Elevated carboxylesterase (CbE) activity has been suggested as a malathion resistance factor in *N. lugens* [4–6]. Among the CbE isozymes identified, E1 CbE exhibited notably higher activity and was present in greater quantities in a resistant population [5]. The NlEST1 gene, which was assumed to encode the E1 CbE, was later cloned and characterized from an OP-resistant *N. lugens*, and NlEST1 overexpression due to gene amplification was determined to be responsible for OP resistance [6].

Along with enhanced CbE activity, the acetylcholinesterase (AChE, EC 3.1.1.7) insensitivity is a primary resistance mechanism to OP and CB insecticides in a wide variety of insect species [7]. The AChE is a key enzyme in the nervous system of both vertebrates and invertebrates, as it regulates the level of the neurotransmitter acetylcholine and terminates nerve impulses [8]. Various point mutations near the catalytic triad and other functionally important locations of AChE cause alteration of the enzyme's structure, resulting in insensitivities to OP and CB insecticides [9]. Two different loci of the AChE gene (*ace1* encoding type-1 AChE, which is paralogous to the *Drosophila* AChE gene; and *ace2* encoding type-2 AChE, which is orthologous to the *Drosophila* AChE gene) have been found in most insects, including aphids, rice green

Abbreviations: CB, carbamate; OP, organophosphate; AChE, acetylcholinesterase; *Nlace1*, *N. lugens ace1* gene; NlAChE1, *N. lugens* AChE1; CbE, carboxylesterase.

* Corresponding author. Address: Department of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Republic of Korea. Fax: +82 2 873 2319.
E-mail address: shlee22@snu.ac.kr (S.H. Lee).

0048-3575/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pestbp.2012.04.003

leafhoppers and white flies [10–14]. In insect species possessing both *ace1* and *ace2*, such as *Plutella xylostella* [15], *Helicoverpa assulta* [16] and *Blattella germanica* [17], *ace1* encodes the predominant catalytic AChE (AChE1), which is likely involved in the target site insensitivity to OP and CB insecticides. In *N. lugens*, however, only *ace2* has been cloned to date, and a point mutation (G185S) in *ace2* has been suggested to be associated with methamidophos resistance [18]. To investigate the primary AChE involved in the target site insensitivity to OP and CB insecticides in *N. lugens*, therefore, it is necessary to identify *ace1* in *N. lugens* and to determine which *ace* locus encodes the main catalytic AChE.

In this paper, we investigated the biochemical and molecular mechanisms of carbofuran resistance in *N. lugens*. In an attempt to examine the target site insensitivity mechanism, we cloned the *ace1* gene from *N. lugens* (*Nlace1*) and screened the point mutations putatively associated with carbofuran resistance. Moreover, we verified the relatedness of AChE1 mutations to phenotypic resistance traits in *N. lugens*.

2. Materials and methods

2.1. Strains

The susceptible strain (SUS), which has been maintained for 28 years without exposure to any insecticides, was obtained from the National Academy of Agricultural Science (Suweon, South Korea). The field strains were collected from Gangjin, Wando, Haenam and Namhae provinces in South Korea in 2009. Four strains were combined and designated as the field mix (FM) strain. The FM strain was selected four times with median lethal concentrations (LC_{50}, 9–45 ppm) of carbofuran GR (a.i. 8%, Gyeongnong, South Korea), and the selected carbofuran-resistant strain was designated as CAS. All strains were reared on rice seedlings (var. Chucheong; 15–20 days after germination) in acrylic cages (26 × 30 × 20 cm) at 26 ± 1 °C with a long-day photoperiod (16L:8D).

2.2. Bioassay

Carbofuran (99.9% ChemService, PA, USA) was serially diluted in acetone and an aliquot (0.2 μl) of the carbofuran solution was topically applied to the abdomen of 17–20 individual females using a PB-600 Repeating Dispenser (Hamilton Company, Reno, NV). The carbofuran-treated *N. lugens* were transferred to rice seedlings, and their mortalities were observed at 24 h post-treatment. The bioassay was conducted in three replicates, and the median lethal dose (LD_{50}) was determined by probit analysis using SPSS (IBM Corp., Armonk, NY).

2.3. Enzyme preparation

Females (50–56 mg) were homogenized in 500–560 μl extraction buffer (0.1 M Tris–HCl, 0.5% Triton X-100, pH 7.8) using a 1-ml glass–glass tissue grinder (Wheaton Science Product, Millville, NJ) and the homogenate was centrifuged at 12,000*g* for 15 min. The supernatant was used as the enzyme source for the determination of esterase (EST), glutathione-*S*-transferases (GSTs) and AChE activities. For the determination of mixed function oxidase (MFO) activities, 1000*g* supernatants from homogenates were used as enzyme sources. Protein concentrations were determined using the bicinchoninic acid (BCA) kit (Sigma–Aldrich, St. Louis, MO) with bovine serum albumin as the standard protein.

2.4. Enzyme activity assays

For the EST assay, the enzyme source (25 μg protein) was incubated with 0.2 mM 1-naphthyl acetate (1-NA, Sigma) for 120 s at 25 °C, and its optical density was determined at 595 nm using a VERSA max microplate reader (Molecular Devices, Inc. Sunnyvale, CA). The same amount of enzyme was also incubated with either 0.2 mM 4-nitrophenyl acetate (4-NPA, Sigma) or 0.2 mM 4-nitrophenyl butyrate (4-NPB, Sigma) for 20 s at 25 °C, and the optical density was measured at 405 nm as described above.

The GST activity assay was conducted using the method of Yu with a slight modification [19]. The enzyme source (50 μg protein) was incubated with 4 mM 1-chloro-2.4-dinitrobenzene (CDNB) and 1 mM GSH (reduced glutathione) for 300 s at 25 °C and optical density measured at 355 nm using a VERSA max microplate reader (Molecular Devices, Inc.).

For the MFO assay, the enzyme source (100 μg protein) was incubated with 0.4 mM 7-ethoxycoumarin (7-EC) and 1 mM NADPH for 30 min at 30 °C in a 96-well microtiter plate. NADPH fluorescence was quenched by oxidizing it to the nonfluorescent NADP+ with the addition of 10 μl of oxidized glutathione (100 mM in water) and 20 μl glutathione reductase (1 unit) [20]. After a 10-min incubation at room temperature (RT), the reaction was stopped with the addition of 125 μl 50% acetonitrile in 50 mM Tris buffer (pH 10). The amount of 7-EC released during incubation was quantified using a SPECTRAmax GEMINI XS spectrofluorometer (Molecular Device, Inc.) at 390-nm excitation and 465-nm emission wavelengths.

AChE activity was measured by the Ellman method with a slight modification [21]. To calculate activity, the enzyme source (50 μg protein) was incubated with 0.4 mM 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB, Sigma) and 0.1 mM acetylthiocholine iodide (ATChI) at 25 °C for 10 min. The AChE activity was measured at 415 nm using a VERSA max microplate reader (Molecular Devices, Inc.).

2.5. AChE inhibition assay

Calculation of the median inhibition concentration (I_{50}) was conducted by preincubating the enzyme source (50 μg protein) with carbofuran solutions (0.003–1 μM) for 10 min prior to the addition of 0.1 mM ATChI and 0.4 mM DTNB. The remaining AChE activity was measured as described above. The I_{50} was determined by probit analysis by SPSS software (IBM Corp., Armonk, NY) using three replicates.

2.6. Native-polyacrylamide gel electrophoresis (PAGE) and AChE activity staining

Enzyme extraction, native-PAGE, Western blotting and AChE activity staining were conducted as described previously with some modifications [22]. Enzymes were extracted from *N. lugens* heads using a micro tissue grinder (Radnoti, Monrovia, CA, USA) with 0.1 M Tris–HCl (pH 7.8) containing 0.5% Triton X-100 (v/v) and centrifuged at 12,000*g* for 15 min at 4 °C. The supernatant was filtered through a glass wool filter to remove excess lipid and stored at −75 °C. Electrophoresis was carried out with a vertical electrophoresis unit (Novex® mini cell, Invitrogen Corp., Carlsbad, CA). Protein preparations from various tissues (20 μg) were separated by native-PAGE (7.5%) at 120 V for 100 min in a cold chamber with a continuous Tris–glycine buffer system. The gel and running buffers contained 0.5% Triton X-100 (v/v). Following native-PAGE, one set of gels was activity-stained to visualize AChE bands according to [23], and the other set was used for Western blot analysis.

2.7. AChE1 and AChE2 detection by Western blotting

Proteins separated on a gel were transferred onto a Hybond-N nitrocellulose membrane (GE Healthcare, Pittsburgh, PA) by