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OPTIMIZATION OF A BIOASSAY METHOD FOR SPECIFIC ACTIVITY OF ACETYLCHOLINESTERASE OF B BIOTYPE *BEMISIA TABACI* (HEMIPTERA: ALEYRODIDAE)

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ABSTRACT

The whitefly *Bemisia tabaci* (Gennadius), a major pest of protected and outdoor crops worldwide, has developed resistance to a wide range of insecticides including organophosphates (OPs) and carbamates (CBs). The biochemical target of OPs and CBs is acetylcholinesterase (AChE). The objective of this study was to optimize AChE bioassay conditions including enzyme concentration, substrate concentration, pH, temperature and reaction time in order to quickly and accurately monitor specific activity of AChE and monitor resistance of *B. tabaci* to insecticides. The results of an orthogonal array test showed that the optimum combination of levels was $A_3B_3C_4D_5E_1$ in which the enzyme concentration was 20 adults/mL, substrate concentration was 7.0×10^{-4} mol/L, pH was 7.5, temperature was 45 °C, and reaction time was 5 min. The specific activity of AChE from B Biotype *B. tabaci* was 41.23 nmol · min^{-1} · mg^{-1} under this set of conditions. This optimum combination proved to be reliable and accurate for testing the specific activity of AChE.

Key Words: B biotype, insecticide resistance, specific activity, orthogonal array

RESUMEN

La mosca blanca *Bemisia tabaci* (Gennadius), una plaga importante en cultivos sembrados en lugares protegidos y en el campo en todo el mundo, ha desarrollado resistencia a una amplia gama de insecticidas, incluyendo organofosfatos (OF) y carbamatos (CBs). El enfoque bioquímico de los OFs y carbamatos es acetilcolinesterasa (AChE). El objetivo de este estudio fue el optimizar las condiciones del bioensayo de AChE incluyendo la concentración de enzima, la concentración de sustrato, pH, temperatura y tiempo de reacción. Los resultados de una prueba de matriz ortogonal mostró que la combinación óptima de los niveles fue $A_3B_3C_4D_5E_1$ en el que la concentración de enzima fue 20 adultos /ml, concentración de sustrato de 7.0×10^{-4} mol/L, pH de 7.5, temperatura de 45 °C y la reacción tiempo de 5 min. La actividad específica de AChE en el biotipo B de *B. tabaci* fue 41.23 nmol · min^{-1} · mg^{-1} bajo estas condiciones. Esta combinación óptima demostró ser fiable y precisa para probar la actividad específica de AChE.

Palabras Clave: biotipo B, resistencia a insecticidas, actividad específica, matriz ortogonal

The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is one of the most destructive insect pests of numerous protected and field crops worldwide, infesting more than 600 plant species (Xu et al. 2011; Pan et al. 2012). The pest has been considered as a species complex that includes more than 24 biotypes such as A, B, Q and Ms, which can be morphologically indistinguishable, and among which biotypes B and Q are the 2 most invasive and widely distributed (Perring 2001; Liu et al. 2007; Chu et al. 2010; Xie et al. 2012).

The pest status of *B. tabaci* has risen considerably in the past 20 yr, because of widespread invasions by the B and Q biotypes (De Barro et al. 2011; Liu et al. 2012). Biotype B has caused especially serious economic losses (Teng et al. 2010). *Bemisia tabaci* directly damages the plants by feeding on phloem sap, and by excreting honeydew on the leaves and fruit (Horowitz et al. 2011), which supports soot mold, which interferes with photosynthesis. Thus *B. tabaci* is considered to be a species complex of diverse cryptic biotypes with differences in host range, host plant adaptability, development rate, insecticide resistance and virus-transmission capability (Bedford et al. 1994; Brown et al. 1995; Wang & Tsai 1996; Berry et al. 2004; Horowitz et al. 2005; Cloyd et al. 2012).

Acetylcholinesterase (AChE; EC 3.1.1.7), an essential hydrolytic enzyme in the cholinergic nervous system, is responsible for catalyzing the degradation of acetylcholine (ACh) into acetate and choline (Dvir et al. 2010; Shi et al. 2012). As a key enzyme in the insect central nervous system, it is a target for the development of insecticides that inhibit it.

Bemisia tabaci has developed resistance to a wide range of insecticides including organophosphates (OPs), carbamates (CBs), synthetic pyrethroids (SPs), cyclodienes, neonicotinoids and insect growth regulators (IGRs) (Cahill et al. 1996; Ahmad et al. 2002; El Kady & Devine 2003; Horowitz et al. 2004; Roditakis et al. 2005; Roditakis et al. 2006; Roditakis et al. 2009; Dennehy et al. 2010; Vassiliou et al. 2011; Yuan et al. 2012). Toxicity of OPs and CBs to animals is attributed to their ability to inhibit the AChE enzyme from breaking down ACh, leading to an increase of both the level and duration of action of the neurotransmitter, ACh (Dulin et al. 2012). Two important classes of inhibitors, OPs and CBs, are analogs of the substrate, ACh, and account for more than 35% of total global insecticide sales (Alon et al. 2008).

Use of chemical insecticides has been the primary strategy for controlling *B. tabaci* (Yuan et al. 2012), and OPs and CBs have played a key role for this purpose for several decades because of their efficacy, fast knockdown activity and low persistence. OP - and CB - resistance in *B. tabaci* is geographically widespread (Cahill et al. 1995). Insensitive AChE has been identified as the most important mechanism of resistance of *B. tabaci* to these 2 classes of insecticides (Byrne & Devonshire 1997).

The optimum conditions for determining the activity of an enzyme activity—such as pH, substrate concentration, temperature, etc.—vary depending on which tissue of an organism is utilized, and which arthropod species is being assayed (Thompson 1999). The primary aim of the present study was to determine the optimum conditions to bioassay the AChE of *B. tabaci* including enzyme concentration, substrate concentration, pH, temperature and reaction time. This information is needed in order to quickly and accurately monitor the specific activity of AChE, and thereby monitor the development of resistance of *B. tabaci* to OP and CB insecticides.

Materials and Methods

Bemisia tabaci Strain

The susceptible reference strain of *B. babaci* was collected from a cucumber field, which had not been subjected to applications of any insecticides, and subsequently maintained for 10 generations in the greenhouse of the Guangdong Entomological Institute, Guangzhou, China. This population was identified as the B biotype by use of a mtDNA COI marker in the laboratory (Luo et al. 2002).

Chemicals

Reagents used in these experiments included acetylthiocholine iodide (ATChI, Sigma), 5,5'-dithio bis-(2-nitrobenzoic acid) (DTNB, Shanghai Yanhui Biotechnology LLC.), eserine (Sigma), albumin bovine V (BSA, Sigma), Coomassie Brilliant Blue G250 (Sigma).

Preparation of Enzyme Solution

Uniformly sized *B. tabaci* adults were selected. They were homogenized in 1 mL 0.1 mol/L of phosphate buffered saline (PBS), containing 0.1% (v/v) Triton X-100. After homogenization, the preparation was centrifuged at 4 °C, 15,000 × g for 20 min and the supernatant was used as the enzyme source.

Measurement of AChE Activity

AChE activity of *B. tabaci* was measured according to the method of Gorun et al. (1978), which is was based on the method of Ellman et al. (1961). In this method the hydrolysis of the substrate analog acetylthiocholine (ATChI) was determined colorimetrically by the absorbance of 2-nitro-5-thiobenzoate at 405 nm, after the reaction of DTNB with the liberated thiocholine. Thus 0.1 mL enzyme preparations, 0.1 mL 0.075 mol/L ATChI solution and 0.9 mL 1×10^{-3} DTNB were combined and mixed. After the reaction was largely complete, 0.1 mL 1×10^{-2} mol/L eserine was added to terminate it. Next a 200 µL solution of the 1.2 mL final volume was placed in a microplate well and monitored colorimetrically at 405 nm. The inactivated enzyme solution served as the control. Each treatment was replicated 3 times.

Determination of the Protein Content of the Enzyme Solution

Determination of the protein content was accomplished by the method of Bradford (1976). A 0.1 mL sample was added to 0.5 mL Coomassie Brilliant Blue G-250. After mixing, the OD_{595} value was measured by a microplate reader at 595 nm between 2 min and 1 h. The protein content of the enzyme solution was calculated based on the standard curve.

Orthogonal Design of Bioassay Method for Specific Activity of AChE

In order to obtain an optimized bioassay method for the specific activity of AChE of *B. tabaci*, a 5-factor 5-level orthogonal test was used (Yang 2002). The adopted 5 factors were: A: enzyme concentration (adults/mL), B: substrate concentration (mol/L), C: pH, D: temperature (°C), and E: reaction time (min). An orthogonal table of $L_{25}(5^6)$ was used. The experimental factors and their levels are shown in Table 1.

Table 1. Experimental factors and their levels in orthogonal tests of acetylcholine esterase of *Bemisia tabaci* B biotype.

	Factors*				
Level	A	B	C	D	E
1	10	1.0×10^{-4}	6.0	25	10
2	15	2.0×10^{-4}	6.5	30	15
3	20	3.0×10^{-4}	7.0	35	20
4	25	4.0×10^{-4}	7.5	40	25
5	30	5.0×10^{-4}	8.0	45	30

*the adopted 5 factors were: A: enzyme concentration (adults/ml), B: substrate concentration (mol/l), C: ph, D: temperature (°C), and E: reaction time (min).

Statistical Analyses

Data were analyzed by one-way analysis of variance (ANOVA) using SPSS software (SPSS Inc., Chicago, Illinois, USA). Statistical analyses were performed on 3 replicates of data obtained per treatment by Tukey's test at $P < 0.05$ and $P < 0.01$ to identify significant and highly significant differences between groups.

Results

The specific activity of AChE from B Biotype *B. tabaci* (Table 2) varied from 0.43 to 38.11 nmol · min^{-1} · mg^{-1}, reflecting the differences of AChE activity in different conditions.

As shown in Table 3, the R-value of range analysis demonstrated that the effect of these variables was reduced in the order of E > A > D > C > B. It was obvious that reaction time was the most potent factor followed in descending order by enzyme concentration, temperature, pH and substrate concentration.

The results of range analysis revealed the optimum level of each factor to be as follows: A = 3, i.e., enzyme concentration of 20 adults/mL; B = 3, i.e., substrate concentration was 7.0×10^{-4} mol/L;

Table 2. Specific activity values determined in the orthogonal array test for acetylcholine esterase of *Bemisia tabaci* B biotype.

		Factors[1]						
No.	Combination[2]	A (adults/mL)	B (mol/L)	C	D (°C)	E (min)	Blank	Specific activity (nmol · min^{-1} · mg^{-1})
1	$A_1B_1C_1D_1E_1$	10	6.0×10^{-4}	6.0	25	5	1	5.70 ± 0.64
2	$A_1B_2C_2D_2E_2$	10	7.0×10^{-4}	6.5	30	10	2	1.62 ± 0.17
3	$A_1B_3C_3D_3E_3$	10	8.0×10^{-4}	7.0	35	15	3	1.61 ± 1.21
4	$A_1B_4C_4D_4E_4$	10	9.0×10^{-4}	7.5	40	20	4	1.48 ± 0.96
5	$A_1B_5C_5D_5E_5$	10	10.0×10^{-4}	8.0	45	25	5	0.43 ± 0.75
6	$A_2B_1C_2D_3E_4$	15	6.0×10^{-4}	6.5	35	20	5	11.95 ± 0.47
7	$A_2B_2C_3D_4E_5$	15	7.0×10^{-4}	7.0	40	25	1	5.53 ± 1.65
8	$A_2B_3C_4D_5E_1$	15	8.0×10^{-4}	7.5	45	5	2	38.11 ± 1.22
9	$A_2B_4C_5D_1E_2$	15	9.0×10^{-4}	8.0	25	10	3	11.23 ± 0.48
10	$A_2B_5C_1D_2E_3$	15	10.0×10^{-4}	6.0	30	15	4	3.88 ± 0.92
11	$A_3B_1C_3D_5E_2$	20	6.0×10^{-4}	7.0	45	10	4	22.16 ± 1.67
12	$A_3B_2C_4D_1E_3$	20	7.0×10^{-4}	7.5	25	15	5	10.13 ± 0.29
13	$A_3B_3C_5D_2E_4$	20	8.0×10^{-4}	8.0	30	20	1	9.22 ± 0.36
14	$A_3B_4C_1D_3E_5$	20	9.0×10^{-4}	6.0	35	25	2	6.18 ± 0.44
15	$A_3B_5C_2D_4E_1$	20	10.0×10^{-4}	6.5	40	5	3	27.99 ± 0.52
16	$A_4B_1C_4D_2E_5$	25	6.0×10^{-4}	7.5	30	25	3	5.33 ± 0.38
17	$A_4B_2C_5D_3E_1$	25	7.0×10^{-4}	8.0	35	5	4	23.74 ± 1.42
18	$A_4B_3C_1D_4E_2$	25	8.0×10^{-4}	6.0	40	10	5	13.59 ± 1.08
19	$A_4B_4C_2D_5E_3$	25	9.0×10^{-4}	6.5	45	15	1	11.65 ± 0.59
20	$A_4B_5C_3D_1E_4$	25	10.0×10^{-4}	7.0	25	20	2	3.69 ± 0.12
21	$A_5B_1C_5D_4E_3$	30	6.0×10^{-4}	8.0	40	15	2	11.62 ± 0.79
22	$A_5B_2C_1D_5E_4$	30	7.0×10^{-4}	6.0	45	20	3	3.69 ± 0.88
23	$A_5B_3C_2D_1E_5$	30	8.0×10^{-4}	6.5	25	25	4	4.41 ± 0.24
24	$A_5B_4C_3D_2E_1$	30	9.0×10^{-4}	7.0	30	5	5	17.71 ± 1.35
25	$A_5B_5C_4D_3E_2$	30	10.0×10^{-4}	7.5	35	10	1	11.78 ± 1.49

[1]The adopted 5 factors were: A: enzyme concentration (adults/mL), B: substrate concentration (mol/L), C: pH, D: temperature (°C), and E: reaction time (min).

[2]The arrangements of A, B, C, D and E were decided by orthogonal design for 5 (factor) × 25 (run number). Every row of A run number represents 1 experimental replicate. Every run was replicated 3 times and every replicate was measured 3 times. Values are mean ± SD.

Table 3. Range analysis of the results of orthogonal projects to optimize determination of acetylcholine esterase of *Bemisia tabaci* B biotype.

	Specific activity of AChE (nmol · min^{-1} · mg^{-1})				
	A	B	C	D	E
K_1	10.84	56.76	33.04	23.21	113.25
K_2	70.70	44.70	57.61	37.76	60.39
K_3	75.67	66.94	50.70	55.25	38.88
K_4	58.00	48.25	66.83	60.20	30.02
K_5	49.20	47.77	56.24	76.04	21.88
k_1	2.17	11.35	6.61	4.64	22.65
k_2	14.14	8.94	11.52	7.55	12.08
k_3	15.13	13.39	10.14	11.05	7.78
k_4	11.60	9.65	13.37	12.04	6.00
k_5	9.84	9.55	11.25	15.21	4.38
R	12.97	4.45	6.76	10.57	18.27
Optimal level	3	3	4	5	1

Notes: K_i is the sum of the specific activity of AChE of the i level of different factors, and k_i represents the average of K_i.
The adopted 5 factors were: A: enzyme concentration (adults/mL), B: substrate concentration (mol/L), C: pH, D: temperature (°C), E: reaction time (min).

C = 4, i.e., pH was 7.5; D = 5, i.e., temperature was 45 °C; and E = 1, i.e., reaction time was 5 min.

Range analysis of the orthogonal test results was relatively simple and intuitively obvious, but did not provide estimates of the magnitude of the error. Therefore more extensive calculations with ANOVA were needed to provide estimates of the magnitude of the error terms. The results of analysis of variance are shown in Table 4. The specific activity of AChE from *B. tabaci* B biotype was significantly affected by the variable factors of enzyme concentration, substrate concentration, pH, temperature and reaction time of which reaction time and enzyme concentration had the greatest effects on the determination of specific activity. In descending order the magnitude of this effect was as follows: E> A > D > C > B, i.e. reaction time > enzyme concentration > temperature > pH > substrate concentration.

In conclusion, the results of range analysis and analysis of variance were consistent. The optimum combination of factor levels was $A_3B_3C_4D_5E_1$: enzyme concentration was 20 adults/mL, substrate concentration was 7.0×10^{-4} mol/L, pH was 7.5, temperature was 45 °C, reaction time was 5 min. However, the best combination ($A_3B_3C_4D_5E_1$) was not included in the orthogonal experimental design table (Table 2). The specific activity of AChE from *B. tabaci* B Biotype was determined under this condition. The result was 41.23 nmol · min^{-1} · mg^{-1}, which was higher than the results of any combination of the orthogonal design table. We found the optimum combination to be reliable and accurate for testing the specific activity of AChE.

Discussion

The relationship of AChE to insecticide resistance of *B. tabaci* needs to be further studied. Variability of insensitivity of the AChE of *B. tabaci* was affected by enzyme concentration, substrate concentration, pH, temperature and reac-

Table 4. Analysis of variance (ANOVA) of the results from orthogonal projects to optimize determination of acetylcholine esterase of *Bemisia tabaci* B biotype.

Variance origin	Sum of square	Degrees of freedom	Mean square	F
A (enzyme conc.)	528.887	4	132.222	15.705**
B (substrate Conc.)	65.407	4	16.352	1.942*
C (pH)	125.348	4	31.337	3.722*
D (Temperature °C)	227.678	4	56.920	6.761**
E (reaction time - min)	1075.926	4	268.981	31.948**
Error	33.677	4	8.419	
Total	4853.852	25		

Notes: In order to improve the accuracy of the analysis, errors of replication and blank row were consolidated in the error item.
"*"and"**"represent significant difference ($P \leq 0.05$) and highly significant difference ($P \leq 0.01$), respectively.

tion time. Reaction time, enzyme concentration, temperature had extremely significant effects on the measurement of AChE in *B. tabaci*. Therefore, these 3 factors should be strictly controlled to improve the accuracy of the determination.

Physicochemical properties of AChE, such as molecular size, shape, solubility, etc., may vary during the course of development of a species. The AChE activities of some insect species were found to increase with each successive instar (Bradford 1976), however, the reverse occurred in some other species (Hill 1989). *Bemisia tabaci* adults used for determining the specific activity of AChE must be consistent in size and age.

The AChE of insecticide-susceptible insects is inhibited by OPs and CBs, whereas that of resistant insects is insensitive to insecticide inhibition. The mechanism of OP - and CB - resistance in *B. tabaci* from different regions of the world has been found to be insensitive AChE (Dittrich et al. 1985, 1990; Byrne & Devonshire 1993, 1997; Byrne et al. 1994; Anthony et al. 1998; Byrne & Toscano 2002; Byrne et al. 2003; Erdogan et al. 2008). We have provided a quick and accurate method of determination of specific activity of the AChE of *B. tabaci*, which is a direct indication of the level of resistance to OPs and CBs.

Endnotes

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