Biochemical Studies of Na⁺,K⁺-ATPase and Acetylcholinesterase Sensitivity to Phenothrin and Thiodicarb Among Different Egyptian Field Populations of Spodoptera littoralis

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ABSTRACT

Enzymatic activity of Na⁺,K⁺-ATPase and AChE of cotton leafworm Spodoptera littoralis collected from different four Egyptian field populations ranged from heavily-sprayed fields and cultivated fields were investigated and compared with a laboratory susceptible population. The highest levels of Na⁺,K⁺-ATPase and AChE activities were found in Alexandria Governorate Egypt. The moderate levels was found in El-Boheira Governorate, Egypt. Na⁺,K⁺-ATPase and AChE and were isolated from brain of S. littoralis larvae (4th instar). The sensitivity of Na⁺,K⁺-ATPase and AChE activity to Phenothrin and Thiodicarb respectively were measured by the $I_{50}$ values. The $I_{50}$ values of Phenothrin on the Na⁺,K⁺-ATPase activity were 0.01, 0.20, 0.36, 0.61 and 0.82µM for lab strain; Borg El-Arab; Abeis; Damanhour and Abou El-Matamir respectively. The $I_{50}$ of Thiodicarb on AChE activity were 0.22, 0.43, 0.54, 0.71 and 0.96µM for lab strain and four field strains respectively. The inhibition constant ($K_i$) values were determined for Na⁺,K⁺-ATPase and AChE inhibitors. Values of $K_i$ in the case of Phenothrin were 5, 18, 20, 30 and 45µM for lab strain; Borg El-Arab; Abeis; Damanhour and Abou El-Matamir respectively on Na⁺,K⁺-ATPase activity. Similarly, Thiodicarb were 20, 28, 30, 40 and 50µM for lab strain and four field strains respectively on AChE activity. The results of the present study may add some forward steps to uses this enzymes indicate of effect this insecticides under study, in the IPM programs of the cotton leafworm.

INTRODUCTION

The Egyptian cotton leafworm Spodoptera littoralis is the major pest attacking several crops and vegetables in Egypt, this pest cause the greatest part of cotton yield losses (Smagghhe and Degheele, 1997; Amin et al., 2001; & Quero et al., 2002). Number of insecticides currently in widespread use: Organophosphates, Carbamates and Pyrethroids are usually used in Egypt (Devonshire and Moores 1982; & Argentine et al., 2002), to suppress the S. littoralis populations, however, most of them dose not give satisfactory results, probably because of development of resistance, (Ishaaya and Klein, 1990; & El-Aw et al., 2002). From this point the need for insect control is essential through chemical control (Pesticides) (Casida and Quistad 2005) so in the present study we began to study a two target in the insect to the knowleage about insecticide susceptibility.

In this work, we describe the development of a biochemical assay system for measuring the sensitivity of Na⁺,K⁺-ATPase and Acetylcholinesterase (AChE) to Phenothrin and Thiodicarb respectively. We also provide enzyme kinetic data for the Na⁺,K⁺-ATPase and AChE in this four field strains Abeis and Borg El-Arab (Alexandria Governorate) Damanhour and Abou El-Matamir (El-Boheira Governorate), and compared them with data obtained of lab strain.

MATERIALS AND METHODS

Insect:

a- The susceptible laboratory strain of Spodoptera littoralis was provided from centeral lab of pesticides, Agricultural Research Center (ARC) Cairo, Egypt which was reared for several years.

b- The field strain was obtained by the collection of the egg masses from cotton fields at Abeis and Borg El-Arab (Alexandria Governorate) Damanhour and Abou El-Matamir (El-Boheira Governorate); the 4th larval instar used for assessments.

Chemical:

Phenothrin (Pyrethroids) provided as technical grade insecticides from U.S.A. Environmental Protection Agency (EPA), USA. Ouabain is a cardiac glycoside which specifically inhibits the Na⁺,K⁺-ATPase (McIlwain,1963). A pure sample was obtained from Sigma Chem., Co. St. Louis. Thiodicarb (Carbamate) provided as technical grade insecticides from JinHung Fine Chem., Co. LTd. Koria. Stock solutions of these compounds were prepared in pure acetone.

Bioassay tests:

Fresh leaves of castor were dipped for 1min in different concentrations of the tested insecticides, all insecticides concentrations were prepared in acetone solution. Control plants were dipped in acetone solution. Treated and control plants were air-dried for 3hrs. The treated leaves were placed in clean glass container at the laboratory conditions of 27±2°C and 65-70%RH. Ten larvae (Lab and Field strains) were used for each test with three replicate at least. Number of alive and dead larvae per replicate was counted 24 and 48hr, after treatment. Concentration-mortality percentages were calculated and corrected for natural mortality according

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to Abbott equation (Abbott, 1925). LC₅₀ values were calculated by using the probit-analysis method of Finney (1971).

**Na⁺,K⁺-ATPase Preparation and Activity Assay:**

Head capsule from *S. littoralis* fourth-instar larvae dissected and homogenized in a solution of 0.32M sucrose, 1mM EDTA and 40mM Tris-HCl buffer (pH 7.4). The homogenate was filtered through two layers of cheese cloth. Mitochondrial ATPase was prepared according to the method reported by Koch (1969), by differential centrifugation of the homogenate at 8000Xg for 10min. The supernatant was then centrifuged at 20000Xg for 30min. The formed pellets were then suspended in the buffer and stored at (-20°C) for use.

The ATPase activity was measurements according to the method reported by Koch (1969), with slight modification by Morshedy (1980) using Tris-HCl buffer instead of imidazole buffer. Absorbancy of inorganic Phosphate (Pi) was measured at λ750nm (Taussky and Shorr, 1953). The method was based on the spectrophotometric determination of the inorganic Phosphate (Pi) liberated from the hydrolysis reaction of the ATP, mediated by the enzyme.

The ATPase activity was measured in a total volume of 1ml. The mitochondrial preparation was mixed with a reaction mixture (700µl) containing 100mM Na⁺, 20mM K⁺, 5mM Mg²⁺ chlorides, 40mM Tris-HCl buffer (pH 7.4), and 5mM ATP. The volume was completed to 850µl with buffer. The mixture was incubated for 15min, in a shaking water bath at 37°C. The reaction was stopped by adding 150µl trichloroacetic acid (TCA, 30%). Hydrolyzed Pi was determined according to the method reported by Koch (1969), by preincubating the enzyme with the inhibitor for 30min. Enzyme specific activity was computed as mg protein/hr.

The protein content in prepared homogenates of *S. littoralis* was assayed spectrophotometrically by the method of Lowery et al., (1951) at λ750nm using Bovine Serum Albumin (BSA ) as a standard protein.

**In Vivo and In Vitro Inhibition and Kinetics of Na⁺,K⁺-ATPase and AChE:**

The inhibition of Na⁺,K⁺-ATPase and AChE activity were determined in all tested sources using the LC₅₀ values of each of the two tested insecticides (Phenothrin and Thiodicarb) as inhibitors. The inhibitor for each of Na⁺,K⁺-ATPase and AChE were evaluated to determine enzyme kinetic parameters. The method of Dixon and Webb (1964) was adopted to draw the Dixon-plots by plotting 1/V versus concentrations of the inhibitor at two concentrations of the substrate. ATP (the substrate of ATPase) concentrations were 3.0 and 5.0mM, while acetycholine iodide (the substrate of AChE) was used at concentrations of 5 and 10mM.

Estimation of I₅₀ value was carried out by preincubating the enzyme with the inhibitor for 30min. Using the following concentrations 0.1; 1; 5; 10; 50 and 100µM. Kᵢ (the inhibition constant) values for each inhibitor were estimated from Dixon-plot.

Michaelis-Menten Kinetics (Kₘ & Vₘₐₓ) values were calculated by a linear regression of 6 points on each Lineweaver and Burk Plot (1934).

**RESULTS AND DISCUSSION**

**Toxicity of Insecticides Against Spodoptera Larvae:**

Toxicity results of the insecticides expressed in terms of LC₅₀ are given in Table (1). Phenothrin LC₅₀ values after 24hr are 0.004, 0.011, 0.031, 0.052 and 0.071ppm for lab strain; Borg El-Arab; Abeis, Damahour and Abou El-Matamir strains respectively. While LC₅₀ values after 48hr for Phenothrin are 0.001, 0.003, 0.011, 0.031 and 0.051ppm for lab strain and the four field strains respectively. Also Thiodicarb LC₅₀ values after 24hr are 0.009, 0.08, 0.05, 0.07 and 0.09ppm for lab strain and the four field strains respectively, while LC₅₀ values after 48hr are 0.006, 0.002, 0.02, 0.04 and 0.06ppm for Thiodicarb against lab strain and the four field strains of *Spodoptera* larvae respectively.

It is clear that the toxicity was higher with the Phenothrin and Thiodicarb for lab strain, Borg El-Arab
and Abeis, while toxicity was low for Damanhour and Abou El-Matamir. Also Phenothrin was more toxic than Thiodicarb in controlling of *S. littoralis*. The present results emphasize that during many years of selection pressure in the field, the resistance and/or tolerance levels to the insecticides had increased due to the intensive application of such insecticides for controlling *S. littoralis* in cotton fields. These results fully agreed with Davis *et al.* (1975), who reported that synthetic Pyrethroids was more toxic other tested insecticides in controlling many species of insects. Hosny *et al.* (1977) mentioned that synthetic Pyrethroids were most superior toxicants against the cotton leafworm better than the tested Organophosphorus insecticides. Moustafa *et al.* (1979) proved that synthetic Pyrethroids were not only superior to Organophosphorus but also to Chlorinated hydrocarbons and Carbamate insecticides in controlling of cotton leafworm. Kaygisiz (1980) and McDonald (1981) reported that synthetic Pyrethroids were the most effective against 4th instar larvae of *S. littoralis*. Ishaaya and Klein (1990) found that *S. littoralis* larvae collected from a cotton field that was heavily sprayed with conventional insecticides showed strong resistance to Organophosphates. Korkor *et al.* (1995) reported that synthetic Pyrethroids were the most effective insecticides against Bollworms. Mascarenhas *et al.* (1998) found that several field strains of beet armyworm, *Spodoptera exigue* (Hubner), exhibited reduce susceptibility to Chlorpyrifos and Thiodicarb.

**Table 1. Toxicity of Phenothrin and Thiodicarb on *S. littoralis* larvae**

<table>
<thead>
<tr>
<th><em>Spodoptera</em> strain locations</th>
<th>LC$_{50}$ (ppm) Phenothrin</th>
<th>LC$_{50}$ (ppm) Thiodicarb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24hr</td>
<td>48hr</td>
</tr>
<tr>
<td>Laboratory</td>
<td>0.004</td>
<td>0.001</td>
</tr>
<tr>
<td>Borg El-Arabi</td>
<td>0.011</td>
<td>0.003</td>
</tr>
<tr>
<td>Abeis</td>
<td>0.031</td>
<td>0.011</td>
</tr>
<tr>
<td>Damanhour</td>
<td>0.052</td>
<td>0.031</td>
</tr>
<tr>
<td>Abou El-Matamir</td>
<td>0.071</td>
<td>0.051</td>
</tr>
</tbody>
</table>

**Specific Activities of Na$^+$.K$^+$-ATPase and AChE:**

Table(2) summarized the specific activity of Na$^+$.K$^+$-ATPase; Mg$^{2+}$-ATPase and AChE Fig (1&2) show the specific activity of the ATPases, isolated from Lab strain and different field strains of *S. littoralis*. The maximum value of specific activity of Na$^+$.K$^+$-ATPase was found in Lab strain and Borg El-Arabi, whereas that the values of Na$^+$.K$^+$- and Mg$^{2+}$-ATPases activities in brain preparations of the *Spodoptera*, were recorded. Total Activities of ATPase were greatest (45.86±0.13 & 41.85±0.11 respectively) in Lab strain and Borg El-Arabi, and least in the Abou El-Matamir (28.51±0.43). Total ATPase activities were modest in Abeis and Damanhour (the values are 38.85±0.06 & 32.94±0.17 respectively). Also observed the Na$^+$.K$^+$-ATPase activity was more than the Mg$^{2+}$-ATPase activity, in all different sources.

Data presented in Table (2) and Fig(3) show the specific activity of the AChE in the brain of the 4th larval instars of lab strain and all tested field strains of *S. littoralis*. The results show that there were significant differences in AChE specific activity between the strains. AChE activity were higher in the lab strain, Borg El-Arabi, and Abeis (the values are 31.86±0.05, 26.56±0.37 & 20.17±0.15 respectively) than Damanhour and Abou El-Matamir (the values are 14.28±0.12 & 10.61±0.09 respectively).

**Table 2. Na$^+$.K$^+$-ATPase and AChE specific activities *Spodoptera* brain larve (4th instar) in different local strains**

<table>
<thead>
<tr>
<th><em>Spodoptera</em> strain locations</th>
<th>Total ATPase</th>
<th>Na$^+$.K$^+$-ATPase</th>
<th>Mg$^{2+}$-ATPase</th>
<th>AChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory</td>
<td>45.86 ± 0.13</td>
<td>36.82 ± 0.04</td>
<td>9.04 ± 0.01</td>
<td>31.86 ± 0.05</td>
</tr>
<tr>
<td>Borg El-Arabi</td>
<td>41.85 ± 0.11</td>
<td>30.92 ± 0.13</td>
<td>7.33 ± 0.10</td>
<td>26.56 ± 0.37</td>
</tr>
<tr>
<td>Abeis</td>
<td>38.85 ± 0.06</td>
<td>28.30 ± 0.14</td>
<td>6.60 ± 0.06</td>
<td>20.17 ± 0.15</td>
</tr>
<tr>
<td>Damanhour</td>
<td>32.94 ± 0.17</td>
<td>25.76 ± 0.15</td>
<td>0.50 ± 0.03</td>
<td>14.28 ± 0.12</td>
</tr>
<tr>
<td>Abou El-Matamir</td>
<td>28.51 ± 0.43</td>
<td>24.21 ± 0.52</td>
<td>4.17 ± 0.08</td>
<td>10.61 ± 0.09</td>
</tr>
</tbody>
</table>

Na$^+$.K$^+$-ATPase specific activity (P1 µmole mg$^{-1}$ Protein hr$^{-1}$)

AChE specific activity ($\lambda_{max}$ 412 mg$^{-1}$ Protein hr$^{-1}$)
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**Fig 1.** ATPase specific activity in *Spodoptera* brain larvae (4\(^\text{th}\) instar) in different local strains

**Fig 2.** Na\(^+\),K\(^+\)-ATPase specific activity in *Spodoptera* brain larvae (4\(^\text{th}\) instar) in different local strains

**Fig 3.** AChE specific activity in *Spodeptera* brain larvae (4\(^\text{th}\) instar) in different local strains
In Vivo Inhibition of Brain *S. littoralis* Na⁺,K⁺-ATPase and AChE Activity:

The *in vivo* inhibitory effect of the LC₅₀ values of two insecticides against the *Spodoptera littoralis* 4th instar lab and field strains larval Na⁺,K⁺-ATPase and AChE is shown in the data given in Table (3). The data revealed that Phenothrin exhibited significant reduction in Na⁺,K⁺-ATPase activity. Percentages of Na⁺,K⁺-ATPase inhibition were 87.3, 84.2, 74.1, 71.4 and 65.5% for lab strain; Borg El- Arab; Abeis; Damanhour and Abou El-Matamir, respectively. On the other hand, in the case of AChE, the significant reduction in its activity was recorded for Thiodicarb, the percentages of AChE inhibition were 82.5, 77.4, 73.6, 68.1 and 56.7% for lab strain and four field strains respectively.

**Table 3. In vivo inhibition of brain *S. littoralis* Na⁺,K⁺-ATPase and AChE activities by two compounds**

<table>
<thead>
<tr>
<th><em>Spodoptera</em> strain locations</th>
<th>% Inhibition of enzymes (LC₅₀) ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺,K⁺-ATPase</td>
</tr>
<tr>
<td>Phenothrin</td>
<td>87.3</td>
</tr>
<tr>
<td>Thiodicarb</td>
<td>84.2</td>
</tr>
<tr>
<td>Laboratory</td>
<td>74.1</td>
</tr>
<tr>
<td>Borg El-Arab</td>
<td>71.4</td>
</tr>
<tr>
<td>Abeis</td>
<td>65.5</td>
</tr>
<tr>
<td>Damanhour</td>
<td>56.5</td>
</tr>
<tr>
<td>Abou El-Matamir</td>
<td>56.5</td>
</tr>
</tbody>
</table>

**Kinetic Parameters of Na⁺,K⁺-ATPase and AChE Inhibition:**

The kinetic studies were conducted to evaluate the effects of Phenothrin on Na⁺,K⁺-ATPase activity and Thiodicarb on AChE activity in both tested strains brain of *S. littoralis* 4th larva. Table (4) shows the obtained Lineweaver-Burk (L-B) plots for Na⁺,K⁺-ATPase and AChE in lab strain and all four tested field strains and the statistical analysis of the obtained values of Kₘ (Michaelis-Menten,constant) and Vₘₐₓ (maximum velocity) of the Na⁺,K⁺-ATPase and AChE. The Kₘ values for Na⁺,K⁺-ATPase and AChE were generally higher in all four tested field strains than lab strain. The changes in Kₘ values of Na⁺,K⁺-ATPase and AChE between the tested field strains indicate changes in the affinities, our result are strongly emphasized by the recent kinetic studies of Gonzalez et al. (1990) found that the calculated Kₘ of 0.22mM for AChE of gastropod *Concholepas concholepas*.

The present results show that the Vₘₐₓ values of Na⁺,K⁺-ATPase and AChE are obviously higher. This points to the higher substrate turnover which may reflect the physiological importance of the Na⁺,K⁺-ATPase in the function of the nervous tissue of the *S. littoralis* larval brain (El-Aw and Hashem, 2001). The Vₘₐₓ values were generally higher in all tested field strains than lab strain. This fact indicated that the number of active sites on the Na⁺,K⁺-ATPase and AChE of the 4th larva brain was increased in the field strains. Such change may be followed by decrease in the insect susceptibility which could be altered by field application of the Pyrethroids and Carbamate insecticides.

**The in vitro inhibition of brain *S. littoralis* Na⁺,K⁺-ATPase and AChE activities:**

To characterize more details about the *in vitro* inhibition of Na⁺,K⁺-ATPase and AChE by the inhibitors, the Kᵢ value of each inhibitor was estimated from the graphical method of Dixon and Weep, (1964) Fig. (4&5) and Table (5). The Kᵢ values were 5, 18, 20, 30 and 45uM for lab strain; Borg El-Arab; Abeis; Damanhour and Abou El-Matamir respectively in the case of Phenothrin while the Kᵢ values were 20, 28, 30, 40 and 50uM for lab strain and four field strains respectively in case of Thiodicarb. The obtained data proved that each of Phenothrin and Thiodicarb showed competitive inhibition on Na⁺,K⁺-ATPase and AChE activity. The present results are accordance with those reported by Zhu and Brindley (1992) who reported competitive inhibition of AChE purified from *Lygus hesperus* by six OPs compounds.

**Table 4. Michaelis-Menten kinetics of the Na⁺,K⁺-ATPase and AChE of larval brain of *S. littoralis* collected from different locations**

<table>
<thead>
<tr>
<th><em>Spodoptera</em> strain locations</th>
<th>Na⁺,K⁺-ATPase</th>
<th>AChE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kₘ(mM)</td>
<td>Vₘₐₓ(mM)</td>
</tr>
<tr>
<td>Laboratory</td>
<td>0.17</td>
<td>5.9</td>
</tr>
<tr>
<td>Borg El-Arab</td>
<td>0.30</td>
<td>3.3</td>
</tr>
<tr>
<td>Abeis</td>
<td>0.36</td>
<td>2.8</td>
</tr>
<tr>
<td>Damanhour</td>
<td>0.40</td>
<td>2.5</td>
</tr>
<tr>
<td>Abou El-Matamir</td>
<td>0.50</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Fig 4. Dixon plot of the effect of Phenothrin on Spodoptera brain larvae (4th instar) Na⁺,K⁺-ATPase activity at 3mM (□) and 5mM (△) ATP.
Fig 5. Dixon plot of the effect of Thiodicarb on Spodoptera brain larvae (4th instar) AChE activity at 5mM (□) and 10mM (▲) of [ASChI]
In comparing the inhibition potency of Phenothrin and Thiodicarb against Na⁺,K⁺-ATPase and AChE activity respectively within the different strains, it is clear that Phenothrin showed to be the strong inhibitor for S. littoralis. On the other hand, the I₅₀ values of each Phenothrin and Thiodicarb in lab strain and Borg El-Arab is more susceptible than that of Abeis, Damanhour and Abou El-Matamir respectively. While the I₅₀ values for Thiodicarb against AChE were 0.22, 0.43, 0.54, 0.71 and 0.96 μM for lab strain and four field strains respectively.

In this work, we describe the development of a biochemical assay system for measuring the sensitivity of Na⁺,K⁺-ATPase and AChE to Pyrethroids and Carbamate insecticides respectively, our primary goal was to develop an assay that could characterize Na⁺,K⁺-ATPase and AChE variants in individual sharpshooters that were under insecticides selection pressure. We also provide enzyme kinetic data for the Na⁺,K⁺-ATPase and AChE in this insect for field strains and compare them with data for the lab strain.

Finally, it may be concluded that Phenothrin (Pyrethroids) is more convenient than Thiodicarb (Carbamate) for the control program of S. littoralis according to its slow effect in inducing resistance. But the induced resistance may be of great concern in the use of synthetic Pyrethroids and Carbamate, for the control program of cotton leafworm.

### REFERENCES


المتخصى العربي

دراسة مدى حساسية أنزيم الصوديوم-بوتاسيوم أدينوسين تراي الفوسفاتيز (Na⁺,K⁺-ATPase) وأنزيم الأسيتايل كولين أستريز (AChE) للفيثولين وоро الفينوثرين في العشائر الحقلية المصرية لدودة ورق القطن

سهام منصور اسماعيل

نتيجة دراسة الاختلافات في نشاط أنزيمين من أهم الأهداف البيولوجية في الحشرة، وهما أنزيم الصوديوم-بوتاسيوم أدينوسين تراي الفوسفاتيز (Na⁺,K⁺-ATPase) وأنزيم الأسيتايل كولين أستريز (AChE) وأيضا مستوى حساسية يرقات العمر الرابع لدودة ورق القطن للفيثولين وоро الفينوثرين، حيث تم استخلاص كل الأنتيؤين من رأس يرقات العمر الرابع لدودة ورق القطن وذلك من عشائر مختلفة من الأزمنة المختارة، تركزت الدراسة على العشائر المنتشرة في المناطق التي ترش بمعدل كثيف من المبيدات (دمهور- أبومطار- أبيس) وأيضا في المناطق الصحراوية المزروعة حديثا (برج العرب) والتي تنتشر فيها زراعة القطن.

وقد أوضحت النتائج أنه قيم التركيزات النصف مميتة (LC₅₀) أظهرت اختلافاً معكوسا حيث كانت سلالة برج العرب أكثر السلالات حساسية بلها سلالة أبيس بينما دمئور وأبومطار كانت أكثر تحملًا وذلك في حالة الأنتيؤين، وقد أوضحت النتائج المتحصل عليها أن أعلى معدل نشاط نوعي للأنزيمين كان في محافظة الأسكندرية (برج العرب) وورق القطن وذلك من قيم النشاط الأنتيؤي لهما.