Some Properties of Acetylcholinesterases Partially purified from Susceptible and Resistant Green Rice Leafhoppers, *Nephotettix cincticeps* UHLER (Hemiptera: Deltocephalidae)

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Properties of acetylcholinesterases (AChE) in the green rice leafhopper were examined using partially purified normal and modified AChEs. The modified enzyme was much less sensitive to inhibition by propoxur and malaoxon than the normal one, but highly sensitive to inhibition by diazoxon. Substrate specificity and effect of pH on activity of the normal enzyme were similar to those of the bovine erythrocyte AChE used as a reference enzyme, but those of the modified AChE were obviously different from those of the other enzymes. On the other hand, difference of the normal and modified AChEs in the Km values of substrates was rather small, i.e., by a factor of less than 2. It may be concluded that the modified AChE alters a binding site, which is related to the reaction with inhibitors, but different from the site for the intrinsic substrate acetylcholine.

**INTRODUCTION**

It has been established that reduced sensitivity of acetylcholinesterase (AChE) to inhibition by inhibitors mostly causes the high level of resistance to most methyl-carbamate insecticides, and also takes part in the resistance to dimethyl organophosphate insecticides such as malathion and fenitrothion (Hama and Iwata, 1971, 1973, 1978; Iwata and Hama, 1972; Yamamoto et al., 1977).

Furthermore, the modified type of AChE, which is much less sensitive to inhibitors, in the carbamate resistant leafhopper could be distinguished from the wild type enzyme or normal AChE in the susceptible leafhopper by DEAE-cellulose column chromatography (Hama, 1976).

In this paper, elution patterns of AChE of the green rice leafhopper were compared among three strains using gel filtration and ion-exchange Sephadex column chromatography, and some properties of partially purified modified AChE and normal AChE were also compared.

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MATERIALS AND METHODS

Insect: Susceptible (S), carbamate resistant (Rmc) and multiple-resistant (N) strains of the green rice leafhopper, *Nephotettix cincticeps* Uhler, were used (HAMA and IWATA, 1971, 1978; HAMA, 1975). S and N strains were collected in Miyagi and Ehime prefectures, respectively, and have been reared with rice-seedlings in the laboratory of National Institute of Agricultural Sciences. The S strain has been used as a standard strain. The N strain showed high level of resistance to both carbamate and organophosphate insecticides (IWATA and HAMA, 1971). The Rmc strain was made from S and N strains according to repeated back-crossings to S strain under selection pressure with Bassa, *o*-isopropoxyphenyl methylcarbamate (HAMA, 1975; HAMA and IWATA, 1978). This strain is resistant to most carbamates as highly as the parent strain N, but has very low resistance to most organophosphate insecticides (HAMA and IWATA, 1978). Both N and Rmc strains have been selected with Bassa in several generations. Females and males of from 3 to 8 days after emergence were used for all the experiments.

Enzyme preparations: Enzyme source was prepared from leafhoppers stored at −20°C (HAMA, 1976). Enzyme preparations were conducted at 4°C. Ten gram of leafhoppers was homogenized with 10-fold distilled water in a glass-homogenizer. The homogenates were centrifuged at 700 g for 10 min to remove debris and the supernate was again centrifuged at 105,000 g for 60 min.

The supernate was used after filtration through a funnel packed with a cotton ball.

The sediment was suspended in a small volume of 0.033 M phosphate buffer, pH 7.2, and 1% Triton X-100 in the phosphate buffer was added to the suspension to

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Fig. 1. Purification of normal and modified AChEs in the green rice leafhopper from aqueous whole body homogenates of susceptible strain (S) and carbamate-resistant strain (Rmc), respectively.
make its concentration 0.4% in order to solubilize AChE. After standing over night at 4°C or several hr at 30°C, the suspension was again centrifuged at 105,000 g for 60 min. The supernate obtained was dialyzed against the phosphate buffer and used as a solubilized-sediment fraction. After this treatment, 50 to 80% of the AChE activity appeared in the supernate.

Dialysis and concentration of enzyme sources were conducted by ultrafiltration with Dia-filter membrane (Bio-Engineering Co., Ltd., Tokyo) through which materials under the molecular weight of 10,000 are filtered.

The normal AChE and the modified AChE of the green rice leafhopper were purified from whole body homogenates of S and Rmc strains, respectively, as shown in Fig. 1 (HAMA, 1976). The solubilized-sediment was used as the enzyme source for most of the experiments. Purification of AChE was 70-fold with 15% recovery. AChE activity of partially purified enzymes was 2–5 μ moles acetylthiocholine/min/mg of protein.

The bovine erythrocyte AChE used as a reference enzyme was purchased from Sigma Chemical Co.

*Determination of AChE activity:* Acetylthiocholine iodide (ATCh), propionylthiocholine iodide (PrTCh) purchased from Sigma Chem. Co., and butyrylthiocholine iodide (BuTCh) purchased from Wako Junyaku Co., Tokyo were used as substrates. Enzyme activity toward these thiocholine esters was determined at 30°C by Ellman’s method (ELLMAN et al., 1961). From a calibration curve made with glutathione as a standard chemical, amounts of esters hydrolyzed were determined (GARRY and ROUTH, 1965).

In some experiments, AChE activity was also determined by radiometric method (SIKOTOS et al., 1969; SAITO et al., 1976) with acetyl-1-14C-choline chloride (14C-ACh, 13.7 mCi/mM) purchased from Amersham, England. Fifty μl of the partially purified enzyme was incubated with 50 μl of 1 × 10−3 M 14C-ACh in 0.067 M phosphate buffer, pH 7.4, for 10 min at 30°C. Then, the reaction mixtures were cooled and dioxane was added to stop the reaction. The reaction mixtures were poured into a Pasteur pipet packed with Amberlite CG-120. 14C-Acetic acid in the mixtures, a metabolite of the substrate, was eluted with 2 ml of dioxane, and its radioactivity was accounted by liquid scintillation spectrometer.

* Determination of sensitivity of AChE to inhibitors:* Propoxur, malaoxon, propaphos (O, O-di-(n)-propyl-O-4-methyl thiophenyl phosphate) and diaxoaxon were used as inhibitors. Propoxur and malaoxon are chemicals which inhibit the normal AChE more strongly than the modified AChE, whereas propaphos and diaxooxon are chemicals which strongly inhibit the modified AChE (HAMA, 1975; HAMA and IWATA, 1978).

Sensitivity of enzymes to inhibitors was determined by pre-incubation with inhibitors as described previously (HAMA and IWATA, 1971; HAMA, 1976). After pre-incubation with an inhibitor for 10 min at 30°C, ATCh or 14C-ACh were added and incubated further for 10 min.

Bimolecular reaction constant (ki) was calculated by the description of ALDRIDGE (1950).

* Determination of protein:* Amounts of protein in enzyme preparations were determined by the Lowry-Folin method (LOWRY et al., 1951) with bovine serum albumin (Daich Chem. Co. Ltd., Tokyo) as a standard protein.

The following five proteins were used to estimate molecular weights of AChE
by gel filtration; apo-ferritin (molecular weight 480,000), γ-globlin (m.w. 160,000), albumin (m.w. 67,000), chymotrypsinogen (m.w. 25,000) and cytochrome C (m.w. 12,400). These proteins except for cytochrome C (Tokyo Kasei Kogyo) were purchased from Miles, England. Detection of proteins was made by absorbance at 280 nm.

Sepharose 6B gel filtration: Gel filtration was conducted under 4°C as described previously (HAMA, 1976). The column (2.64 φ × 100 cm) packed with Sepharose 6B (Pharmacia Fine Chemical Inc.) was prepared with 0.033 M phosphate buffer, pH 7.2, containing 0.05 M KCl. An enzyme source was introduced from the top of the column and eluted with the same phosphate buffer at 4.5 m/ hr. Eight-ml fractions were collected. The void volume for the column was checked by the elution of dextran blue.

DEAE-Sephadex column chromatography: The column (1.5 φ × 30 cm) packed with DEAE-Sephadex 50 (Pharmacia Fine Chemical Inc.) was prepared with 0.05 M Tris-HCl buffer, pH 7.54. An enzyme source was introduced from the top of the column, and eluted with the same buffer of a linear gradient of KCl concentration to 0.6 M at 15 m/ hr at 4°C. Five-ml fractions were collected. KCl concentration was checked by the Mohr titrimetric method using 5% K₂CrO₄ and 0.1 N silver nitrate solution.

RESULTS

Sepharose 6B gel filtration

Figs. 2 and 3 show elution patterns of AChE activity in 105,000 g-supernate and solubilized-sediment fractions prepared from S and N strains using Sepharose 6B gel filtration. Three AChE peaks were detected in the supernate; a small peak (AChE 3) at the void volume followed by another small peak (AChE 2) and a main peak (AChE 1), whereas there was only one peak, AChE 1, in the solubilized-sediment.

There were no evident differences in the elution patterns of AChE activity in each fraction among S, N and Rmc strains by gel filtration (data on Rmc were omitted).

Five known proteins were also eluted by Sepharose 6B gel filtration under the

![Fig. 2. Elution patterns of AChE activity in 105,000 g supernate prepared from aqueous whole body extracts of susceptible (S) and multiple-resistant (N) strains using Sepharose 6B gel filtration. Activity of enzyme sources introduced from the top of the column was not the same among the strains. Fraction volume: 8 ml/fraction, arrow: void volume.](image-url)
same condition. The relative elution speeds of each protein (fraction numbers divided by the fraction number of dextran blue) were plotted against each molecular weight. As shown in Fig. 4, plots were on a straight line. The molecular weights of the enzymes were estimated to be 120,000–150,000 for AChE 1, 350,000–450,000 for AChE 2 and ≥2,000,000 for AChE 3.

![Fig. 3. Elution patterns of AChE activity in 105,000 g solubilized-sediment prepared from aqueous whole body extracts of susceptible (S) and multiple-resistant (N) strains using Sepharose 6B gel filtration. Activity of enzyme sources introduced from the top of the column was not the same among the strains. Fraction volume: 8 ml/fraction, arrow: void volume.](image)

![Fig. 4. Relationship between molecular weight of proteins and their relative elution speed (fraction numbers divided by the fraction number of dextran blue) on Sepharose 6B gel filtration.](image)
DEAE-Sephadex column chromatography

Fig. 5 shows elution patterns of AChE activity in a major fraction of the supernate, AChE 1, using DEAE-Sephadex column chromatography. Although AChE was eluted as one peak in both S and Rmc strains, in the S strain it was eluted around 0.44 M KCl, which was later than that in the 0.38 M KCl elution of the Rmc strain. In the N strain, there seemed to be two peaks, which coincided with each peak of Rmc and S strains, as judged by KCl concentrations at which the enzymes were eluted.

After pre-incubation with methylcarbamate propoxur at $2 \times 10^{-5}$ M or dipropyl organophosphate propaphos at $2 \times 10^{-4}$ M for 10 min at 30°C, the AChE activity in each fraction of DEAE-Sephadex column chromatography was plotted in Fig. 5. The AChE of the S strain was inhibited completely by propoxur, but not by propaphos, and that of the Rmc strain was not inhibited by propoxur, but was relatively inhibited by propaphos. In the N strain, the first peak eluted was not inhibited by propoxur, but was inhibited by propaphos, and the second peak eluted was inhibited completely by propoxur, but not by propaphos.

These results indicate that AChE in the S strain is the normal AChE, most of AChE in the Rmc strain is the modified AChE, and the enzyme in the N strain consists of the normal AChE and the modified AChE with the ratio of about 1:1 in their activity.

The elution patterns of AChE activity in the solubilized-sediment and the supernate were not different in each strain using DEAE-Sephadex column chromatography.

Sensitivity of AChE to inhibitors

Sensitivity of partially purified normal and modified AChEs to propoxur, malaoxon and diazoxon is shown in Figs. 6, 7 and 8. The modified AChE was much less sensitive to inhibition by propoxur and malaoxon, but it was much more sensitive to inhibition by diazoxon than the normal AChE.

The bimolecular reaction constants, $k_i$, between AChE and malaoxon were $3 \times 10^{6}$ M$^{-1}$ min$^{-1}$ in the normal AChE and $2 \times 10^{5}$ M$^{-1}$ min$^{-1}$ in the modified AChE, whose $k_i$ was decreased by a factor of 15. The $k_i$ values for diazoxon were $8.7 \times 10^{5}$ M$^{-1}$ min$^{-1}$ in the normal AChE and $1.7 \times 10^{6}$ M$^{-1}$ min$^{-1}$ in the modified AChE, whose $k_i$ was increased by a factor of 2.

Substrate specificity of AChE

The relative activity of normal AChE and modified AChE of the leafhopper, and bovine erythrocyte AChE toward ATCh, PrTCh and BuTCh is shown in Fig. 9. The activity pattern of the normal AChE was similar to that of the bovine erythrocyte AChE, but that of the modified AChE was obviously different from those of the other two AChE sources.

The relative activity of the modified AChE toward PrTCh was much lower than that of the other AChE sources, but it was rather high toward BuTCh.

The activity of both the normal AChE and the bovine erythrocyte AChE was suppressed or inhibited at more than $3 \times 10^{-3}$ M of ATCh or PrTCh, but in the modified AChE no evident inhibition of activity occurred within the concentrations of substrates tested. In another test, however, the AChE activity was suppressed at more than $10^{-2}$ M of ATCh even in the modified AChE (data not shown).

The optimum concentration of ACh for the modified AChE also shifted toward
Fig. 5. Elution patterns of a major component, the supernate AChE 1, by DEAE-Sephadex column chromatography following Sepharose 6B gel filtration.

- ●: AChE activity without an inhibitor, ○: AChE activity after pre-incubation with \(2 \times 10^{-5}\) M propoxur for 10 min at 30°C, ×: AChE activity after pre-incubation with \(2 \times 10^{-4}\) M propaphos for 10 min at 30°C. Fraction volume: 5 ml/fraction.
Fig. 6. Sensitivity of partially purified leafhopper normal and modified AChEs to propoxur. ○: values determined by radioassay using $^{14}$C-ACh, ●: values determined by Ellman's method using ATCh.

Fig. 7. Sensitivity of partially purified leafhopper normal and modified AChEs to malaoxon.

Fig. 8. Sensitivity of partially purified leafhopper normal and modified AChEs to diazoxon.

higher concentration than that for the normal AChE (Fig. 10).

The affinity of each AChE for ATCh and PrTCh was evaluated by the Lineweaver-Burk plot (Table 1). The $K_m$ values of both ATCh and PrTCh for the bovine erythrocyte AChE were in the order of $10^{-4}$ M higher than those for the leafhopper AChE, which were in the order of $10^{-5}$ M. The values of ATCh and PrTCh for the modified
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**Fig. 9.** Relative activity of bovine erythrocyte AChE and partially purified leafhopper normal and modified AChEs toward ATCh (●), PrTCh (○) and BuTCh (×).

**Fig. 10.** Relative activity of partially purified leafhopper normal and modified AChEs toward \(^{14}\text{C}-\text{ACh}\).

AChE were somewhat higher than those for the normal AChE.

**Effect of pH on AChE activity**

Fig. 11 shows effect of pH on AChE activity of normal and modified AChEs and
Table 1. Km Values of ATCh and PrTCh for Bovine Erythrocyte AChE, and Partially Purified Leafhopper Normal and Modified AChEs

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Km (ATCh)</th>
<th>Km (PrTCh)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine erythrocyte AChE</td>
<td>$1.72 \times 10^{-4}$ M</td>
<td>$2.0 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>Leafhopper normal AChE</td>
<td>$4.76 \times 10^{-5}$</td>
<td>$4.17 \times 10^{-5}$</td>
</tr>
<tr>
<td>Leafhopper modified AChE</td>
<td>$7.14 \times 10^{-5}$</td>
<td>$7.14 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

Fig. 11. Effects of pH on activity of bovine erythrocyte AChE, and partially purified leafhopper normal and modified AChEs. ●: 0.1 M phosphate buffer, ○: 0.05 M Tris-HCl buffer.

bovine erythrocyte AChE. When tested with 0.1 M phosphate buffer, no evident difference appeared among enzyme sources. When 0.05 M Tris-HCl buffer was used, however, AChE activity of the modified AChE increased with increasing pH, although activity of the normal AChE and the bovine erythrocyte AChE were suppressed in the range of 8–9.

DISCUSSION

AChE activity in the green rice leafhopper is distributed in both 105,000 g-sediment and -supernate fractions, although more than half of the activity exists in the sediment fraction such as with many other sources (HAMA, 1976). However, it has been shown that the sediment AChE and the supernate AChE prepared from S or Rmc strains are not intrinsically different in their sensitivity to inhibitors (HAMA, 1977), and in their elution patterns of gel chromatography on Sepharose 6B or DEAE-cellulose columns (HAMA, 1976).

AChE activity in the supernate was separated into three peaks by Sepharose 6B gel filtration (Fig. 2), although two small peaks, AChE 2 and AChE 3, did not always appear.

In a previous paper (HAMA, 1976), AChE peak corresponding to AChE 2 was not observed using Sepharose 6B gel filtration in the same manner, except for the column
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size, 2.64 φ × 45 cm instead of 2.64 φ × 100 cm.

It is known that AChE of the electric eel and other sources is an oligomer enzyme, and that aggregation and deaggregation of oligomers are strongly affected by various factors such as salt concentration, pH and detergents (GRAFIUS and MILLAR, 1965, 1967; MASSOUÍ and RIEGER, 1969; KRYSAN and KRUCKEBERG, 1970; DUDAI et al., 1972; STEELE and SMALLMAN, 1976).

On the basis of the distribution of molecular weights of AChE 1, AChE 2 and AChE 3 of the leafhopper, the relative instability of AChE 2 and AChE 3 being larger than the main peak AChE 1, and no detection of AChE 2 and AChE 3 in the sediment solubilized with Triton X-100 (Fig. 3), it is suggested that AChE 2 and AChE 3 are an aggregating form of AChE 1. The main AChE 1 in the leafhopper showing a molecular weight of 120,000–150,000 was almost the same as that of the house fly AChE in their molecular weight (KRYSAN and CHADWICK, 1966; TRIPATHI and O'BRIEN, 1977), although a house fly AChE showing a molecular weight of 80,000 was detected and proposed to be a 'fundamental unit' (STEELE and SMALLMAN, 1976).

It has been found that there exist at least two types of AChE in the leafhopper; the modified AChE and the normal AChE, which can be distinguished by DEAE-cellulose column chromatography (HAMA, 1976). Such results were confirmed by the patterns of AChE activity after pre-incubation with selective inhibitors, methyl-carbamate propoxur and n-dipropyl organophosphate propaphos by DEAE-Sephadex column chromatography (Fig. 5).

The modified AChE in the leafhopper was much less sensitive to methylcarbamates such as propoxur and carbaryl, and dimethyl organophosphates such as malaoxon and fenitroxon (HAMA and IWATA, 1971, 1978; IWATA and HAMA, 1972; YAMAMOTO et al., 1977). On the other hand, the following chemicals inhibited the modified AChE more strongly than the normal AChE; diethyl and n-dipropyl organophosphates such as diaoxon, pyridafenoxon and propaphos (HAMA, 1975; MIKAGE et al., 1977; HAMA and IWATA, 1978), and n-propyl carbamates (YAMAMOTO et al., 1977). Such a tendency was confirmed with partially purified normal and modified AChEs (Figs. 6, 7 and 8).

The optimum concentration of ATCh or ACh for the modified AChE was higher than that for the normal AChE (Figs. 9 and 10).

It was reported that the AChE activity of the Ridgeland resistant strain of the cattle tick, *Boophilus microplus*, which had a modified AChE, was not inhibited even by 1.73 × 10^{-2} M ATCh differently from those of the other strains including resistant strains having a modified AChE (NOLAN and SCHNITZERLING, 1975). It has also been observed that the optimum concentration of ATCh for AChE in the dimethoate resistant house fly having a modified AChE was higher than that for AChE in the susceptible house fly (DEVERSHIRE, 1975).

It has been proposed that the suppression of AChE activity by an excess amount of substrates may be caused by the inhibition of deacylation of the acylated AChE because of the combining of the free substrates at the acylated AChE (KRUPKA and LAIDLAR, 1961), and that may be related with sites other than the active site, at which intrinsic substrate ACh binds (ALDRIDGE and REINER, 1969).

Evident differences in substrate specificity and effects of pH between the modified AChE and the normal AChE indicate structural alterations of the modified AChE in its active site.
On the other hand, it has been observed that decreased sensitivity of AChE to carbamates in the leafhopper does not always take with increase of AChE activity (Hama, 1977), although AChE activity of the N strain was higher than that of the susceptible strain (Kojima et al., 1974; Yamamoto et al., 1977). Also, difference in the Km values of ATCh and PrTCh between the normal AChE and the modified AChE was rather small (Table 1).

Consequently, it may be concluded that the modified AChE in the leafhopper alters in a binding site, which is related to the reaction with inhibitors but different from the binding site for intrinsic substrate ACh, as proposed by Tripathi and O'Brien (1973, 1975) and O'Brien et al. (1978) with the house fly.

REFERENCES


1 "Cicadellidae" in our previous papers should be read "Deltocephalidae".


