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Cholinesterase Effects of Pesticides in Rabbit Embryos and their Potential for Use in Hazard Assessment

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Abstract

Cholinesterase are often taken as biomarkers of exposure and effect. Then acetylhydrolase isozymes and acylcholine acylhydrolase were examined for their suitability as biomarkers of pesticide exposure. The study of effects of embryos of the rabbit is already an accepted tool in monitor food waste levels, but effects of various groups of substances to the rabbit embryo remain to be studied. A 43% inhibition of acetylhydrolase enzyme activities was reached by 250 mg/kg malathion, while 35% inhibition of acylcholine acylhydrolase enzyme activities was reached by 500 mg/kg malathion indicating the possible use of these enzymes as biomarkers of exposure. There are found some significant specific activities of the acetylhydrolase ranged from 1.5-7.1 U mg^{-1} protein. The specific activity of acylcholine acylhydrolase in the range of 0.9-4.5 U mg^{-1} protein in the respective developmental stages. Substrate-specificity was analyzed using specific inhibitors (iso-OMPA and BW284c51). The results showed that the observed cholinesterase's activities in the whole embryo may be attributed mainly to acetylhydrolase with a partial capability to use butyrylthiocholine (BuSCh) as an additional substrate.

Keywords: Acetylhydrolase; Acylcholine acylhydrolase; Rabbit embryo; Biomarker; Malathion, Pesticide

Introduction

Several major classes of insecticides are applied in the field as inactive esters which are then hydrolyzed within rabbit tissues to release the alcohol or phytotoxic acid [1]. The toxic effects of insecticides to rabbit can vary between acute lethality to sub-lethal effects. The sub-lethal effects include differential inhibition of several enzymes, of which the inhibition of acetylhydrolase is the most recognizable. As rabbit are an important component of the food chain and are simply obtained, different toxicity tests have been performed within this group to assess the risk of unintentional intoxication [2]. Acetylhydrolase is the main known sites of action for organophosphate (OP) and carbamate insecticides [3]. Acetylhydrolase severs the transmitter acetylcholine. The recognition of inhibition of acetylhydrolase by insecticides is accepted as a biomarker of exposure [4,5]. Acetylhydrolase also is remarkably important for the neural and muscular development [5,6] in the embryos of the rabbit. The OP (Malathion) is the metabolically activated metabolite the parent substance malaoxon and was chosen as model test substance [2]. It is an OP which forms covalent chemical bonds with the isozymes of the cholinesterases type (acetylhydrolase and acylcholine acylhydrolase) thereby inhibiting it. Headed for being able to use cholinesterases enzyme as biomarkers, the different types of cholinesterases were considered, which may show different sensitivities to OP [7]. For the exact composition of the isozymes of cholinesterases in the rabbit is not known and different substrates and inhibitors were used to characterize the composition of isozymes cholinesterases in whole homogenates of the rabbit embryo [8]. The inhibition of acylcholine acylhydrolases as alternative phosphorylation sites [9] by insecticides is faster than that of acetylhydrolase [10]. Consequently, the acetylhydrolase might be protected stoichiometrically [11] by acylcholine acylhydrolase which are then considered an OP harmfulness buffer cholinesterases enzyme [12]. Hence, acylcholine acylhydrolase assay was included in the enzyme analysis to examine if acylcholine acylhydrolase might be a more sensitive biomarker of exposure to insecticides (malathion a descriptive in this study) than the cholinesterase isozymes. In the current paper, the acetylhydrolase and acylcholine acylhydrolase of the rabbit in the first five days post-fertilization of development and the effects of malathion on the cholinesterase isozymes activities were analyzed.

Materials and Methods

Chemicals

Cholinesterases substrates, [acetyl-β-methylthio-choline iodide (AβMSCh); butyrylthiocholine iodide (BuSCh); propionylthiocholine iodide (PrSCh)]; Ellman reagent, 5, 5′-dithiobis (2-nitrobenzoic acid); and OP (malathion), diethyl 2-[(dimethoxyphosphorothioyl)sulfanyl] butanedioate were supplied by G. L. Industries (E) Ltd., Guwahati, India. The specific inhibitors, tetramonoiso-propylpyrophosphortetramide (iso-OMPA); and 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284c51) were purchased from by the Sigma Chemical Company. All other reagents and solvents used in this work were of analytical grade.

Animals

Rabbit obtained from the local market (Kirkuk), recommended in the rabbit embryo test guideline [8]. The light/dark regime was set to 12 h/12 h. The rabbit was fed three times a day with commercial food ad libitum. Embryos were collected using a grid covered dish and successively cleaned with distilled water. The present study has conducted and approved at the College of Veterinary Medicine at the University of Kirkuk (Iraq).

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Sample preparation

Rabbit embryos were collected and washed twice with distilled water and exposed to the insecticide without regard of fertilization. Unfertilized embryos were differentiated from fertilized embryos after 1 h and excluded from the test. Stock solutions were prepared freshly the day before the test. The embryos were exposed to the malathion for 12 h. Malathion was chosen as the model pesticide in this study. Although numerous data exist about the malathion and its effects to many different vertebrates [13,14], g malathion was preferred in this study, because it is not known, if the embryos of the rabbit are able to metabolize malathion.

Enzyme analyses

The sample (embryo) was removed using a scalpel, cut into small pieces (1- 2 mm3), and rinsed until the blood was fully removed. The sample was then placed on ice in 10 mL tubes (5 mm internal diameter) and homogenized using a mechanically driven homogenizer with phosphate buffer (0.1M, pH 8) with a 1: 10 ratio (w/v) of 1 part of sample tissue to 9 parts of buffer and a speed of 10000 rpm. Homogenization required 5 min; after every one min or so of homogenization, the mixture was rested for 30 s to allow cooling. The homogenate was then centrifuged at 10000 g for 15 min and the supernatant fraction was retained and used for subsequent analyses. Cholinesterases activities were determined at room temperature 25°C by the Ellman method [15] and different thiocholine substrates (AβMSCh, BuSCh, and PrSCh) were used for measuring enzymes activity. Following combination of thiocholine derivatives with 5, 5′-dithiobis (2-nitrobenzoic acid) forms the yellow anion 5-thio-2- nitrobenzoic acid, which absorbs strongly at 412 nm [16]. Substrate solutions were prepared and used on the same day and kept on ice during use. Protein was assayed by the method of Bradford using bovine serum albumin as a standard [17]. All measurements in this study were carried out in duplicate.

Properties of cholinesterases enzyme by using specific inhibitors

Cholinesterases activities in homogenate supernatants of untreated rabbits and substrate-specificity assays with (AβMSCh, BuSCh, and PrSCh) in concentrations from 0.125 to 8 mM were carried out with pools of five days old embryos. Cholinesterases from the whole-body homogenates from embryos (two different experiments) were characterized using specific substrates and inhibitors [8]. The homogenate supernatants were incubated with the inhibitors in with 16 mM concentrations of BW284c51 and iso-OMPA at 25°C before the reaction was terminated by addition of excess substrate and following the analysis of residual enzyme activity. BW284c51 and iso-OMPA served as inhibitors of acetylhydrolase and acylcholine acylhydrolase, respectively. Stock solutions of iso-OMPA were dissolved in ethanol, while BW284c51 was dissolved in water.

Reaction rate versus enzyme concentration

Proportionality between the initial velocity of the reaction millioptical density units per minute (mOD/min) and the concentration of the enzyme was assayed using decreasing volumes of homogenates of the rabbit. Activities of three pools with 10 embryos each (five days of age) in a density 1 part of sample tissue (embryo) to 9 parts of buffer (0.1 M, pH 8). Enzyme assays were done with the three substrates (AβMSCh, BuSCh, and PrSCh) as described in the above section.

Statistical analysis

Statistical software package SigmaPlot version 11 (Systat software, Inc.) was used to perform all analyses. Normality and homogenate supernatants were checked before undertaking tests. Differences between times were evaluated by performing a one-way ANOVA (analysis of variance) and malathion concentration as fixed factors. A significant difference was acknowledged when $P<0.05$.

Results

Substrate-specific activities in rabbit embryos

To analyze the normal range of the enzyme activity in rabbit embryos, the relative contribution of the acetylhydrolase and their preferences for the different substrates were quantified. For this reason, increasing substrate concentrations from 0.125 to 8 mM were tested with supernatant homogenates of pools of five days old embryos. The specific activity of cholinesterases as a function of the different substrate concentrations (AβMSCh, BuSCh, and PrSCh) is shown in Figure 1. The results indicate that acetylhydrolase is the dominant cholinesterases enzyme of rabbit embryos. An increase of substrate concentrations from 0.5 mM produced an increase of activity to 2 U. Further increase of substrate concentrations to 2 mM was followed by a plateau phase in enzyme activity indicating that saturating concentration of substrate was reached. The increase of substrate concentration up to 8 mM caused a comparatively steep increase of the enzyme activity to 7.1 U. The activities measured with the other three substrates (AβMSCh, BuSCh, and PrSCh) and embryo (five days old post-fertilization) depending on specific substrate concentrations.

Cholinesterases characterization

Different cholinesterases enzyme specific inhibitors were used to characterize the contribution of the acetylhydrolase and acylcholine acylhydrolase in the rabbit embryo. In this study supernatant was incubated with 16 mM of BW284c51 (which inhibits acetylhydrolase) and iso-OMPA (which inhibits acylcholine acylhydrolase) were used at the same concentration in the above. After 1 h of incubation, the

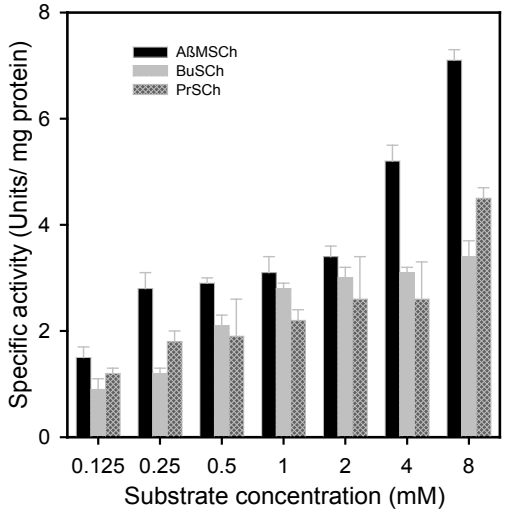

Figure 1: Specific cholinesterases enzyme activities of in the developing rabbit embryo (five days old post-fertilization) depending on specific substrate concentrations (0.125 to 8 mM). Data are depicted as mean ± S.E. The substrates used were (AβMSCh, BuSCh and PrSCh).

reaction was stopped with the excess of AβMSCh and the residual activity was assayed. In addition, increasing substrate concentrations from 0.125 to 8 mM were used to test for residual activity of non-specific cholinesterases which might convert the substrates (Figure 2). The concentration of 16 mM BW284c51 and iso-OMPA almost totally inhibited cholinesterases activity with substrate concentrations up to 4 mM. With substrate concentrations above 4 mM an increase of activity was seen independent from the different inhibitors. With the acetylhydrolase activity of the controls (set to 100%) the different residual activities made up 5-15% at 0.5 mM, 23-25% at 1 mM, 35-42% at 2 mM, 56-62% at 4 mM and 69-81% at 8 mM substrate. So, regardless of the high inhibitor concentrations, the substrates were converted by enzymes.

Reaction rate versus enzyme concentration

The increase in optical density with time was directly proportional to the sample volume i.e., enzyme concentrations in the homogenates (r^2 ranged between 0.997 and 0.98 with the substrates (AβMSCh, BuSCh, and PrSCh), respectively). Data are described in Figure 3. Consequently, Michaelis-Menten kinetics can be assumed for the measurement in the range between 10 and 160 µL sample volume for the substrates AβMSCh/BuSCh and between 2.5 and 40 µL for PrSCH with homogenates of five days old embryos.

In vivo inhibition of the acetylhydrolase and acylcholine acylhydrolases

All enzymes were inhibited from 250 to 2000 mg/kg with intra dermal injection with malathion. Acetylhydrolase and acylcholine acylhydrolases (using AβMSCh, BuSCh, and PrSCh substrates) were significantly decreased by malathion compared to the control and ranged between 43.38-91.78%, 14.72-80.65 and 5.98-71.76% by using AβMSCh, BuSCh, and PrSCh substrates, respectively (Table 1). Inhibitory effect of 2000 mg/kg malathion to the activity of the cholinesterase isozymes and acylcholine acylhydrolase in supernatants of whole embryo homogenates (five days post-fertilization) (Table 1).

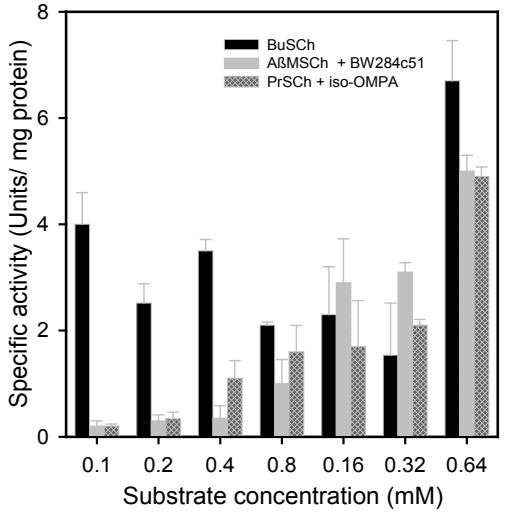

Figure 2: Specific enzyme activities of cholinesterases assayed with and without inhibitors of acetyl hydrolase and acyl choline acyl hydrolase by using BW284c51 and iso-OMPA as specific inhibitors. Data are represented as mean ± S.E.

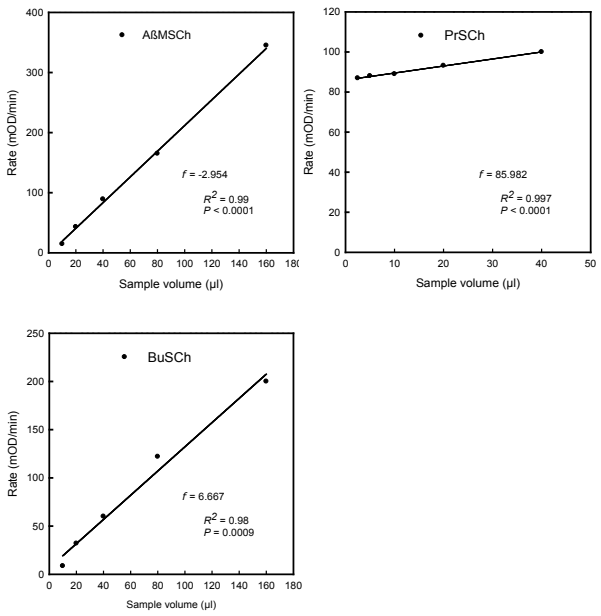

Figure 3: Correlation between the amount of protein/enzyme (µL) and increase of millioptical density units per minute (mOD/min). Data are depicted as mean ± S.E. with n=5 for each concentration analysed in quadruplicate. Parameters for linear regressions were used (Sigma Plot)..

% inhibition of malathion	AβMSCh	BuSCh	PrSCh
250 mg/kg	43.38	14.72	5.98
500 mg/kg	88.93	35.14	35.33
1000 mg/kg	92.47	76.58	43.64
2000 mg/kg	91.78	80.65	71.76

Data in the table is supernatants of whole embryo homogenates (five days post-fertilization), n=5

Table 1: Inhibitory effect of malathion from 250 to 2000 mg/kg to the activity of the acetyl hydrolase and acyl choline acyl hydrolases in supernatants of whole embryo homogenates (Five days post-fertilization), by using three substrates (AβMSCh, BuSCh and PrSCh).

Discussion

The biggest share of the acetylhydrolase in the rabbit embryo is contributed by acetylhydrolase, which seems to have also a potential to convert PrSCh and BuSCh. As the specific activity of the acetylhydrolase is decreasing with increasing substrate concentration, substrate inhibition is assumed, which is a characteristic feature for acetylhydrolase [8]. This finding is in good agreement with earlier rabbit (Netherland Dwarf) data. Tennyson et al. [18] did not find an acylcholine acylhydrolase enzyme activity in rabbit embryos but could prove the existence of an acetylhydrolase gene.

Jbilo et al. [19] found a very low content of liver and plasma acylcholine acylhydrolase to acetylhydrolase in rabbits. While acylcholine acylhydrolase have been found in heart rabbits [20]. The reason for the different occurrence of acylcholine acylhydrolase in various rabbit organs is not known but [21] hypothesized that the duplication of the ancestral gene that gave rise to acetylhydrolase and acylcholine acylhydrolase in some rabbits [19] was subsequently lost in New Zealand rabbits.

From the data of this work, it can be concluded that the enzyme activities with AβMSCh concentrations above 0.5 mM (Figure 2) are

due to unspecific cholinesterases which cannot be inhibited by high concentrations of 16 mM of either iso-OMPA or BW284c51. As a consequence of the statements above, the concentration of AβMSCh should not be increased above 0.5 mM to be sure about the specific activity measurements of acetylhydrolase in rabbit embryos. The findings of unspecific activity are in good agreement with observations of Sine et al. [22], who found residual enzyme activity of 21.3-34.7% even though using 10^{-5} M of BW284c51. The concentration of AβMSCh was slightly higher than used here (2 mM versus 0.125 mM). Still, this is comparable with our data in which 23-25% of activity were measurable with 4 mM substrate and 16 mM iso-OMPA (Figure 2). The specific activity of acylcholine acylhydrolase might be only one out of several isozymes in rabbit. Several studies indicated the existence of multiple acylcholine acylhydrolase isozymes among rabbit species [23-25].

The acetylhydrolase enzyme inhibition (43%) measured in the rabbit embryos by 250 mg/kg malathion and 35% inhibition of acylcholine acylhydrolase enzyme activities was reached by 500 mg/kg in this work is comparable to the literature (Table 1). Silver [8] tested five different OPs for their acute toxicity to the rabbit. A concentration of only 25 μM malathion decreased the acetylhydrolase enzyme activity in the brain of rabbit to 50% of the control activities after five days old exposure. Others hypothesized that a significant reduction of 10-20% in acetylhydrolase activity is generally considered indicative of OP exposure in the guinea pig [26] But this might not be true for all species as in contrast to the above data [27] showed that significant inhibition of acetylhydrolase invertebrates were only seen with lethal concentrations of physostigmine and neostigmine. So, they postulated that acetylhydrolase activity might only be used for the detection of acute toxicity but not for the detection of sublethal exposure. With the use of acylcholine acylhydrolase as an additional biomarker of exposure to OP insecticides, it was tested if acylcholine acylhydrolase actually might be able to buffer the inhibition of cholinesterases enzyme. Acylcholine acylhydrolase might help as a stoichiometric buffer system at least at the very early stages of development. As the enzyme activity of acylcholine acylhydrolase is as high or even slightly lesser than the cholinesterase units at later growing stages (five days post-fertilization) and due to the results of the malathion test did not show that acylcholine acylhydrolase was much more inhibited than the acetylhydrolase (Table 1) the theory of buffering acylcholine acylhydrolase can approve at these later stages of development. The use of specific acylcholine acylhydrolase inhibitors in mixture with cholinesterases inhibitors (malathion) could prove if acylcholine acylhydrolase is a significant path for (detoxifying) insecticides through stoichiometric buffering. However, further tests with lower concentrations then tested here would clarify, whether the "buffer" role might only show up at concentrations below a certain level of insecticide.

Conclusions

In conclusion, this study indicated that both acetylhydrolase isozymes and acylcholine acylhydrolase activities were inhibited by BW284c51 and iso-OMPA, and the inhibition of malathion was more obvious. To the best of our understanding, this is the first paper showing cholinesterase activity inhibition by malathion in rabbit embryo. This result also supports the hypothesis that diverse classes of environmental pollutants can inhibit cholinesterases activity in various vertebrates. Additionally, molecular biology studies are needed to define how OP can directly or indirectly alter cholinesterases activities at the biochemical level. Finally, despite this further studies are necessary under different laboratories, in order to improve and strengthen these results and to increase our knowledge about this very interesting enzyme as a potential biochemical marker for pesticide intoxication, as well as results obtained from these studies also confirm the suitability of tissues for Ellman cholinesterase determinations in rabbit samples.

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