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Influence of pesticides on the pH regulatory enzyme, carbonic anhydrase, from European Seabass liver and bovine erythrocytes

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ABSTRACT

The objective of this study was to assess the inhibitory effects of six commonly used pesticides, cyhalothrin, cypermethrin, dichlorvos, methamidophos, chlorpyrifos and methylparathion, on the pH regulatory enzyme carbonic anhydrase (CA) of Dicentrarchus labrax (European Seabass) liver (dCA) and bovine erythrocytes (bCA). Results of the study showed that the pesticides displayed quite variable inhibition profiles with K_I values ranging from 0.376 to 26.164 μM against dCA, and from 1.174 to 53.281 μM against bCA. Methylparathion was the most effective inhibitor for both enzymes. Overall data show that all of the tested pesticides inhibit both dCA and bCA at low concentrations indicating that indiscriminate use of these pesticides might cause disruption of acid base regulation resulting in animal deaths. Our results also point out that susceptibility to these pesticides varies among CAs from different organisms.

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1. Introduction

Carbonic anhydrase (CA; carbonate hydrolyase, EC 4.2.1.1) enzyme, which exists commonly in living organisms, has various izoenzymes according to conditions and necessities of the medium. It is one of the most studied enzymes and CA-I and CA-II are the most common isoenzymes (Tripp et al., 2001). A number of different CA isozymes have been described in higher vertebrates. Sixteen izozymes have been defined up to now, that differ in their subcellular place, catalytic activity and emotional different types of inhibitors. Some of these enzymes are cytolosolic (CA I, CA II, CA III, CA VII and CA XIII), others are membrane bound (CA IV, CA IX, CA XII and CA XIV), two are mitochondrial (CA VA and CA VB), and one is secreted in saliva (CA VI) (Supuran, 2008). It has been clarified that there is no expression of CA XV in human and other primates, but it is plentiful in rodents and other higher vertebrates (Ozensoy et al., 2004; Hilvo et al., 2005).The enzyme has esterase activity besides its hydratase activity, but physiologically hydratase activity is of great importance (Innocenti et al.,

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2005). For instance, it plays an important role in the regulation of acid-base balance in the organisms. Where this balance is impaired, for example, in intraocular tension, therapeutic intervention to carbonic anhydrase enzyme activity is a common applied method. In this respect, compounds of carbonic anhydrase inhibitors gains a clinical importance (Puscas et al., 2000).Pesticides are chemical substances used as biological agent, antimicrobial, disinfectant or device against any pest. Some of them are persistent organic pollutants and contribute to soil contamination. They are also one of the considerable causes for especially fresh water pollution (Hayes, 1990). Cypermethrin, a chemical, used to protect from many pests, including moth pests of cotton, fruit, and vegetable crops. It is also used for crack, crevice, and spot treatment to control insect pests in stores, warehouses, industrial buildings, houses, apartment buildings, greenhouses, laboratories, on ships, buses, trucks, and aircraft (Hayes, 1990; Ecobichon, 1996; Pretty and Hine, 2005; Ekinci and Senturk, 2010).Pesticides can interfere into fresh water such as river and lake by rainwater or irrigation water. This situation may be hazardous for living systems, including specific enzymes. It is well known that enzymes catalyze almost all chemical reactions in the metabolism of the living systems (Senturk et al., 2009; Ekinci and Beydemir, 2010; Ceyhun et al., 2010). These chemical substances including pesticides, drugs and metal ions influence metabolism at low concentrations by decreasing or increasing enzyme activities (Ekinci and Beydemir, 2010; Ceyhun et al., 2010). Cyhalothrin, cypermethrin, dichlorvos, methamidophos, chlorpyrifos and methylparathion are well known to interfere with a number processes since they have neurotoxic, hematotoxic, genotoxic, hepatic and renal effects on vertebrate (Ekinci and Senturk, 2010). However, little is known about how their affects on specific enzymes in organisms, such as fish and bovine. For this reason, we aimed in this study to evaluate the influences of selected pesticides on carbonic anhydrase enzyme from dicentrarchus labrax and bovine erythrocytes.

2. Materials and methods

2.1. Chemicals

Sepharose 4B, protein assay reagents and 4-nitrophenylacetate were obtained from Sigma–Aldrich Co. All other chemicals were of analytical grade and obtained from Merck.

2.2. Purification of carbonic anhydrase II from dicentrarchus labrax liver and bovine erythrocytes by affinity chromatography

Fish livers were purified from frozen fish tissues obtained from a commercial fish farm in Black Sea region in Turkey. The purification of dCA isozyme was performed with a simple one step method by a Sepharose-4B-aniline-sulfanilamide affinity column. Bovine blood was sampled from the caudal vein using a 10 ml plastic heparinized syringe (5 IU/ml) and transferred to tubes. Blood sample were centrifuged at 2500 × *g* for 15 min. The plasma was removed by drip. After the package of red cells was washed with KCl solution (0.16 M) three times, the samples were centrifuged at 2500 × *g* each time and supernatants were removed. The erythrocytes were hemolyzed with five volumes of ice-cold water and centrifuged (+4 °C, 10,000 × *g*) for 30 min to remove the ghosts and intact cells (Ceyhun et al., 2010). The bovine erythrocytes hemolysate was adjusted to 8.7 with solid Tris. Sepharose-4B-aniline-sulfanylamide affinity column was equilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7) and bCA II enzyme was eluted with and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6). All procedures were performed at 4 °C (Senturk et al., 2009) (Table 1).

2.3. Esterase activity assay

The esterase activity was assayed by following the change in absorbance of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion at 348 nm over a period of 3 min at 25 °C using a spectrophotometer (Beckman Coulter UV-VIS) according to the method described by Verpoorte et al. (1976). The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05 M Tris–SO_4 buffer (pH 7.4), 1.0 mL 3 mM 4-nitrophenylacetate, 0.5 mL H_2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution.

2.4. Protein determination

During each purification steps, protein determination was performed spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard (Bradford, 1976).

2.5. SDS polyacrylamide gel electrophoresis

After the purification steps, SDS polyacrylamide gel electrophoresis was performed to verify enzyme purity. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli procedure (Laemmli, 1970).

2.6. In vitro inhibition effect of pesticides on these enzymes activity

In this study, CA was purified from dicentrarchus labrax liver and bovine erythrocytes activities of the effluents were performed using the esterase activity method by using 4-nitrophenyl acetate (NPA) as substrate. For cyhalothrin, cypermethrin, dichlorvos, methamidophos, chlorpyrifos and methylparathion, inhibition studies were performed by using esterase activity. All compounds were tested in triplicate at each concentration used. Five different inhibitor concentrations were treated. In Table 2, we reported K_I values at three different inhibitor concentrations by using esterase activity method.

2.7. Statistical analysis

Student *t*-test was used for statistical analysis and $p < 0.05$ was considered statistically significant.

Table 1 – Summary of purification procedure for dicentrarchus labrax liver CA.

Purification steps	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity	Specific activity (EU/mg)	Yield %	Purification fold
Homogenate	135	40	13.72	548.8	5400	9.84	100	1
Affinity chromatography	427	6	0.54	3.24	2562	790.74	47.44	80.36

Table 2 – Summary of purification procedure for bovine erythrocyte CA II.

Purification steps	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity	Specific activity (EU/mg)	Yield %	Purification fold
Homolysate	167	30	4.72	141.6	5010	35.38	100	1
Affinity chromatography	323	8	0.08	0.64	2584	4037.5	51.57	114.12

3. Results and discussion

The isozymes of CA play important roles in different tissues (Supuran et al., 2001; Ekinci et al., 2011). The similarities of CAs from various sources have been determined from their crystal structures. It is known that carbonic anhydrase has been purified many times from different organisms (Demirdag et al., 2011) and the effects of various chemicals, pesticides and drugs on its activity have been investigated (Gervais and Tufts, 1999; Ekinci and Beydemir, 2010; Ceyhun et al., 2011). Hundreds of pollutants in the form of metals, acids, bases and other toxic compounds are being added to rivers, seas and the atmosphere and this causes a situation which has results in destruction of the natural balance.

The present work examined the effects of pesticides on dCA and bCA II. Both hydratase and esterase activities of the enzyme were investigated.

Today, all living things can inevitably be exposed to the effects of different chemical substances. This situation can be arisen from people's environment like their work environment. Although there is no connection with the atmosphere of such a work environment, people and other organisms are primarily affected by the waste created by people. In particular, the elimination of waste materials from factories in various countries has been made by burying into the ground or releasing to the air. As a result, many toxic substances such as pesticides, have been passing into the soil, water, plants, and then from there to animals and people. So today, especially enzyme activity studies made by using these substances remains its popularity (Ekinci and Senturk, 2010; Ceyhun et al., 2011).

Ceyhun et al. (2010) found that deltamethrin, diazinon, propoxur and cypermethrin, have inhibitory effect on rainbow trout gill carbonic anhydrase. In another study about enzyme inhibiton reported by Ekinci and Beydemir (2010) the effects of some pesticide and fungicides on the activities of rainbow trout liver, muscle, kidney and brain carbonic anhydrase enzymes were investigated *in vitro*.Carbonic anhydrase enzyme has Zn^{2+} ion in its active site and, therefore, we assume that the pesticides inhibit the enzyme at very low concentrations due to the electronegative atoms in their chemical structures (Fig. 1). Dogan (2006) observed that IC_{50} value of deltamethrin was 40 times lower than that of Lambda-cyhalotrin. Deltamethrin has Br atom while Lambda-cyhalotrin has Cl. Similarly, we calculated in this study that deltamethrin has three times lower IC_{50} value than Cypermethrin which includes Cl atom (Table 3).Our groups recently investigated the interaction of rainbow trout erythrocyte glucose-6-phosphate dehydrogenase and rainbow trout gill carbonic anhydrase enzyme with several types of pesticides, such as cypermethrin and deltamethrin. Cypermethrin and propoxur inhibited glucose-6-phosphate dehydrogenase enzyme *in vitro* and deltamethrin inhibited both *in vivo* and *in vitro*. The obtained IC_{50} values for deltamethrin, cypermethrin and propoxur were 0.63, 1.02 and 12 mM, respectively (Senturk et al., 2009) (Fig. 2).Consequently, the inhibitory effects of some pesticides on the enzymatic activity of dicentrarchus labrax liver and bovine erythrocytes CA were

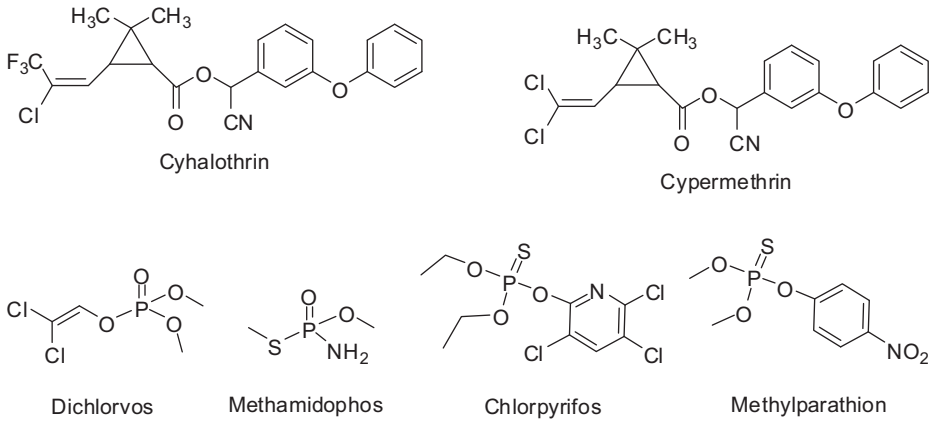


Fig. 1 – Molecular structures of used pesticides.

Table 3 – K_I and IC_{50} values of dCA and bCA II.

Inhibitors	dCA (μM)		bCA II (μM)	
	IC_{50}	K_I	IC_{50}	K_I
Cyhalothrin	1.895	1.074	2.336	1.863
Cypermethrin	1.248	0.832	28.442	16.173
Dichlorvos	23.187	14.178	10.178	5.872
Methamidophos	38.731	26.164	2.129	1.636
Chlorpyrifos	2.643	2.175	84.124	53.281
Methylparathion	0.853	0.376	2.146	1.174

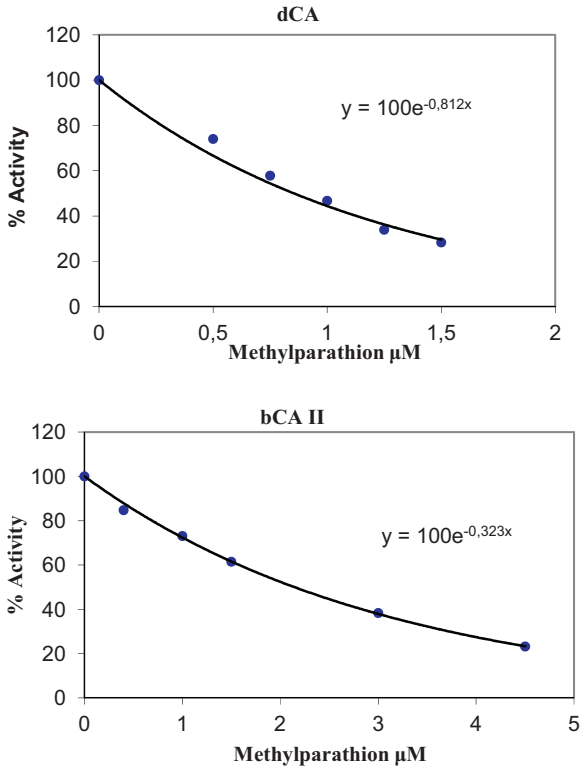


Fig. 2 – IC_{50} graphics for dCA and bCA II with methylparathion (MP).

determined. The results indicate that among the six pesticides used in this study, the most effective one is methylparathion which is widely used both at homes and in agricultural fields. Methylparathion inhibits these enzymes at very low doses, indicating that fish in natural and cultural environments are susceptible to this pesticide and that methylparathion contaminations would result in collective fish and bovine deaths. This would lead to the increase in food insufficiency for increasing populations and cause disruption of ecological balance. For this reason, the usage of especially methylparathion, cyhalothrin and cypermethrin must be well controlled.

Conflict of interest statement

The authors state no conflict of interests.

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