JOURNAL OF ENZYME INHIBITION AND MEDICINAL CHEMISTRY, 2017
VOL. 32, NO. 1, 47–50
http://dx.doi.org/10.1080/14756366.2016.1232255

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RESEARCH ARTICLE

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Carbonic anhydrase from *Apis mellifera*: purification and inhibition by pesticides

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ABSTRACT

Carbonic anhydrase (CA) enzymes have been shown to play an important role in ion transport and in pH regulation in several organisms. Despite this information and the wealth of knowledge regarding the significance of CA enzymes, few studies have been reported about bee CA enzymes and the hazardous effects of chemicals. Using *Apis mellifera* as a model, this study aimed to determine the risk of pesticides on *Apis mellifera* Carbonic anhydrase enzyme (Am CA). CA was initially purified from *Apis mellifera* spermatheca for the first time in the literature. The enzyme was purified with an overall purification of ~35-fold with a molecular weight of ~32 kDa. The enzyme was then exposed to pesticides, including tebuconazole, propoxur, carbaryl, carbofuran, simazine and atrazine. The six pesticides dose-dependently inhibited *in vitro* AmCA activity at low micromolar concentrations. IC_{50} values for the pesticides were 0.0030, 0.0321, 0.0031, 0.0087, 0.0273 and 0.0165 μM, respectively. The AmCA inhibition mechanism of these compounds is unknown at this moment.

ARTICLE HISTORY
Received 6 August 2016
Accepted 31 August 2016

KEYWORDS
Apis mellifera; carbonic anhydrase; inhibitor; pesticide

Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc metalloenzymes, which regulates pH and CO_2 levels in all living organisms[1,2]. A number of different CA isozymes have been described in higher vertebrates, whereas in other animals, e.g. insects, these enzymes are by far less investigated. Among the vertebrate CA isozymes, there are cytosolic (such as CA I, CA II, CA III, CA VII), membrane-bound (CA IV, CA IX, CA XII and CA XIV), mitochondrial (CA VA and CA VB), secretory forms (CA VI) and several acatalytic forms (CA VIII, CA X and CA XI)[3].

The physiological function of the CA isozymes is to facilitate the interconversion of CO_2 and HCO_3; therefore, they play key roles in diverse processes, such as physiological pH control and gas balance, calcification and photosynthesis. In addition, CA plays an important role in ion transport and pH regulation in eye, kidney, central nervous system (CNS) and inner ear[4].

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 and contamination. They are also one of the considerable causes for plant pollution[5]. In many cases, pesticides and fungicides can interfere into rain water, irrigation water or river, plants and may be hazardous for specific enzymes. It is well known that enzymes catalyze almost all chemical reactions in the metabolism of the living systems, and many chemical substances including pesticides, fungicides, drugs and metal ions influence metabolism at low concentrations by decreasing or increasing enzyme activities[6].

Tebuconazole, propoxur, carbaryl, carbofuran, simazine and atrazine are well-known to interfere with a number processes since they have neurotoxic, hematotoxic, genotoxic, hepatic and renal effects on vertebrate. Nevertheless, little is known about their effects on specific enzymes in organisms especially honey bee. For this reason, we aimed in the current study to analyze the interactions between some pesticides and fungicides and carbonic anhydrase enzymes. To this end, we selected *Apis mellifera* as a model animal. We also aimed in this study to purify and characterize honey bee spermatheca CA enzyme for the first time. using affinity chromatography.

Materials and methods

Chemicals

Sepharose-4B, protein assay reagents, and chemicals for electrophoresis were purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany).

Preparation of the homogenate

Apis mellifera samples were washed three times with 50 mM Tris-HCl +0.1 M Na_2SO_4 (pH 8.0). And each of bees were homogenized by liquid nitrogen, transferred to the same buffer and centrifuged at 4 °C, 15 000 *g* for 60 min. Supernatant was used in further studies.

Purification of carbonic anhydrase from honey bee by affinity chromatography

CNBr activated Sepharose-4B was washed with ddH_2O. After that, aniline was attached to the activated gel as a spacer arm and

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finally diazotized sulfanilamide clamped to the para position of aniline molecule as ligand.

The homogenate was applied to the prepared Sepharose 4B-tyrosine-sulfanylamide affinity column equilibrated with 25 mM Tris-HCl/0.1M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris-HCl/22 mM Na_2SO_4 (pH 8.7). The AmCA was eluted with 1M NaCl/25 mM Na_2HPO_4 (pH 6.3). All procedures were performed at 4 °C[7].

Hydratase activity determination

Enzyme activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson[8]. CO_2-Hydratase activity as an enzyme unit (EU) was calculated by using the equation $(t_0–t_c/t_c)$, where t_0 and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

Protein determination

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method[9], with bovine serum albumin as a standard.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The control of enzyme purity was carried out using Laemmli's procedure[10] in 3 and 8% acrylamide concentrations for running and stacking gel, respectively. 10% SDS was added to the gel solution. The gel was stabilized in a solution containing 50% propanol +10% TCA +40% distilled water for 30 min. Staining was performed for about 2 h in a solution of 0.1% Coommassie Brilliant Blue R-250 + 50% methanol +10% acetic acid. Finally, washing was carried out in a solution of 50% methanol +10% acetic acid +40% distilled water until the protein bands were cleared.

In vitro *inhibition assays*

The effects of increasing concentrations of tebuconazole, propoxur, carbaryl, carbofuran, simazine and atrazine on AmCA activities were determined colorimetrically using CO_2-hydratase assay (Figure 1).

The pesticides were also tested in the hydratase activity assay in triplicate at each concentration used. Different concentrations of pesticides were examined in preliminary assays. Enzyme activities were measured in the presence of different concentrations of tebuconazole, propoxur, carbaryl, carbofuran, simazine, and atrazine. Control enzyme activity in the absence of a pesticide was taken as 100%. For each pesticide, an activity % versus inhibitor concentration tube was drawn using conventional polynominal regression software (Microsoft Office 2000, Excel). Pesticide concentrations that produced 50% inhibition (IC_{50}) were calculated from graphs.

Results and discussion

Here, we isolated the CA enzyme from *Apis mellifera* spermatheca for the first time. We achieved to purify the enzyme in a single step using affinity chromatography on Sepharose 4B tyrosine-sulfanilamide. The enzyme was purified 35-fold with a recovery ratio of 17.87% compared to the homogenate (Table 1). After the sample had completely passed through, the column was washed with 25 mM Tris-HCl/22 mM Na_2SO_4 buffer whose pH was 8.7. During washing, absorbencies of fractions were spectrophotometerically measured at 280 nm and 348 nm. These values of the absorbance showed that some proteins, bound to the affinity material, have been removed from the column by the washing solutions. Then, the enzyme was eluted with 1 M NaCl/25 mM Na_2HPO_4 pH 6.3. At the end of the last step, a highly purified enzyme was obtained exhibiting a single band on SDS-PAGE (Figure 2). We used only one chromatographic technique, Sepharose 4B tyrosine-sulfanilamide affinity chromatography by modification of washing and elution conditions.

The molecular weight was determined to be ~37 kDa. Similar results have been observed for the enzyme from different sources. For example, human erythrocyte CA is 29 kDa, bovine erythrocyte CA is 29 kDa and European seabass liver CA is 30.2 kDa[3–6].

In addition to purification of the enzyme, tebuconazole, propoxur, carbaryl, carbofuran, simazine and atrazine were chosen to investigate their inhibitory effects honey bee spermatheca CA and I_{50} parameters of these pesticides were determined. Pesticides inhibited the enzyme activity at low concentrations.

Researches on influences of various chemicals on CA enzymes have gained a great attention recently[2,11–17]. For instance, *in vitro* effect of some heavy metals on enzymes, such as intestinal and branchial carbonic anhydrase and Na^+–K^+–ATPase, which play a key role in salt and osmoregulation and acid–base balance in the teleost fish, was studied. CA activities in gill and intestinal homogenates were significantly inhibited by heavy metals[18]. In another study, freshwater rainbow trout exposed to 10 μg l^{-1} Ag for 48 h had significantly lower activities of the branchial enzymes Na^+/K^+ ATPase (85% inhibition) and carbonic anhydrase (28% inhibition). The results suggested that a disturbance of branchial ionoregulation, as a result of inhibition of branchial enzymes involved in ion transport, is the principal mechanism of the physiological toxicity of silver nitrate to freshwater fish. Another study demonstrated that Cl^- uptake in *P. promelas* acclimated to soft water occurs through both a Na^+:K^+:$2Cl^-$ co-transporter and a Cl^-/HCO_3^- exchanger, but is not dependent on carbonic anhydrase[19].

CA which is a widespread metalloenzyme, which has previously been purified and characterized from many living organisms including animals. The isozymes of CA play important roles in different tissues[2,11–18]. The similarities of CAs from various sources have been determined from their crystal structures[2,11–18]. Hundreds of pollutants in the form of metals, acids, bases and other toxic compounds are being added to rivers, seas and the atmosphere a situation which has resulted in the destruction of the natural balance[19]. However, we have not found any data on AmCA enzyme in the literature and thus our study includes the investigation of the *Apis mellifera* CA enzyme for the first time. The inhibition mechanisms of these compounds against this α-CA are not known at this moment, also considering the fact that they do not incorporate functionalities usually present in most classes of reported inhibitors[20–22]. It should be also stressed that except *Drosophila melanogaster*, for which a β- and two α-CAs were described in detail[23,24], the investigation of this enzyme superfamily in insects is still on its infancy, with very few literature reports available to date.

Conclusions

CA was purified from *Apis mellifera* spermatheca by affinity chromatography on Sepharose 4B-tyrosine-sulfanilamide (Table 1). SDS-PAGE gels revealed that CA migrated as a single band.

Table 1. Summary of purification procedure of CA from honey bee, AmCA.

Purification step	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Spesific activity (EU/mg)	Yield (%)	Purification factor
Homogenate	5	12.1	141	705	5	100	1
Affinity Chromatography	3	0.24	0.72	126	175	17.87	35

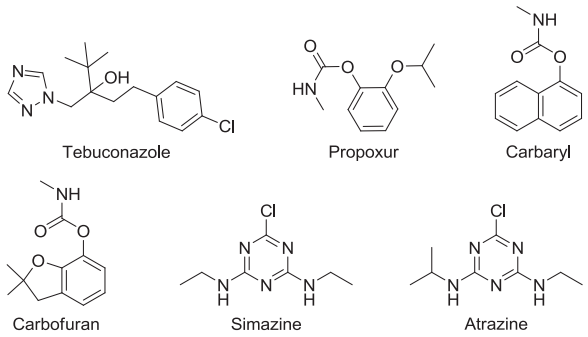

Figure 1. Structures of pesticides tested for the inhibition of AmCA activity.

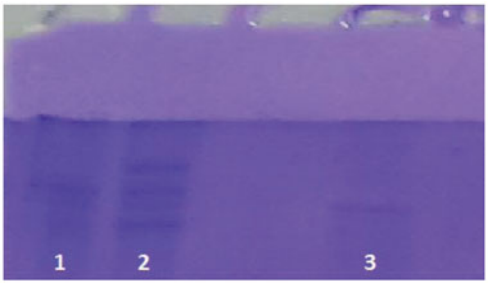

Figure 2. SDS-PAGE photograph. Lane 1: truncated β-galactosidase (83 kDa). Lane 2: standard proteins: truncated β-galactosidase (83 kDa), bovine albumin (66 kDa) and bovine carbonic anhydrase (29 kDa). Line 3: AmCA estimated molecular weight 37 kDa.

Table 2. AmCA inhibition data with studied pesticides (IC_{50} values), by an esterase assay with 4-nitrophenylacetate as substrate.

Inhibitor	hbCA (μM)
Tebuconazole	0.0030
Propoxur	0.0321
Carbaryl	0.0031
Carbofuran	0.0087
Simazine	0.0273
Atrazine	0.0165

The overall purification yield of CA was 17.87%, specific activity was 175 EU/mg protein and purification range was ∼35-fold (Table 1). Figure 2 shows the *in vitro* effects of tebuconazole. Tebuconazole has higher inhibition effects than propoxur, carbaryl, carbofuran, simazine, and atrazine. IC_{50} values were determined as 0.0030, 0.0321, 0.0031, 0.0087, 0.0273 and 0.0165 μM, for tebuconazole, propoxur, carbaryl, carbofuran, simazine and atrazine, respectively (Table 2). Our results showed that pesticides inhibit hbCA activity with rank order tebuconazole > carbaryl > carbofuran > atrazine > simazine > propoxur in *in vitro* conditions. Our findings indicate these pesticides are potent inhibitors for AmCA enzymes, and might cause undesirable results by disrupting acid–base regulation as well as salt transport, although their inhibition mechanism is unknown at this moment.

Disclosure statement

The authors report no declarations of interest.

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