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RESEARCH ARTICLE

Inhibition effects of some pesticides and heavy metals on carbonic anhydrase enzyme activity purified from horse mackerel (*Trachurus trachurus*) gill tissues

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Abstract

The gill tissue is the main site of metabolic enzymes or compensation, with the kidney tissue playing a supporting role. At the gill tissue, carbonic anhydrase enzymes (CAs) catalyze the hydration of CO_2 to HCO_3^- and H^+ for production to the H_2O. In this work, the CA enzyme was purified from horse mackerel (*Trachurus trachurus*) gill with a specific activity of 21,381.42 EU/mg, purification fold of 150.61, total activity of 2347.68 EU/mL, and a yield of 16.13% using sepharose 4B-L-tyrosine-sulfanilamide affinity gel chromatography. For recording the enzyme purity, gel electrophoresis was performed, and single band was seen. The molecular weight of this enzyme was found approximately 35 kDa. Also, the inhibitory effects of different pesticides such as thiram, clofentezine, propineb, deltamethrin, azoxystrobin, and thiophanate and heavy metal ions such as Fe^{2+}, Cu^{2+}, Co^{2+}, Pb^{2+} Hg^{2+}, and As^{3+} on horse mackerel gill tissue CA enzyme activities were investigated. Our results indicated that these pesticides and metal ions showed inhibitory effects at low nanomolar and millimolar concentrations for fish gill CA enzymes, respectively.

Keywords Carbonic anhydrase · Horse mackerel · *Trachurus trachurus* · Gill · Heavy metal · Pesticides

Introduction

Pesticides are chemical compounds that are employed to control the organisms thought to be hazardous. The most commonly employed ones are fungicides, herbicides, rodenticides, and insecticides (Koksal et al. 2018). They are the most common contaminants of the soil, air pollution environment, and water. It is clear that a number of compounds, pesticides, and metal ions can be dangerous for the living organisms (Özaslan et al. 2018). Among these pesticides, propineb has been extensively employed fungicide due to its low production cost and activity against fungal plant diseases (Abbaci et al. 2014). Thiram is commonly employed as a fungicide to prevent harvested crops from deterioration in transport or storage (Walia et al. 2009). Deltamethrin is an insecticide employed worldwide in home pest control, agriculture, disease vector control, and protection of foodstuff (Yousef et al. 2006). Clofentezine is widely employed as a potent contact ovicide and played a key role to act by interfering with cell differentiation and cell growth (Krämer et al. 2012). Azoxystrobin has been widely employed as fungicides due to its large spectrum control of fungal diseases (Butler et al. 2018). Thiophanate has a large-

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spectrum benzimidazole fungicide and commonly applied for the control of a number of vegetable and fruit pathogens (Ye et al. 2008).

Heavy metals are important toxic substances due to their biological accumulation, toxicity, and persistence in aquatic ecosystems (Kirici et al. 2016; Rai 2009). Development in agriculture and industry has caused a rise in their levels (Demir et al. 2017). This rise has become one of the most significant problems of human health and environmental toxicology (Tchounwou et al. 2012). It is clear that this condition is significant for living organisms in aquatic environments including specific enzymes. Scientific studies clarify that contaminants such as pesticides and metal ions show impact by decreasing or increasing enzyme activity at very low concentrations (Demir et al. 2016). Copper possesses a significant function in living organisms. It is found in the structure of many proteins, but much concentration of this metal ion can be toxic to living organisms (Kirici et al. 2017; Manyin and Rowe 2009). Mercury is an environmental pollutant and dangerous industrial, which induces severe damage in various organs in human and animals (Caglayan et al. 2019b; Gado and Aldahmash 2013). Arsenic is an environmental contaminant found in food, soil, water, and air. Chronic exposure to high levels of arsenic is related to a number of adverse impact, such as peripheral vascular diseases, neurological effects, skin lesions, and reproductive toxicity (Bustaffa et al. 2014; Turk et al. 2019). Lead is well-known to be neurotoxic to humans and possesses a number of harmful health impacts at high levels of exposure (Jarvis et al. 2018). Cobalt is acutely cumulative, toxic in extensive doses, and long-term exposure even at a low level and can lead to adverse health impacts connected to several tissues and organs (Simonsen et al. 2012). Iron is an important trace element of the body, being found in functional form in myoglobin, hemoglobin, and cytochrome enzymes with iron sulfur complexes (Pari et al. 2015). However, the much concentration of it causes cellular damage, mutation, and malignant transformations which in turn cause an array of diseases (Jaishankar et al. 2014).

Carbonic anhydrase (CA) has been found in all living organism cells and includes Zn^{2+} in its active site. It is first recorded in bovine erythrocytes (Aslan et al. 2018; Boztas et al. 2014; Sağlık et al. 2019; Taslimi et al. 2017). CA enzymes have a key role in a number of diverse metabolic processes like acid–base regulation, transportation, and respiration of carbon dioxide and bicarbonate, calcification, bone resorption, homeostasis, electrolyte secretion, and so on (Caglayan 2019; Gündoğdu et al. 2019; Taslimi et al. 2018a; Taslimi et al. 2019). CAs are extensively found in most living organisms and are classified in seven groups: α-, β-, γ-, δ-, η-, ζ-, and θ-CAs (Bayindir et al. 2019; Caglayan et al. 2019a; Taslimi et al. 2018b). Only the α-CAs exist in cytoplasm of green plants, algae, vertebrates, and bacteria. Thus, CA enzyme is discovered in tissues of fish (Atmaca et al. 2019; Kucuk and Gulcin 2016). Until now, 16 diverse CA isoforms have been recorded in animal cells and plants. CAs I, II, III, VII, and XIII are cytoplasmic, CA V is mitochondrial, CA VI is secretory, CAs IV, IX, XII, and XIV are membrane-associated, and CAs VIII, X, and XI are noncatalytic isoenzymes (Bayrak et al. 2017; Kose et al. 2016; Öztaskın et al. 2017).

In the present study, we investigated the in vitro toxic effects of some heavy metal ions, including Fe^{2+}, Cu^{2+}, Co^{2+}, Pb^{2+}, Hg^{2+}, and As^{3+} and some pesticides including propineb, thiram, deltamethrin, clofentezine, azoxystrobin, and thiophanate on the CA enzyme purified from the horse mackerel gill tissues.

Materials and methods

Chemicals

Thiram (Birgin Forte, 80 WP), deltamethrin (Dentis 25 EC), propineb (Antracol WP 70, Bayer), clofentezine (Apofen), thiophanate (Sumitop WP), and azoxystrobin (Efdal Azbin SC) (Fig. 1) were obtained from an agricultural pesticide shop. $Pb(CH_3COO)_2$, $NaAsO_2$, $CoCl_2$, $FeCl_2$, $CuSO_4{\cdot}5H_2O$, Sepharose-4B, protein assay reagents, chemicals for electrophoresis and all another chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Preparation of gill homogenate

Horse mackerel was obtained from the Marmara Sea in the northwest Turkey. The gill tissues were cut into a piece of 20 g and kept at − 80 °C for later use. To obtain gill tissue homogenate, the tissue was grinded using liquid nitrogen and homogenized in a buffer solution of 25 mM Tris HCl/0.1 M Na_2SO_4 (pH 8.7). The suspension was centrifuged for 30 min at 13500× g. and this operation was performed three times. The supernatant was used for next analysis.

Purification of CA from horse mackerel gills

The pH of the homogenate obtained from horse mackerel gills was adjusted to 8.7 with solid Tris. Homogenate was applied to the column and washed with a solution of Tris-HCl and 400 mL of 25 mM Na_2SO_4 (pH 8.7). Thus, enzyme purification was performed as in previous studies (Caglayan and Gulcin 2018; Kucuk and Gulcin 2016; Soyut and Beydemir 2008).

Measurement of CA enzyme activity

CA enzyme activity can be measured in two ways: first one is esterase activity that can be carried out in vitro and followed

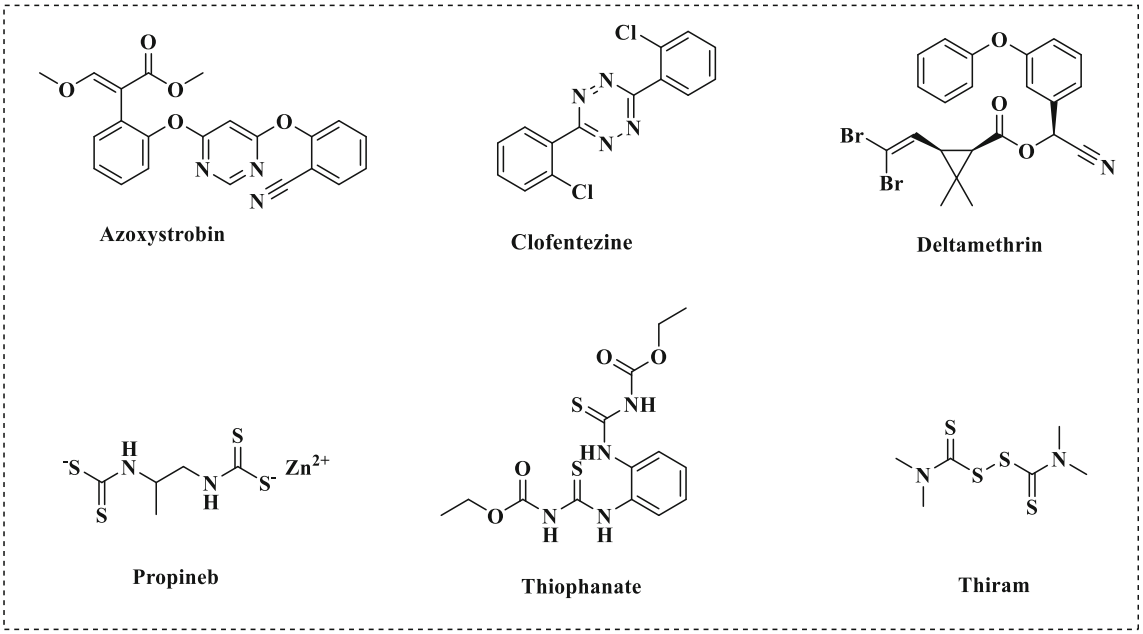

Fig. 1 The chemical structures of used pesticides including azoxystrobin, clofentezine, deltamethrin, propineb, thiophanate, and thiram

spectrophotometrically, and the second is CO_2-hydratase activity, the physiological activity of the CA. Enzymes hydratase activities were performed according to the assay explained by Wilbur and Anderson (1948). The CA enzyme esterase activity was performed as in previous studies (Topal and Gülçin 2014; Türkeş et al. 2019).

Protein determination

Quantitative amounts of total CA enzyme were designated conforming to the Bradford (1976) procedure at 595 nm, spectrophotometrically. Bovine serum albumin was used as positive controls (Öztaşkın et al. 2019).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to check the purity of the isozymes and calculate the molecular weights (Turkan et al. 2019). For this reason, 3% and 10% acrylamide, respectively, for stacking and running containing 0.1% SDS were prepared according to Laemmli procedure (1970). Denaturized enzyme samples and protein markers were applied to the electrophoresis medium. The electrophoresis gel was held in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid in one night. The method was performed according to our previous studies (Beydemir and Demir 2017; Demir and Beydemir 2015). Used protein marker was the product with a catalog number of Thermo 26616.

Kinetic studies

Optimum and stable pHs

For designation of optimum pH value, CA activities were measured in 1.0 M Na-phosphate buffers ranging from pH 5.0–8.0 and 1.0 M Tris-HCl ranging from pH 7.5–9.0, 1.0 M glycine-NaOH ranging from pH 9.0–10.5. On the other hand, for the designation of stable pH value, the CA enzymes activities were measured in these buffers. The activity measurements were performed at 24-h period during 3-day incubation using p-nitrophenylacetate (PNA) as the substrate under standard situations.

Optimum ionic strength and temperature

For obtaining the optimum ionic strength value of the enzyme, activities used various concentrations of glycine/NaOH buffer (pH 9.0) ranging from 0.1 M to 1.1 M. The horse mackerel gills CA enzyme activities were measured at various temperatures with an increase of 10 °C ranging from 0 to 80 °C for determining the optimum temperature.

Inhibition studies

The inhibitory effects of metal ions (Fe^{2+}, Cu^{2+}, Co^{2+}, Pb^{2+} Hg^{2+}, and As^{3+}) and pesticides (propineb, thiram, deltamethrin, clofentezine, azoxystrobin, and thiophanate) were evaluated on CA enzyme activity purified from horse mackerel gill tissue. Metal ions were dissolved in water, the effects of inhibition on the enzyme were investigated, IC_{50} and K_i studies

were performed, and the values were calculated and plotted (Caglayan and Gulcin 2018, Kucukoglu et al. 2019).

Results

Characterization studies for CA enzyme

In this work, the CA enzyme was purified from horse mackerel gill with a specific activity of 21,381.42 EU/mg, purification fold of 150.61, total activity of 2347.68, and a yield of 16.13% using sepharose 4B-L-tyrosine-sulfanilamide affinity chromatography (Table 1). For determining the enzyme purity and molecular mass, SDS-PAGE was performed, and single band was observed. The molecular mass was found approximately 35 kDa (Fig. 2). The recorded molecular mass was similar to CA enzymes purified from other tissues as previous studies. For instance, rainbow trout liver 29.4 kDa, flounder gills 29 kDa (Sender et al. 1999), zebrafish erythrocyte 29 kDa (Peterson et al. 1997), and sea bream gills 30.5 kDa (Kaya et al. 2013) were determined, respectively. For horse mackerel gill tissue, quantitative protein determination in enzyme solutions obtained by Bradford method was performed. A standard graph was prepared, and quantitative protein determination of enzyme solutions obtained by affinity chromatography was found using this standard graph. SDS-PAGE method was utilized to check the purity of the eluates obtained by affinity of fish CA enzyme (Burmaoglu et al. 2019). Therefore, the electrophoresis system was installed, and the enzyme samples were loaded into the wells in sequence and then carried out. The photograph showing the bands obtained is shown in Fig. 2. Optimal pH study was performed for CA enzyme purified from fish, and their pH was determined spectrophotometrically using buffer solutions of 5–11 (Fig. 3a). The optimum pH was determined as 9.0 for CA enzyme purified from gill tissue. In order to designate the optimal ionic strength for CA enzyme activity purified from fish gill, solutions of different concentrations of glycine/NaOH buffer, which were determined to be suitable in previous studies, were prepared. Activity measurements at different glycine/NaOH concentrations were made, and a graph of glycine/NaOH concentration and activity values was plotted. As a result of the studies, the most suitable ionic strength for CA enzyme purified from fish gill was determined as glycine/NaOH (pH 9.0, 1.0 M) buffer (Fig. 3b). In this study, a stable pH study was performed to designate the stable pH of purified CA enzyme. The results are shown in Fig. 3c. As a result of these studies, stable pH was determined as pH 8.0. Indeed, 1.0 M glycine/NaOH (pH 9.0) buffer solution with optimum pH and appropriate ionic strength were used to designate the optimum temperature of the purified CA enzyme. Activity measurements were performed at 0 °C to 80 °C every 10 °C. The results are shown in Fig. 3d. As a result of these studies, the optimum temperature was determined as 30 °C.

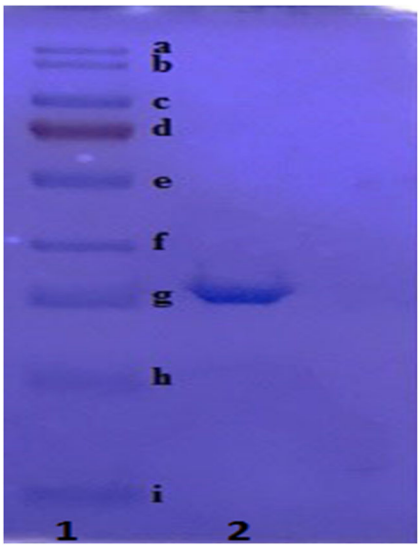

Fig. 2 SDS polyacrylamide gel electrophoresis of horse mackerel gills CA purified by Sepharose 4B-L-Tyrosine-sulfanilamide affinity gel. Line 1: the standard proteins (a, 180 kDa; b,130 kDa; c, 100 kDa; d, 70 kDa; e, 55 kDa; f, 40 kDa; g, 35 kDa; h, 25 kDa; i, 15 kDa). Line 2: horse mackerel gills CA

Inhibition results

In present study, we examined the CA inhibition effects of pesticides and some metal ions. The used of some pesticides had IC_{50} values in the range of 3.25–36.76 nM. IC_{50} values of some pesticides exhibited the following order: thiram (3.25 nM, r^2: 0.9801) < clofentezine (3.55 nM, r^2: 0.9489) < propineb (8.45 nM, r^2: 0.9130) < deltamethrin (12.37 nM, r^2: 0.9602) < azoxystrobin (30.97 nM, r^2: 0.9598) < thiophanate

Table 1 Summary of purification of CA enzyme from horse mackerel gill tissues

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU/mL)	Specific activity (EU/mg)	Yield (%)	Purification fold
Homogenate	632.86	23	4.458	102.534	14,555.78	141.96	100.00	1.00
Sepharose-4B-L- tyrosine sulfanilamide affinity chromatography	782.56	3	0.036	0.109	2347.68	21,381.42	16.13	150.61

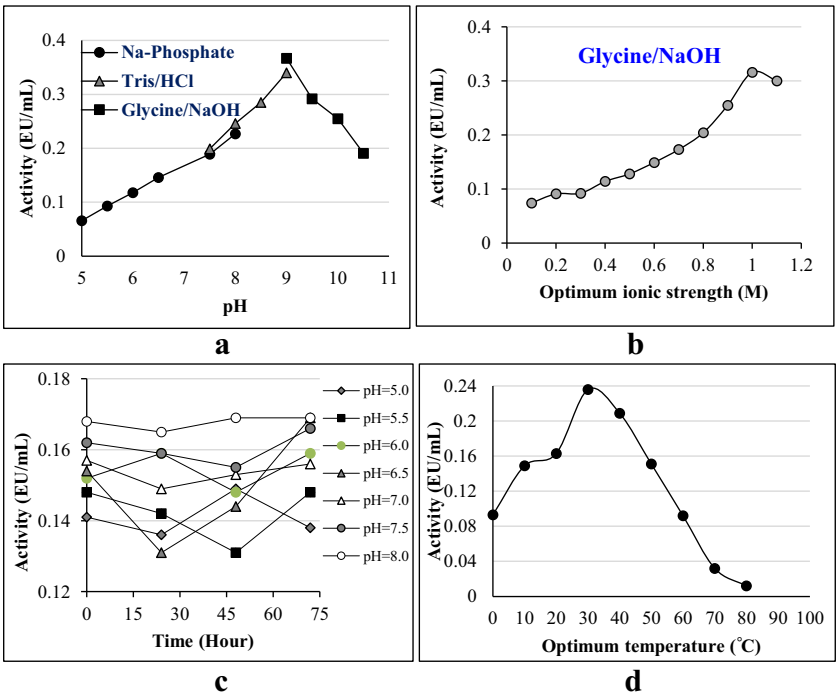

Fig. 3 (**a**) Determination of optimum pH for from horse mackerel gills in 1.0 M phosphate, 1.0 M Tris-HCl buffer, and 1.0 M glycine/NaOH buffer (**b**) determination of optimum ionic strength (M, glycine/NaOH buffer) from horse mackerel gills. (**c**) Determination of stable pH graph of from horse mackerel gills for 3 days. (**d**) The effect of temperature on CA enzyme activity from horse mackerel gill tissues

(36.76 nM, r^2: 0.9491). On the other hand, it demonstrated K_i values in the range of 4.38 ± 1.98–48.38 ± 14.01 nM. K_i values of these some pesticides exhibited the following order: thiram (4.38 ± 1.98 nM) < clofentezine (5.27 ± 2.16 nM) < propineb (11.17 ± 5.03 nM) < deltamethrin (11.96 ± 3.11 nM) < azoxystrobin (13.98 ± 3.23 nM) < thiophanate (48.38 ± 14.01 nM) (Table 2 and Fig. 4).

The used metal ions had IC_{50} values in the range of 0.66–7.12 mM. IC_{50} values of some metal ions exhibited the following order: Fe^{2+} (0.66 mM, r^2: 0.9884) < Pb^{2+} (0.87 mM, r^2: 0.9571) < Hg^{2+} (2.53 mM, r^2: 0.9883) < Co^{2+} (3.22 mM, r^2: 0.9408) < As^{3+} (7.01 mM, r^2: 0.9205) < Cu^{2+} (7.12 mM, r^2: 0.9770). On the other hand, these metal ions demonstrated Ki values in the range of 0.58 ± 0.11–10.12 ± 1.01 mM. K_i values of these metal ions exhibited the following order: Fe^{2+} (0.58 ± 0.11 mM) < Pb^{2+} (1.85 ± 0.22 mM) < Co^{2+} (2.74 ± 0.18 mM) < Hg^{2+} (3.70 ± 0.31 mM) < As^{3+} (6.53 ± 0.64 mM) < Cu^{2+} (10.12 ± 1.01 mM) (Table 3 and Fig. 5). The metal ions used in this study showed inhibitory effects on CA enzyme activity. A group of scientists investigated the concentration of heavy metal ions in commercially significant species of shellfish, fish, and fish products from fish markets in around the Cochin area. The study explained that diverse metals (Pb, Cd, Cr, Hg, Zn, As, Co, Cu, Ni, Mn, and Se) were available in the samples at diverse levels (Sivaperumal et al. 2007).

Table 2 IC_{50} and K_i values and inhibition types of some pesticides on CA activity obtained from horse mackerel gill tissues

Pesticides	IC_{50} (nM)	r^2	K_i (nM)	Inhibition type
Azoxystrobin	30.97	0.9598	13.98 ± 3.23	Competitive
Clofentezine	3.55	0.9489	5.27 ± 2.16	Competitive
Deltamethrin	12.37	0.9602	11.96 ± 3.11	Uncompetitive
Propineb	8.45	0.9130	11.17 ± 5.03	Uncompetitive
Thiophanate	36.76	0.9491	48.38 ± 14.01	Uncompetitive
Thiram	3.25	0.9801	4.38 ± 1.98	Uncompetitive

Discussion

The fish gill tissue is an intricate organ, known to be involved in ion transport, acid-base regulation, and respiratory gas exchange. CA, present in gill cells, is assumed to play an important role in these mechanisms. Today, pesticide compounds are playing a significant role in new agriculture. Indeed, they decrease the total product losses of pesticide compounds, and it is durable, relatively trusty, and complete control. On the other hand, pests with minimal costs and effort have enhanced crop yields by killing. Thus, pesticide is one of the powerful pollutant of the water environment (Murty 1986). Pesticide compounds are defined as strong pollutants of the water environment with officious effects on non-aim organisms like water and fish animals (Khan et al. 2012). Fishes are test organisms and ideal sentinels for behavioral methods of toxic chemicals like pesticide and heavy metals and diverse stress factors exposure due to their ecological correlation in plenty of natural systems (Vakonaki et al. 2013). Pesticide compounds are materials, which used to control or kill the organisms investigated to be detrimental. The maximum extensively used

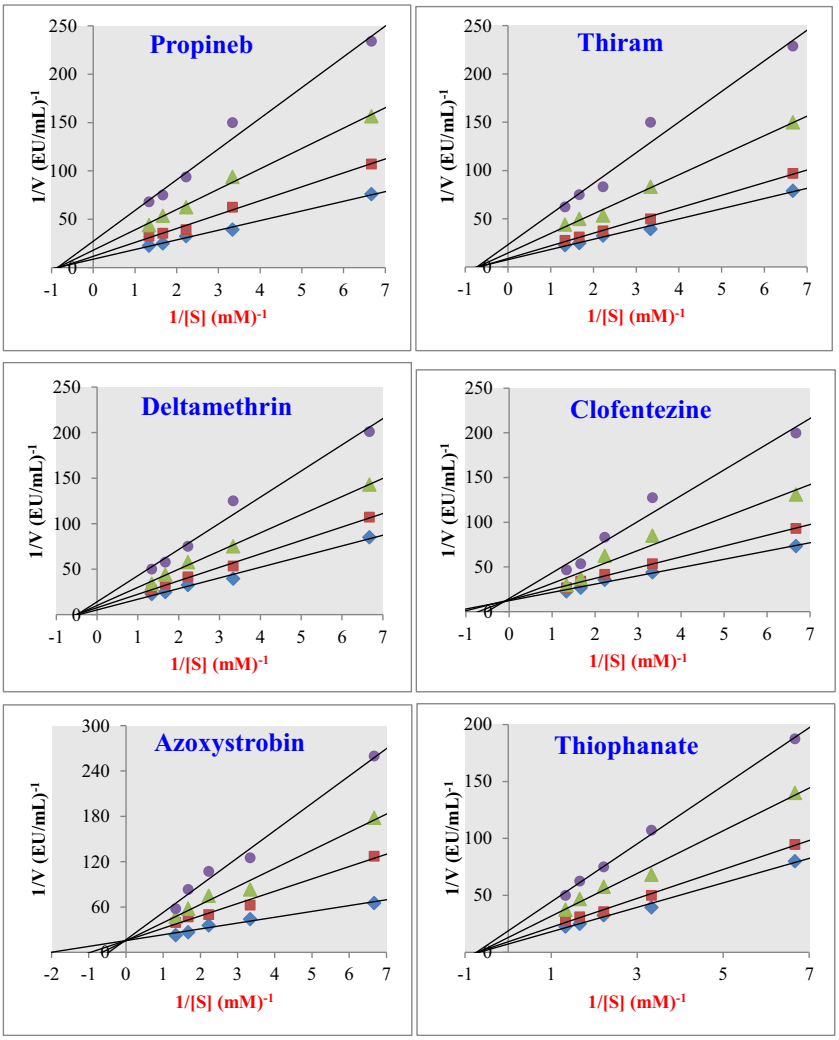

Fig. 4 K_i graphs of pesticides for horse mackerel gill tissues

ones are herbicides, fungicides, insecticides, and rodenticides. It is recorded that plenty of molecules, pesticide molecules, and metal ions can be perilous for the living organism cells. Some studies on pesticide compounds clarify that these molecules define their toxic effects by inhibiting the important enzymes even at low concentrations like nanomolar or micromolar (Ceyhun et al. 2010). Additionally, pesticide molecules can participate in freshwater like lake and river by irrigation water or rainwater. This condition can be perilous for living some systems, like specific metabolic enzymes.

Table 3 IC_{50} and K_i values and inhibition types of some heavy metal CA acitivity obtained from horse mackerel gill tissues

Metal ions	IC_{50} (mM)	r^2	K_i (mM)	Inhibition type
Fe^{2+}	0.66	0.9884	0.58 ± 0.11	Competitive
Cu^{2+}	7.12	0.9770	10.12 ± 1.01	Uncompetitive
Co^{2+}	3.22	0.9408	2.74 ± 0.18	Competitive
Pb^{2+}	0.87	0.9571	1.85 ± 0.22	Uncompetitive
Hg^{2+}	2.53	0.9883	3.70 ± 0.31	Uncompetitive
As^{2+}	7.01	0.9205	6.53 ± 0.64	Uncompetitive

It was known that enzymes catalyze more chemical reactions in the metabolism of the living systems (Erdemir et al. 2019; Huseynova et al. 2018; Zengin et al. 2018). Indeed, these chemical compounds like metal ions, drugs, and pesticides influence metabolism at low concentrations by reducing or enhancing enzyme activities. Thus, the inhibitory acts of several pesticide compounds on the enzymatic activity of rainbow trout CA were recorded. The outcomes demonstrate that between the four pesticide compounds which utilized in this paper, the most effective pesticides are thiram, clofentezine, propineb, and deltamethrin which are widely utilized at agricultural and in homes fields. Deltamethrin pesticide inhibits the CA enzyme at low doses, exclusively in vivo situations, indicating that fish in cultural environments and natural are sensitive to this pesticide and that contaminations would result in plural fish deaths. This would show enhance in food inadequacy for enhancing people and reason cutting of

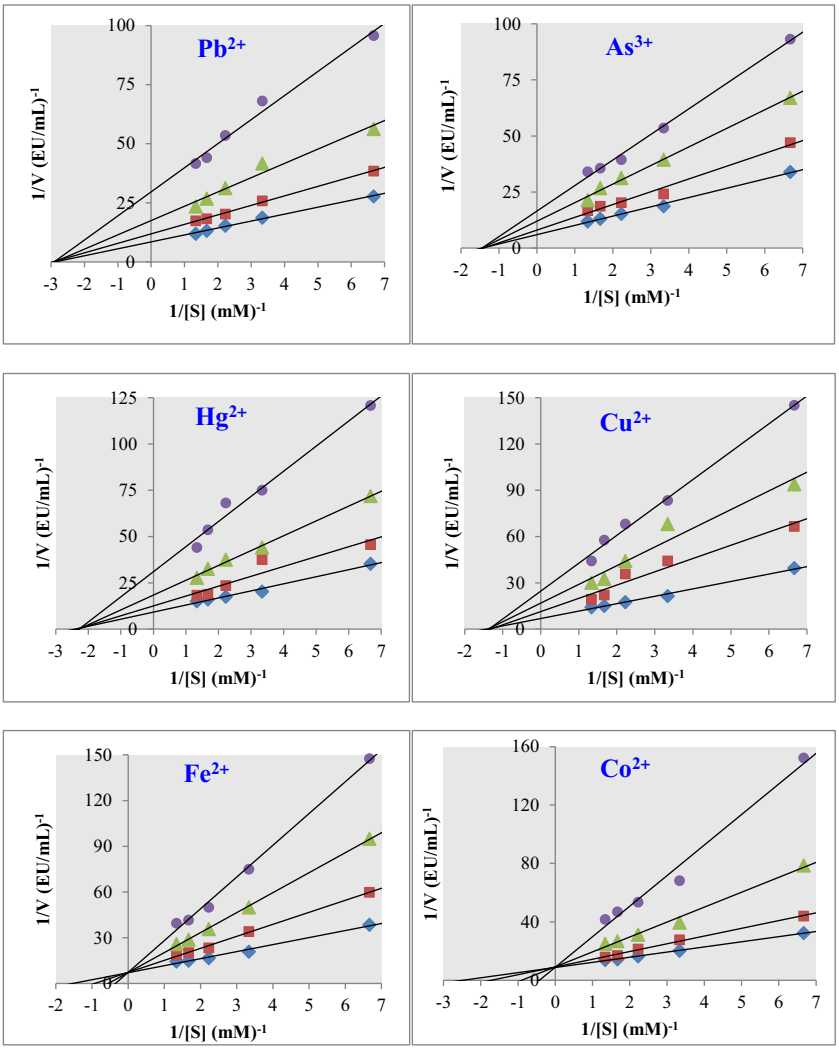

Fig. 5 K_i graphs of metal ions for horse mackerel gill tissues

ecological balance. Thus, the utilization of deltamethrin compound must be well controlled.

The heavy metal is in the third or higher period of the periodic table is a nonscientific term used for found metals. In general, all metals that are toxic and cause environmental pollution are called heavy metals. The definition of heavy metal in terms of physical property is the metals with a density higher than 5 g/cm^3 in living organism. This group includes more than 60 metals including lead, cadmium, chromium, iron, cobalt, copper, nickel, mercury, and zinc. Aquaculture is an easily digestible food with superior biological value (Kaya et al. 2013). It is a healthy food with protein, vitamins, mineral substances and low fat content. In terms of nutritional physiology fish, meat and milk are important source of animal protein. There are also environmental factors that adversely affect fish, and the first of these are biotoxins, parasites, infectious microorganisms, physical-chemical factors, pesticides, hydrocarbons, and heavy metals (Söyüt and Beydemir 2012). Marine organisms, such as fish, accumulate heavy metals in layers and layers at higher concentrations than water or sediment. The uptake of heavy metals in the aquatic environment by fish, which forms an important link in the food chain, is through structures such as the digestive system, body surface, and gills. Most uptakes are through gills. This is because heavy metal-containing breathing water interacts with gill lamellae, which have a large surface area. Surplus essential elements and non-essential heavy metals cause structural and functional disturbances at the cellular and molecular level in fish (Söyüt et al. 2012).

For example, a group of scientists purified the CA enzyme from the liver of the teleost fish *Dicentrarchus labrax* (Ceyhun et al. 2011). The purification consisted of a single step affinity chromatography on Sepharose. The CA was purified 78.8-fold with a yield of 46% and a specific activity of 751.72 U/mg proteins. It has an optimum pH at 7.5, an optimum temperature at 25 °C , an optimum ionic strength at 10 mM, and a stable pH at 8.5. In another study, Ceyhun et al. (2010) investigated the effects of the pesticides,

diazinon, deltamethrin, cypermethrin, and propoxur, on the activity of rainbow trout (*Oncorhynchus mykiss*) gill CA. The CA was purified from rainbow trout gills using Sepharose affinity chromatography. The overall purification was approx. 214-fold. SDS–polyacrylamide gel electrophoresis showed a single band corresponding to a molecular weight of approx. 29 kDa, which our study is in harmony with these studies.

Conclusion

In conclusion, the horse mackerel gill CA was purified for the first time in one step with a high specific activity. Its kinetic properties were investigated. In addition, the inhibitory effects of some pesticides and heavy metal ions on the enzyme activity were determined.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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