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ORIGINAL PAPER

Inhibitory effects of Pb^{2+}, Fe^{2+}, Cd^{2+} and Co^{2+} on carbonic anhydrase enzyme from muscle of the Kangal fish (*Garra rufa*)*

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Abstract

The Kangal fish (*Garra rufa*), known as the "doctor fish", lives in the Kangal Spring in Sivas, Turkiye. In this study, carbonic anhydrase (CA) from the muscle tissues of the Kangal fish (*Garra rufa*) was purified and characterized for the first time. To this end, CA was purified using a Sepharose-4B-L-Tyrosine-sulfanilamide affinity column (STAC) with specific activity of 34.36 EU mg^{-1}, yield of 17.98% and 201.0 purification fold. To control the CA enzyme purity, SDS-PAGE was performed and a single band was found. The Michaelis constant (K_m) and maximum velocity (V_{max}) were determined for CA. Also, p-nitrophenylacetate (PNA) was used as CA substrate. Furthermore, inhibition constants (K_i) and half maximal enzyme inhibitory concentration (IC_{50}) for each metal ion were determined using by Lineweaver-Burk graphs. Additionally, optimum ionic strength was found to be 1.0 M (Tris-SO_4), optimum pH was calculated as 9.0 (Tris-SO_4) and stable 8.5 pH was found (phosphate buffer) for the CA from the muscle tissues of the fish. Furthermore, activation enthalpy (ΔH), activation energy (Ea), optimum temperature and Q_{10} values were obtained from the Arrhenius plot of CA from *Garra rufa* muscle tissue as 6.70 kcal mol^{-1}, 7.32 Kcal mol^{-1}, 35.0°C, 1.37, respectively. K_{cat} and V_0 values of CA from *Garra rufa* muscle CA were calculated as 19.21 s^{-1} and $1.8x10^4$ mM s^{-1}, respectively. Finally, Ki values of some heavy metal ions (Co^{2+}, Pb^{2+}, Cd^{2+}, and Fe^{2+}) in the Kangal fish muscle CA were calculated in the range of 0.25-26.09 mM using the esterase activity assay.

Keywords: *Garra rufa*, enzyme purification, Kangal fish, enzyme inhibition, carbonic anhydrase

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INTRODUCTION

There are different areas of business which supply constant income and which show the potential for progress in the country's economy. An example is tourism. It is true, however, that if the focus is solely on mass tourism, the benefits will be below the expectations, even though some contribution to the country's economy will be made. The course of development in global tourism is changing, from holiday understood as the sea, sand and sun to catering to other preferences. In view of this situation, countries are beginning to activate alternative tourism sources. Turkiye has quite a rich offer of alternative tourisms. Health tourism is certainly worth considering because the value added it creates is higher than that of mass tourism. Health tourism divides into two important sub-categories: medical and thermal tourisms. In Turkiye, the major area of health tourism is thermal tourism, also called SPA tourism. Psoriasis is an example of illness which can be treated in popular hot springs (Sayili et al. 2007, Ozcelik et al. 2000).

Kangal Fish Spring is located 13 km away from Kangal in Sivas. It is one of the most important SPA resorts in that region. The hot spring, supplying pools and a stream, is inhabited by the so-called doctor fish, *Garra rufa*, which are used for ichthyotherapy, especially in treatment of psoriasis. While the fish eat the dead skin on people's feet, they remove psoriatic scales are turn to plaque, which are softened by the warm water. This causes slight bleeding and superficial ulcerations. It has been reported that a high level of selenium in water, which is present in Kagal Fish Spring, is an important factor in healing these scars (Ozer et al. 1987, Akpınar, Aksoylar, 1988).

Carbonic anhydrase (CA, E.C.4.2.1.1) is a Zn^{2+}-containing metalloenzyme, which catalyzes the fast and reversible dehydration of carbon dioxide (CO_2) and water to bicarbonate (HCO_3^-) and proton (Coban et al. 2009, Gul et al. 2016, Sujayev et al. 2017).

$$CO_2 + H_2O \overset{CA}{\Leftrightarrow} H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

CAs are involved in a variety of bioprocesses, including biosynthesis, respiration, gas balance, physiological pH regulation, acid-base regulation, calcification, and bone resorption (Hisar et al. 2005*a*, Kocyigit et al. 2017, Topal et al. 2017). CAs were first isolated from mammalian erythrocytes (Kocak et al. 2016, Turan et al. 2016), and since then they have also been purified from fish, rat and human erythrocytes, rat saliva, cattle leukocytes, bovine bones, plant sources, and microbes that have been studied extensively (Artunc et al. 2016, Mutlu et al. 2023). In mammalian cells, the molecular mass of CA was found to be around 30 KDa (Hisar et al. 2005*b*, Gilmour 2010, Cincinelli et al. 2015, Ozgeris et al. 2016). CA enzymes have been examined and divided into eight different and distinct classes α-, β-, γ-, δ-, ζ-, η-, θ-, and *t*-CAs (Güney et al. 2023, Karimov et al. 2023). One of these

families, α-CA is found in bacteria, green plants' cytoplasm, vertebrates and algae. Additionally, CAs are found in different tissues of fishes (Genc Bilgicli et al. 2019, Küçükoğlu et al. 2019, Karagecili et al. 2023*a*). All of these isoenzymes have zinc ions in their active sites (Boztas et al. 2015, Ozmen Ozgun et al. 2016, Oztaskin et al. 2019). Each CA family has a catalytic function that is comparable. Zn^{2+} ions have an important role in the processes catalyzed by CA enzymes (Bayrak et al. 2019, Burmaoğlu et al. 2019, Ozmen Ozgun et al. 2019).

Heavy metals an aquatic environment usually occur in trace amounts, but with a rapid population growth in recent years, industrialization, agricultural activity, industrial activity, for example by the seaside, wastewater from human settlements, etc. pollution with heavy metals is increasing (Ozbey et al. 2016, Taslimi et al. 2017, Gulcin, Alwasel 2023). As a result, aquatic organisms, including fish, are exposed to increasing concentrations of pollutants (Küçük and Gulcin, 2016, Sarı et al. 2018). Heavy metals in ambient concentrations tend to accumulate in fish tissue and organs. Also, it has been reported that heavy metals affect different blood parameters, enzyme activation, growth and development. The level of a metal that accumulates in fish tissues and organs depends on its ambient concentration and duration of its effect (Küçük, Gulcin 2016, Huseyinova et al. 2018, Biçer et al. 2019).

We carried out this study to purify for the first time the CA enzyme from the muscle tissue of Kangal fish (*Garra rufa*). The choice of this fish, which is especially used in the treatment of psoriasis, is because is different from other fish species in this respect. Characterizing the CA purified from the Kangal fish's muscle for the first time and determining its kinetic properties is another aspect of the study. The effect of some heavy metal ions (Pb^{2+}, Fe^{2+}, Cd^{2+} and Co^{2+}) on this enzyme was also determined *in vitro*.

MATERIALS AND METHODS

Chemicals

$Pb(NO_3)_2$, $FeCl_2H_2O$, $CoCl_26H_2O$, and $CdCl_2H_2O$, as well as *p*-nitrophenyl acetate, protein assay reagents, CNBr-activated-Sepharose-4B and the other analytical grade compounds were provided Sigma-Aldrich (GmbH, Germany).

Preparation of homogenate from Kangal fish (*Garra rufa*)

Cold chain protocols were followed when Kangal fish (*Garra rufa*) samples were delivered to the lab from the Kangal fish resort in Sivas. Muscular tissues from the fish were removed and kept at -80°C (Akıncıoglu et al. 2014, Kirici et al. 2016, Küçük, Gulcin 2016).

Purification of CA by Affinity Chromatography

The muscle tissues of Kangal fish (*Garra rufa*) were washed with a 0.9 percent NaCI isotonic saline solution. Liquid nitrogen was used to lyse the tissues for approximately 85 minutes (Çetinkaya et al. 2014, Topal, Gülcin, 2014). Then, muscle tissue samples were centrifuged at 10.000*xg* as an intermediate speed. They were separated into the supernatant and precipitate. The supernatant was added to a buffer solution comprising Tris-HCl/Na_2SO_4 (25 mM 0.1 M^{-1}) for kinetic studies at pH 8.7 (Şentürk et al. 2009, Bulut et al. 2023). The pH-corrected homogenate was put onto a STAC column and washed with Na_2SO_4 (22 mM) in a Tris-HCl solution (pH 8.7, 25 mM), again. Then, the CA enzyme connected to the chromatographic column was eluted at a column flow rate 20 mL h^{-1} with $NaClO_4$/$NaCH_3COO$ (pH 5.6, 0.5 M / 0.1 M). All chromatographic procedures were carried out at 4°C (Gulcin et al. 2004, Atasaver et al. 2013).

CA enzyme activity measurement

The activity of CA from Kangal fish (*Garra rufa*) muscle tissues was determined according to Verpoorte's method (1967) as described in prior studies (Göksu et al. 2014, Zengin et al. 2018, Zahedi et al. 2023). For this assay, the transformation of PNA to *p*-nitrophenolate was recorded on a spectrophotometer for at 348 nm 3 min and 25°C (Coban et al. 2008, Koçyiğit et al. 2017, Yiğit et al. 2023). In a total volume of 1 mL, the enzymatic reaction included 0.4 mL of Tris-SO_4 buffer solution (0.05 M, pH 7.4), 0.36 mL PNA (3 mM), 0.22 mL H_2O, and 0.2 mL CA solution (Coban et al. 2007, Turkan et al. 2019).

Determination of protein

Protein concentrations were determined by measuring the absorbance of fractions at 280 nm (Oztürk Sarıkaya et al. 2010, Arabaci et al. 2014, Oztaskin et al. 2023). Bovine serum albumin (BSA) was used as standard protein (Köksal, Gülçin 2008, Akbaba et al. 2014, Bora et al. 2022). The protein concentration was spectrophotometrically measured at 595 nm during the purification phases, using BSA as the standard in the Bradford method (1976) as described in detail (Scozzafava et al. 2015, Yıldırım et al. 2015).

CA purity control by SDS-PAGE

After purification of the CA enzyme, for enzyme purity SDS-PAGE was performed according to Laemmle's protocol (1970) as given in precious studies (Hisar et al. 2005c, Ekinci et al. 2011, Gul et al. 2017). The stacking and running gels contained 3% and 10% acrylamide, respectively, and 0.1% SDS. The electrode buffer included Tris-glycine (0.25 M/ 2 M, pH 8.3). The buffer solution was added by mixing 4 mL SDS (%10), 1 mL Tris-HCl (1 M pH 6.8), 1.5 mL bromophenol blue (BP, 0.1%), 1.5 mL glycerol, 1 mL

β-mercaptopethanol, and 5 mL water. A 20 µg sample was placed in 50 µL buffer solution. The mixture solution was heated in a water bath at 100°C for 5 minutes. Each area of the stacking gel was filled with pure enzyme samples (Halıç Poslu et al. 2024, El Ati et al. 2024). To begin, an electric potential of 80 V was applied to the flowing gel until bromophenol blue was attained. For 5 min, the mixture was warmed in a water bath at 100°C. Each region of the stacking gel was loaded with pure CA samples. To begin, an electric potential of 80 V was used to transfer BP to the flowing gel (Gulcin et al. 2005, Köksal, Gülçin, 2008, Gumus et al. 2022).

Kinetic studies

Determination of optimum pH and ionic strength and stable pH

To determine optimum pH for CA enzyme, the esterase assay was tested in 1.0 M Tris-SO_4 buffers in a range of pH 7.0-9.0 and 1.0 M phosphate buffers between pH 5.0-8.0 (Küçük, Gülçin 2016, Kuzu et al. 2021). The CA enzyme activity from Kangal fish (*Garra rufa*) was also tested in Tris-SO_4 (1.0 M) buffers between pH 7.0-9.0 and 1.0 M phosphate buffer between pH 5.0-8.0 to determine stable pH. Under optimum conditions, enzyme activity measurements were taken every 12 h throughout the course of a 4-day incubation period using the PNA substrate. Using varied quantities of Tris-SO_4 buffer (pH 9.0), between 0.1-1.0 M, the optimum ionic strength of CA enzyme Kangal fish (*Garra rufa*) activity was determined (Küçük, Gülçin 2016).

Optimum temperature, activation energy (E_a), activation enthalpy (H) and Q_{10}

The optimal temperature, activation energy (E_a), activation enthalpy (H), and Q_{10} values of Kangal fish (*Garra rufa*) muscle CA activities were determined at different temp. ranging from 0°C to 60°C with a 10°C increment. To begin, an ice water bath was used to create a temp. range of 0 to 20°C. Then, using constant temperature, a range of temperatures over 20°C was achieved. All measurements of CA activities were integrated to an Arrhenius plot of the log k^{-1}/T to record the activation enthalpy, activation energy, ideal temperature, and Q_{10} values (Küçük, Gülçin 2016).

Kinetic studies

Several concentrations (0.15, 0.30, 0.45, 0.60 and 0.75 mM) of PNA with the optimal pH at 25°C temp. was used to calculate K_m and V_{max} values for kinetic experiments. The CA activities were spectrophotometrically measured at 348 nm. Then, K_m and V_{max} were calculated from Lineweaver-Burk graphs (Genc et al. 2016, Ceylan et al. 2017). The k_{cat} value represents the enzyme's turnover rate. k_{cat} was estimated using the equation V_{max} / ET, where ET represents total CA and V_0 represents the specificity constant of CA activity (Lineweaver, Burk, 1934).

In vitro inhibition studies

Iron, cobalt, lead, and cadmium were tested for their inhibitory effects on the enzyme function. Heavy metals were studied in varying substrate concentrations to assess their effects (Gocer et al. 2017, Gul et al. 2017). The CA activities were measured for different concentrations of Fe^{2+} ions (5, 8, 10, 12 and 15 mM), Pb^{2+} ions (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM), Co^{2+} ions (8, 10, 12, 13 and 15 mM), Cd^{2+} (20, 25, 30, 35, 40, 45, 50 and 55 mM) in 1.0 mL cuvettes. In the chart, the control of CA activity without the use of an inhibitor was set to 100%. For each inhibitor, an CA activity was plotted against the concentration of the metal ions. The graphs revealed that CA activity was reduced by 50% when the metal concentration (IC_{50}) was employed (Kocyigit et al. 2017, Akıncıoğlu et al. 2021). K_i values were recorded with determining three different concentrations for each metal ion: Pb^{2+}: 0.2, 0.4 and 0.6 Mm; Fe^{2+}: 5, 8 and 10 Mm; Co^{2+}: 10, 12 and 15 mM; and Cd^{2+}: 30, 45 and 55 mM. Additionally, for each metal ion, PNA was used at five different concentrations (0.15, 0.30, 0.45, 0.60 and 0.75 mM). For determining the inhibition type and K_i, Lineweaver-Burk graphs were used (1934).

RESULTS AND DISCUSSION

SPA treatment has long been used for chronic skin diseases. It has been stated that the Kangal Balıklı Thermal Spring, which is one of these thermal springs, can be used for supportive treatment purposes. SPA treatment has long been used in various health issues, such as atopic dermatitis and psoriasis, eczema (lichen planus), a skin disease known as loss of pigment in the skin (vitiligo), in which light-colored patches on the skin occur, ichthyosis, parapsoriasis, alopecia areata, pityriasis rubra pilaris, acne, prurigo and rosacea. Thus, it is used in the treatment of many dermatological diseases. If approved by dermatologists, SPA treatments can be considered as a supportive or alternative treatment to traditional treatments (Kaya Erdogan et al. 2019). It is thought that Kangal fish in the Kangal Fish Spring facilities open up the diseased skin and help the absorption of the SPA water, hence the healing occurs via the content of the water. However, knowing the physiology of these fish will help to protect the species (Uysal et al. 2019).

The fish functionally combines the hydration of carbon dioxide (CO_2) flowing through the gills to provide bicarbonate (HCO_3^-) and protons (H^+) ions for branchial NaCl uptake and ionic regulation associated with effective regulation of the acid-base level (Aytac et al. 2023, Gok et al. 2023, Zengin et al. 2024). This process in fish is mainly based on the modulation of Na^+ / H^+ and Cl^- / HCO_3^- modifiers at the gills so as to adjust the plasma HCO_3^- concentration (Atalar et al. 2023, Guven et al. 2023, Güven et al. 2024). It is obvious that CA contributes to acid-base regulation; surprisingly, however, there are few studies about the role of branchial CA in this regula-

tion process (Celik Onar et al. 2023, Durmaz et al. 2023*a*). Apart from the cited studies, it has been observed that acetazolamide application significantly reduces branchial acid excretion in rainbow trout (*Oncorhynchus mykiss*). In addition, it has been determined that this effect is stronger in trout (*Oncorhynchus mykiss*) that are exposed to environmental hypercapnia and causes respiratory acidosis. Additionally, significant changes in mRNA and protein levels have been noted in branchial CA in response to acid-base changes (Durmaz et al. 2023b, Ozden et al. 2023).

In the current work, using a STAC affinity column, CA was isolated from muscle tissue of Kangal fish (*Garra rufa*) and then characterized. The enzyme was purified approximately 203.3-fold with a yield of 17.98% and specific activity of 34.76 EU mg^{-1} (Table 1). The SDS-PAGE method was used to record the subunit and purity molecule mass of the CA, and a single band was obtained (Figure 1). CA has molecular mass of approximately 33.39 KDa (Figure 2).

Table 1

Summary of purification steps of carbonic anhydrase enzyme from Kangal fish (*Garra rufa*) muscle tissues

Purification steps	Activity (EU mL^{-1})	Total volume (mL)	Protein (mg mL^{-1})	Total protein (mg)	Total activity (EU mL^{-1})	Specific activity (EU mg^{-1})	Yield (%)	Purification fold
Homogenate	0.189	40.00	34.55	44.32	7.56	0.171	100	1.00
Sepharose-4B-L--Tyrosine--sulfanilamide affinity chromatography	0.536	2.50	0.016	0.039	1.34	34.36	17.98	203.3

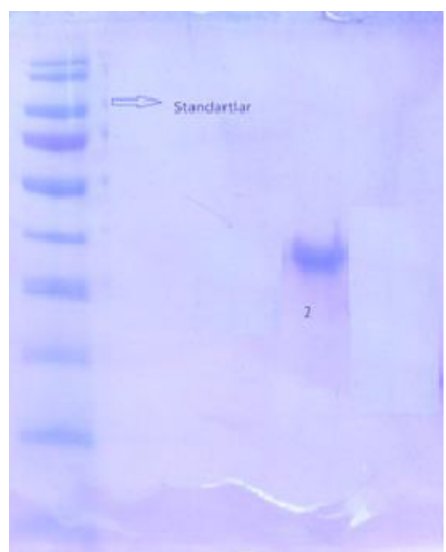

Fig. 1. Photo of SDS-PAGE of carbonic anhydrase enzymes purified by STAC affinity chromatography *2: muscle tissue CA (1: 250 KDa 2: 150 KDa 3: 100 KDa, 4: 70 KDa 5: 50 KDa 6: 30 KDa 7: 15 KDa)

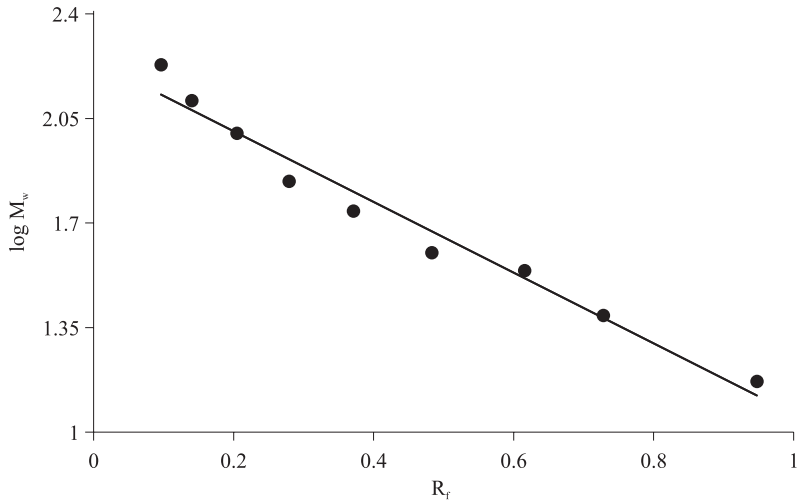

Fig. 2. Standard R_f–log M_w graph of the molecular weight of CA from Kangal fish (*Garra rufa*) muscle using the SDS-PAGE results

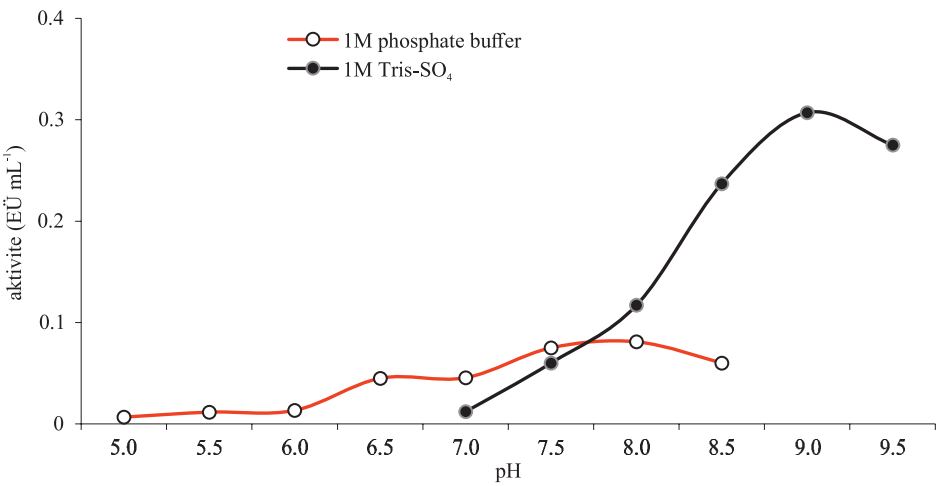

Fig. 3. Determination of optimum pH for from Kangal fish (*Garra rufa*) muscle in 1.0 M phosphate and 1.0 M Tris-SO_4 buffers

In the CA characterization studies, the optimum pH (Figure 3) and stable pH (Figure 4) were recorded as 8.5 and 9.0, respectively, for PNA substrate of CA enzyme in 1.0 M Tris-SO_4. Additionally, ΔH, optimum temperature, Q_{10} and E_a values were calculated as 6.70 kcal mol^{-1}, 35°C, 1.37 kcal mol^{-1} and 6.70 kcal mol^{-1} (Figure 4), respectively. In this study, kinetic parameters, including K_m, V_{max} and k_{cat} were measured as 1.10 mM, 0.44 EU mL^{-1} and 19.21 s^{-1} respectively. Additionally, V_0 is selfsame of CA activity, was first time determined as 1.8×10^4 mM×s^{-1} in this work (Table 2).

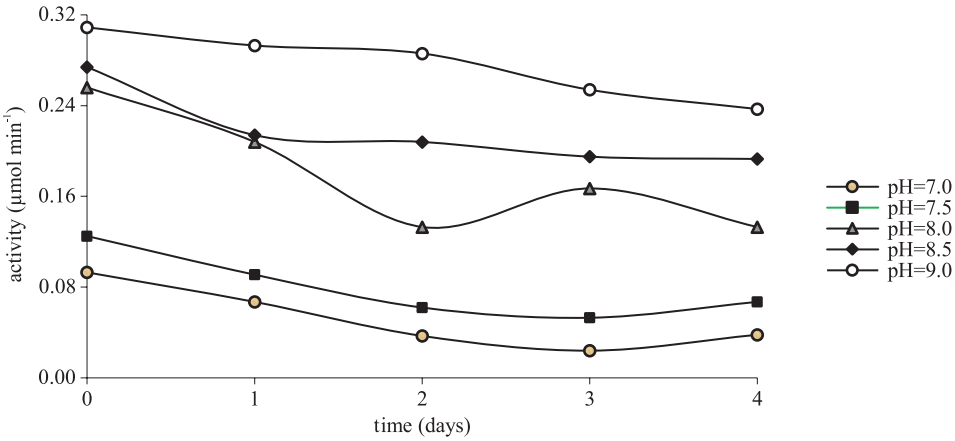

Fig. 4. Determination of stable pH of Kangal fish (*Garra rufa*) muscle for five days

Table 2

Kinetic parameters of CA from Kangal fish (*Garra rufa*) muscle by PNA

Kinetic parameters	Values
Optimum pH (Tris–SO_4, 1.0 M)	9.0
Optimum ionic strength (Tris–SO_4, M)	1.0
Stable pH (Phosphate, 1.0 M)	8.5
Optimum temp. (°C)	35
E_a (kcal mol^{-1})	7.32
ΔH (kcal mol^{-1})	6.70
Q_{10}	1.37
K_M (mM)	1.10
V_{max} (EU mL^{-1})	0.44
k_{cat} (s^{-1})	19.21
V_0 (mM s^{-1})	$1.8x10^4$
Molecular mass (KDa)	33.39

In a previous study conducted by Peterson and co-workers, CA was purified from kidney of black sea trout with specific activity of 603.77 and yield of 35.5%. The molecular mass of CA from fish muscle was found to be approximately 29.7 KDa, similarly to CAs from other sources. The obtained molecular mass was similar of CAs purified from many other living tissues. For instance, molecular mass of flounder gills, zebrafish (*Danio rerio*) erythrocyte (Peterson et al. 1997), Antarctica icefish (*Chinodraco hamatus*) gills (Rizzello et al. 2007), and Rainbow trout (*Oncorhynchus mykiss*) liver (Soyut et al. 2012) molecular mass of sea bream (S*parus aurata*) gills (Rizzello et al. 2007) were determined as 29.0, 29.0, 28.0, 29.4 and 30.5 KDa, respectively. In a kinetic study for K_m 1.10 mM, V_{max} 0.44 EU mL^{-1}, k_{cat} 19.21 s^{-1} and

V_0 1.8x10^4 Mm s^{-1}, the values were recorded with using PNA substrate. Also, optimum temp. (35°C), E_a (7.32 kcal mol^{-1}), ΔH (6.70 kcal mol^{-1}) and Q_{10} (1.37) results were determined as in other previous research reported in the literature.

In the part of our study related to characterization of CA, the optimum pH (Figure 3) and stable pH (Figure 4) were recorded as 8.5 and 9.0, respectively, for PNA substrate of CA enzyme in 1.0 M Tris-SO_4. Additionally, ΔH, optimum temperature, Q_{10} and E_a values were calculated as 6.70 kcal mol^{-1}, 35°C, 1.37 kcal mol^{-1} and 6.70 kcal mol^{-1} (Figure 5) respectively. In this

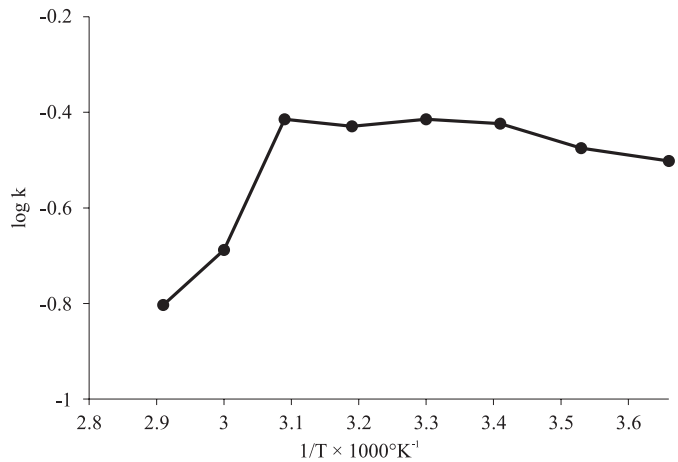

Fig. 5. The effect of temperature on CA enzyme activity Kangal fish (*Garra rufa*) muscle

study, kinetic parameters including K_m, V_{max} and k_{cat} were measured as 1.10 mM, 0.44 EU mL^{-1} and 19.21 s^{-1}, respectively. Additionally, V_0 is self-same of CA activity, and it was determined as 1.8×10^4 mM×s^{-1} in our work (Table 2).

One of inhibition parameters is IC_{50} amount. It was determined for Pb^{2+} Fe^{2+}, Cd^{2+} and Co^{2+} from enzyme activity-metal ion concentrations. Another important inhibition parameter is K_i, which was calculated from Lineweaver--Burk plots (Figures 6 and 7), and the inhibition types were determined based on the same graphs for each metal (Table 3), as described previously (Kiziltas et al. 2022*a*, *b*).

Table 3

Inhibition kinetics (inhibition types, IC_{50} and K_i values) of some heavy metals related to CA obtained from Kangal fish (*Garra rufa*) muscle

Metal ions	IC_{50} (mM)	K_i (mM)	Inhibition type
Co^{2+}	7.97	14.10±7.19	Uncompetitive
Cd^{2+}	46.21	26.09±9.04	Competitive
Pb^{2+}	0.33	0.25±0.05	Competitive
Fe^{2+}	6.42	17.58±8.74	Uncompetitive

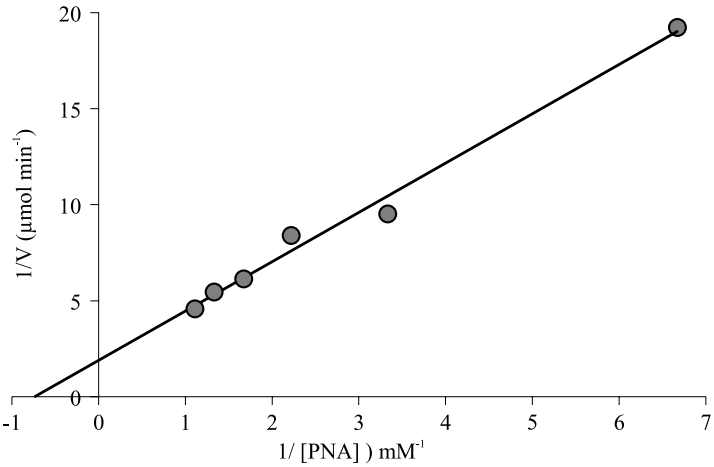

Fig. 6. Determination of Lineweaver-Burk graph for CA enzyme purified from Kangal fish (*Garra rufa*) muscle at five different concentrations of PNA

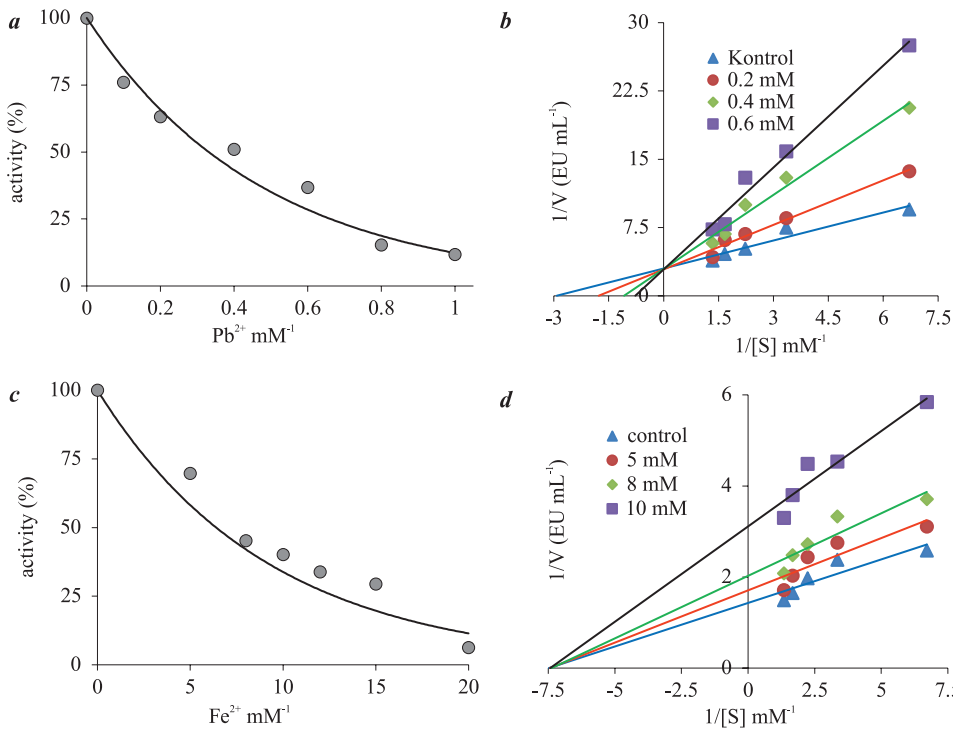

Fig. 7. The effects of Pb^{2+} and Fe^{2+} ions on CA from Kangal fish (*Garra rufa*) muscle CA activity: ***a*** and ***b*** are IC_{50} and Lineveawer-Burk graphs of Pb^{2+} ions, ***c*** and ***d*** are IC_{50} and Lineveawer-Burk graphs of Fe^{2+} ions

Metals are common in nature and have a high resistance to oxidation. Metals have geological and biological movements in nature. Rainwater dislodges them from mineral and rock deposits and transports them to the soil, seas, rivers, and sediments (Taysi et al. 2021). Plants and animals accumulate metals in the a food chain, which is part of the biological cycles. With the degradation of dead organisms, they re-enter ecosystems (Caglayan et al. 2019, Taysi et al. 2021). Heavy metals emitted by factories and technical infrastructure have a variety of effects on animals, people, and other living beings. Furthermore, heavy metals can alter the catalytic activity of membrane transport systems (Caglayan et al. 2019, Donkın et al. 2020). The average content of some metals in the water where the Kangal fish live was found as follows: Fe 0.13 mg L^{-1}, Cu 0.024 mg L^{-1}, Pb 0.07 mg mL^{-1}, Al 0.018 mg L^{-1} (Degirmenci et al. 2021). With the effect of the thermal power plant situated in the region, the ecological balance is disturbed. Heavy metal ratios in soil, air and water are changing day by day. For this reason, the effect of some heavy metals (Pb^{2+}, Fe^{2+}, Cd^{2+} and Co^{2+} ions) on the purified CA was investigated *in vitro* in order to contribute to the elucidation of the physiology of the fish, which is important for the region. All results were compared to the IC_{50} and K_i values of CA enzyme for heavy metal concentrations in Table 3. The heavy metals had effective inhibitory impact on CA, with K_i values between 0.25±0.05-26.09±9.04 mM. All of the heavy metals had similar inhibitory properties, although the most active one is Pb^{2+}, which achieved K_i of 0.25±0.05 mM. Also, IC_{50} values of heavy metals were increased in the following order: Pb^{2+} (0.33 mM) < Co^{2+} (14.10 mM) < Fe^{2+} (17.85 mM) < Cd^{2+} (26.09 mM). Enzyme inhibition is essential for the metabolism of all living creatures. The majority of medications and other substances, including heavy metals, work by interfering with enzymes. As a result, much research has been done on the effects of heavy metals and organic chemicals on CA activity (Ozcelik, Hayta et al. 2015). It was observed that heavy metals inhibited CA purified from Kangal fish muscle tissue. It is thought that the increased heavy metal concentration in the water in the Kangal spa may cause damage to the species.

CONCLUSION

In this study, the CA enzyme was isolated from Kangal fish muscle tissue for the first time. The mass of the molecules was measured using the SDS-PAGE method. The p-nitrophenyl acetate substrate was used to determine kinetic parameters. The optimum temperature, E_a, ΔH and Q_{10} were calculated from Arrhenius plot. On the other hand, the other kinetic values (V_{max}, K_m, k_{cat} and V0) were measured separately. Finally, the inhibition parameters (IC_{50}, K_i and inhibition type) were obtained from Lineweaver--Burk graphs.

Author contributions

U.M.K. – investigation, methodology, resources, I.G. – investigation, methodology, resources, software, conceptualization, data curation, formal analysis, funding acquisition, writing – original draft preparation, writing – review & editing. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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