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Characterization and anions inhibition studies of an α-carbonic anhydrase from the teleost fish *Dicentrarchus labrax*

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ABSTRACT

Carbonic anhydrase (CA; EC 4.2.1.1) was purified from the gill of the teleost fish *Dicentrarchus labrax* (European seabass). The purification procedure consisted of a single step affinity chromatography on Sepharose 4B-tyrosine-sulfanilamide. The enzyme was purified 84.9-fold with a yield of 58%, and a specific activity of 838.9 U/mg proteins. It has an optimum pH at 8.0; an optimum temperature at 10 °C. The kinetic parameters of this enzyme were determined for its esterase activity, with 4-nitrophenyl acetate (NPA) as substrate. The following anions, $H_2NSO_3^-$, I^-, SCN^-, NO_3^-, NO_2^-, N_3^-, Br^-, Cl^-, SO_4^{2-}, and F^- showed inhibitory effects on the enzyme. Sulfamic acid, iodide, and thiocyanate exhibited the strongest inhibitory action, in the micromolar range (K_is of 87–187 μM). NO_3^-, NO_2^- and N_3^- were moderate inhibitors, whereas other anions showed only weak actions. All tested anions inhibited the enzyme in a competitive manner. Our findings indicate that these anions inhibit the fish enzyme in a similar manner to other α-CAs from mammals investigated earlier, but the susceptibility to various anions differs significantly between the fish and mammalian CAs.

1. Introduction

Carbonic anhydrases (CAs; EC 4.2.1.1) are metalloenzymes which catalyze the interconversion between CO_2 and HCO_3^-. They play key roles in diverse physiological processes, such as pH control, gas balance, calcification, ion transport, secretion of electrolytes, tumorigenesis, etc.[1] Sixteen CA isozymes have been described in mammals, that differ in their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors. Some of these isozymes are cytosolic (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 (CAVA and CA VB), and one is secreted in saliva (CA VI).[1]

Up to now, CA has been purified from many different vertebrate tissues including human erythrocytes,[2–4] rainbow trout brain and liver,[5] fish gills.[6] Considering the role of gill CA in osmoregulatory processes, the relation between branchial CA activity and/or distribution and ambient salinity has been also investigated in several species.[7–14]

The teleost fish *Dicentrarchus labrax* (European seabass), is a primarily ocean-living fish that sometimes enters brackish and fresh water. Its habitats include estuaries, lagoons, coastal waters and rivers. It is found in the waters in and around Europe, including the eastern Atlantic Ocean (from Norway to Senegal), the Mediterranean Sea and the Black Sea.[15] It has economically great importance due to its meat quality in addition to being one of the most cultured animals among other seawater fish.

Although there are studies regarding purification of CA from various tissues, no reports have been found on purification and characterization of the enzyme from European seabass (*Dicentrarchus labrax*). In the present study, we purified and characterized an α-CA from *Dicentrarchus labrax* gill for the first time, and investigated its kinetic properties and inhibitory effects of anions including $H_2NSO_3^-$, I^-, SCN^-, NO_3^-, NO_2^-, N_3^-, Br^-, Cl^-, SO_4^{2-}, and F^- on enzyme activity.

2. Results and discussion

2.1. Purification and characterization of carbonic anhydrase from European seabass gill

Carbon dioxide, produced in fish tissues, is hydrated rapidly by CA, being converted into bicarbonate, and transported in the

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Table 1
Summary of purification steps for European seabass gill carbonic anhydrase enzyme

Purification steps	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Hemolysate	132	30	13.15	394.5	3900	9.88	100	1
Sepharose-4B-tyrosine-sulfanilamide affinity column chromatography	453	5	0.54	2.7	2265	838.89	58	84.9

blood.[1,12,13] Approximately 98% of the transported and stored carbon dioxide is in bicarbonate form, in all vertebrates investigated to date. However, few studies in nona-mammalian species are available in the literature and this is the reason why in this study, we investigated the European seabass (*Dicentrarchus labrax*), and isolated a gill CA. Purification procedure was carried out by affinity chromatography on Sepharose 4B-tyrosine-sulfanilamide, as this zinc enzyme has high affinity for sulfonamides, which bind in deprotonated for to the metal ion from the enzyme active site.[1]

The enzyme was purified up to 84.9–fold with a recovery ratio of 58% compared to homogenate (Table 1). After the sample had completely passed through, the column was washed with 10 mM Tris–HCl/0.1 M Na_2SO_4 buffer whose pH was 7.5. During washing, absorbencies of fractions were measured at 280 and 348 nm by means of spectrometer. These values 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 7.0. At the end of the last step, a highly purified enzyme was obtained exhibiting a single band on SDS–PAGE (Fig. 1).

We used a single step chromatographic technique, employing Sepharose 4B-tyrosine-sulfanilamide affinity chromatography which strongly binds α-CAs.[16] The optimum pH for the purification of the enzyme was determined to be 8.0; the optimum temperature of 10 °C; optimum ionic strength 400 mM. The stable pH profile of the enzyme was determined at four different pHs in 50 mM Tris–HCl and five different pHs in 50 mM K-phosphate buffer. The enzyme maintained 97% of the maximum catalytic activity at the end of 14 days in 50 mM Tris–HCl buffer (pH 6.5), proving it to be stable, similar to many other α-CAs.[1,16]

To determine the native molecular weight of the enzyme, gel filtration was carried out. For this purpose, K_{av} values for the enzyme and standard proteins were calculated, and a K_{av}-LogMW graph was obtained. The molecular weight was determined to be 29 kDa. Similar results have been observed for the enzyme from different sources. For example, human erythrocyte CA is 29 kDa,[2] bovine erythrocyte CA is 29 kDa[16], and rainbow trout liver CA is 31 kDa.[16] The molecular weight was proved to be 28.7 kDa by SDS–PAGE (Fig. 1).

α-CAs have some catalytic versatility, acting also as esterases, phosphatases, and even paraoxonases.[17,18] Thus, we have investigated the esterase activity of the fish enzyme with 4-nitrophenyl acetate (NPA) as substrate (Fig. 2). The K_M and V_{max} values were calculated for NPA hydrolysis catalyzed by the fish enzyme by means Lineweaver–Burk graphs (Fig. 2). The Michaelis–Menten constant K_M constant was calculated to be of 1.595 mM, and V_{max} was 0.509 μmol × min^{-1} for NPA. This enzyme has thus comparable esterase activity with the human isoforms hCA I and II, investigated earlier.[18]

2.2. CA inhibition

It is well- known that metal complexing anions bind to CAs, representing a rather well investigated class of inhibitors.[19] Most anions coordinate directly to the metal ion from the enzyme active site, replacing the coordinated water molecule/hydroxide ion, or add to the coordination sphere, leading to trigonal bipyramidal geometries of the Zn (II) ion.[1,19–22]

In our study, we examined the inhibitory actions of anions on the CA from *Dicentrarchus labrax* gill, by assaying the inhibition of the esterase activity mentioned above. Inhibition data against are shown in Table 2.

As seen from data of Table 2 and Figure 3, all investigated anions show inhibitory activity, but sulfamic acid was the strongest one, with K_i value of 0.0874 mM, which is very similar to that of the human (h) enzyme hCA V, but quite different from hCA I, II, and IV[19–21] reported earlier. Iodide was also an excellent fish CA inhibitor, with a potency intermediate between that of sulfamic acid on one part, and thiocyanate on the other one. Thiocyanate exhibited quite effective inhibition that is similar for hCA I and hCA IX, but rather different than hCA II, IV, and V. Interestingly, nitrate showed much more effective inhibition on fish CA compared to human isoforms. Azide had a K_i value of 1.5627 mM, although it was found to be 0.0012 mM for hCA I and 0.005 mM for hCA IX.[21] Nitrite showed similar capacity with azide which is much lower than those obtained for hCA I, II, IV, and V. Bromide and chloride were found to be rather weak inhibitors of the fish

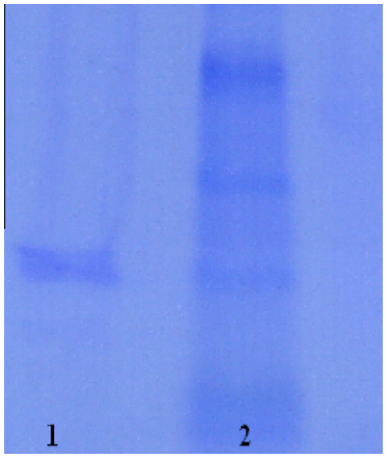

Figure 1. SDS–PAGE photograph. Lane 1: Affinity chromatography. Lane 2: Standard proteins: bovine albumin (66 kDa), chicken ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa) and chicken egg white lysozyme (16.5 kDa).

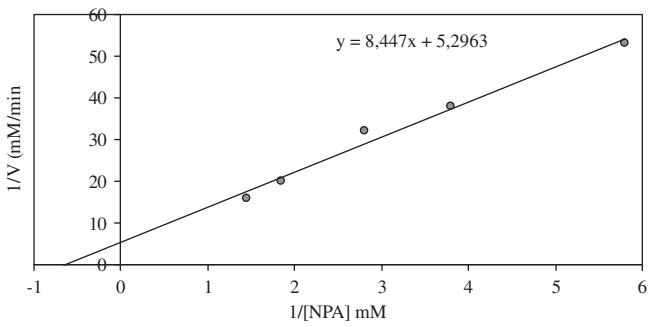

Figure 2. Lineweaver–Burk curves for 4-NPA hydrolysis catalyzed by the fish enzyme, at five different concentrations of substrate.

Table 2
Inhibition data of the fish CA with anions

Anion	K_i (mM)					
	Dicentrarchus labrax CA	hCA I[a]	hCA II[a]	hCA IV[b]	hCA V[c]	hCA IX[a]
$H_2NSO_3^-$	0.087	0.021	0.39	9.3×10^{-4}	0.12	–
I^-	0.155	0.3	26	0.08	25	7
SCN^-	0.187	0.2	1.6	39	0.74	0.13
NO_3^-	0.712	7	35	58.7	16	46
NO_2^-	1.142	8.4	63	30.8	16	–
N_3^-	1.563	0.0012	1.5	65.1	0.3	0.005
Br^-	3.522	4	63	0.09	50	16
Cl^-	4.872	6	>200	0.09	156	33
SO_4^{2-}	14.21	63	>200	9	680	–
F^-	39.20	>300	>300	0.07	241	48

K_i values were obtained from regression analysis graphs for fish gill CA in the presence of different anion concentrations.
[a] From Refs. [19,21].
[b] From Refs. [20,21].
[c] From Ref.[20].

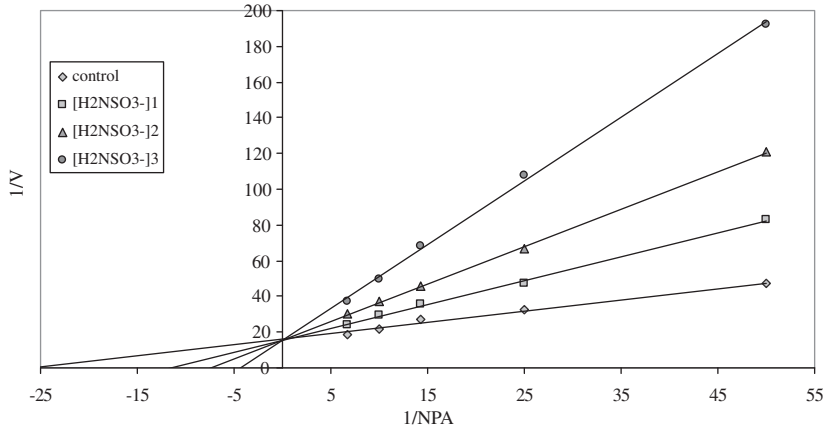

Figure 3. Lineweaver–Burk curves at five different substrate (NPA) and three different sulfamic acid concentrations.

CA with K_i value of 3.5221 and 4.8714 mM, which are very close to that of hCA I, whereas they are much lower than those of hCA II, VA and IX.[22] Sulfate was found to be a weak inhibitor against the fish CA, and similar results were obtained for hCA I, II, IV, and V. Fluoride was the weakest inhibitor for fish CA (K_i: 39.215 mM), and the same result was reported for hCA I, II, V, and IX; however, it was determined to be effective hCA IV inhibitor with K_i value of 0.07 mM.[21]

It should be noted the important difference of affinity of these anions for fish and human CA enzymes. All tested anions here exhibited competitive inhibition which might indicate that these anions are in competition with *p*-nitrophenyl acetate binding site. However, there are no X-ray crystal structures allowing us to know where the ester binds within the CA active site.

3. Conclusions

We purified CA from the European seabass (*Dicentrarchus labrax*) gill which has not been reported earlier, and analyzed the features of this enzyme. The kinetic data for the esterase activity of this enzyme with 4-NPA as substrate are in good agreement with others reported in the literature. The inhibitory effects of several anions on the enzyme activity were reported. Our findings indicate that these anions inhibit the fish enzyme in a similar manner to other α-CAs from mammals investigated earlier, but the susceptibility to various anions differ between the fish and mammalian CAs. All tested anions exhibited competitive inhibition with the ester substrate, which indicates that these anions are in competition with *p*-nitrophenyl acetate for binding to the active site. These results might be useful for toxicology researchers of fish living in fresh and sea water, as the species considered here. Since the fluvial systems draining Eurasia and central Europe introduce large volumes of sediment and dissolved substances into the Black Sea, aquatic organisms in this area are at great risk of chemical toxicity. Indeed, some of the investigated showed inhibition of the fish enzyme in the micromolar range, which may interfere with the physiological function of this CA.

4. Experimental

4.1. Chemicals

H_2NSO_3H, KNO_3, $NaNO_2$, NaN_3, $NaHSO_4$, NaSCN, KI, KCl, KBr, KF, 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.

4.2. Purification of carbonic anhydrase from fish gill by affinity chromatography

Fish gill carbonic anhydrase was purified from frozen fish tissue obtained from a commercial fish farm in Black Sea region in Turkey. Tissue samples were centrifuged at 10,000 rpm for 30 min, and plasma and precipitate were removed. The pH of the homogenate was adjusted to 7.5 with solid Tris. The homogenate was applied to the prepared Sepharose 4B-L-tyrosine-sulfanilamide

affinity column which had been equilibrated with 10 mM Tris–HCl/0.1 M Na_2SO_4 (pH 7.5). The affinity gel was washed with 10 mM Tris–HCl/22 mM Na_2SO_4 (pH 7.5), and the enzyme was eluted by 1.2 M NaCl/25 mM Na_2HPO_4 (pH 6.3). All procedures were performed at 4 °C.

4.3. Esterase activity assay

Carbonic anhydrase esterase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25 °C using a spectrophotometer (CHEBIOS UV–VIS) according to the method described by Verpoorte et al.[23] The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL of 0.05 M Tris–SO_4 buffer (pH 7.4), 1 mL of 3 mM 4-nitrophenylacetate, 0.5 mL of H_2O and 0.1 mL of enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. The inhibitory effects of $H_2NSO_3^-$, I^-, SCN^-, NO_3^-, NO_2^-, N_3^-, Br^-, Cl^-, SO_4^{2-}, and F^- were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. European seabass gill CA enzyme activities were measured for H_2NSO_3H (0.015–0.125 mM), KI (0.025–0.135 mM), NaSCN (0.025–0.250 mM), KNO_3 (0.3–1.3 mM), $NaNO_2$ (0.45–1.5 mM), NaN_3 (0.5–1.75 mM), KBr (1–6.5 mM), KCl (1.5–8 mM), $NaHSO_4$ (2–14 mM), and KF (5–50 mM) at cuvette concentrations. Control cuvette activity in the absence of inhibitor was used as 100%. For each inhibitor, an activity (%) – [Inhibitor] graph was drawn. To determine K_i values, three different inhibitor concentrations were tested. In these experiments, 4-nitrophenylacetate was used as substrate at five different concentrations (0.10–0.60 mM). K_i values were calculated from Lineweaver–Burk curves.[24]

4.4. Protein determination

Protein quantity was determined spectrophotometrically at 595 nm during the purification steps according to the Bradford method, using bovine serum albumin as the standard.[25]

4.5. SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis was performed after purification of the enzyme. 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.[26] A 20 μg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without the dye.

4.6. Molecular weight determination

4.6.1. Sephadex G-200 gel filtration

The molecular weight of the enzyme was determined by gel filtration chromatography.[27] At first, for establishing the void volume, Blue Dextran (2000 kDa) was passed through the column; then, bovine erythrocyte carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), and alcohol dehydrogenase (150 kDa) were used as standard proteins (Sigma MW-GF-200). Finally, the sample was applied to the column and the molecular weight was determined by K_{av}-LogMW graph.

4.7. In vitro effects of anions

In order to determine the effects of anions on fish gill CA, different concentrations of anions were added to the reaction medium. The enzyme activity was measured, and an experiment in the absence of inhibitor was used as control (100% activity). The IC_{50} values were obtained from activity (%) versus anion concentration plots.

To determine K_i constants in the media with inhibitor, the substrate (NPA) concentrations were 0.01, 0.02, 0.035, 0.05, and 0.07 mM. Inhibitor solutions (salts) were added to the reaction medium, resulting in three different fixed concentrations of inhibitors in 1 mL of total reaction volume. Lineweaver–Burk graphs[24] were drawn by using $1/V$ versuss $1/[S]$ values, and K_i constant were calculated from these graphs. Regression analysis graphs were drawn for IC_{50} using inhibition% values by a statistical package (SPSS-for windows; version 10.0) on a computer (student t-test; $n = 3$).

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