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Rosmarinic acid: a potent carbonic anhydrase isoenzymes inhibitor

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Abstract: Rosmarinic acid is a water-soluble ester of caffeic acid and 3,4-dihydroxyphenyllactic acids, and is mainly found in plant species including Boraginaceae and Lamiaceae. In this research, we determined the inhibition property of rosmarinic acid on carbonic anhydrase isoenzymes I and II (hCA I and II) purified from human erythrocytes by using Sepharose-4B affinity column chromatography. hCA I and II isoenzymes were obtained with a yield of 57.9% and 67.2% and 76.5- and 509.3-fold purification of each isoenzyme, respectively. In order to show the purity of the isoenzymes, SDS-PAGE was performed and one band was observed. In vitro inhibition of both hCA I and II isoenzymes by rosmarinic acid using CO_2-esterase activity gave Ki values of 86.0 μM and 57.0 μM, respectively.

Key words: Carbonic anhydrase I, carbonic anhydrase II, rosmarinic acid, enzyme purification, enzyme inhibition

1. Introduction

Enzymes, biological molecules responsible for thousands of metabolic processes, are synthesized by living cells and speed up in chemical reactions during the metabolism of living organisms.[1] Carbonic anhydrases (CAs; Carbonate hydrolyase, E.C.: 4.2.1.1) are metalloenzymes widespread in most living organisms and systematized by 5 historical irrelevant gene families: the α-, β-, γ-, δ- and ζ-CA.[2] The α-CA is found in mammals, β-CA is found in plants and some prokaryotes, and γ-CA exists only in archaea bacteria. Two other rare classes (δ-CA and ζ-CA) are similar to the β-CA class found in diatoms.[3] The 3 main classes (α-, β-, and γ-) of CA are structurally discrete and are thought to have evolved independently, possibly as a result of convergent evolution.[3] CA enzyme greatly increases the rate of the reaction between 10^4 and 10^6 reactions per second. Most CAs contain a zinc ion in their active site. The α-, β-, and δ-CAs contain a Zn^{2+} ion at the active site. On the other hand, the γ-CAs are likely Fe^{2+} enzymes, while the metal ion is generally replaced by Cd in the ζ-CAs.[2] CA was first isolated from bovine erythrocytes in 1933. The discovery of this CA in erythrocytes was over 80 years ago.[4] Subsequently, it was purified and characterized from many different plant and animal tissues. Mammalian CA's molecular mass is about 30 kDa. CA catalyzes the conversion of carbon dioxide (CO_2) to bicarbonate (HCO_3^-) ion and proton (H^+). CA has a ping-pong catalytic mechanism for both hydration and dehydration of CO_2 and HCO_3^-.[5]

$$\text{Enzyme} \sim Zn^{2+}-OH^- + CO_2 \Longleftrightarrow \text{Enzyme} \sim Zn^{2+}-HCO_3^-$$

$$\text{Enzyme} \sim Zn^{2+}-HCO_3 + H_2O \Longleftrightarrow \text{Enzyme} \sim Zn^{2+}-H_2O + HCO_3^-$$

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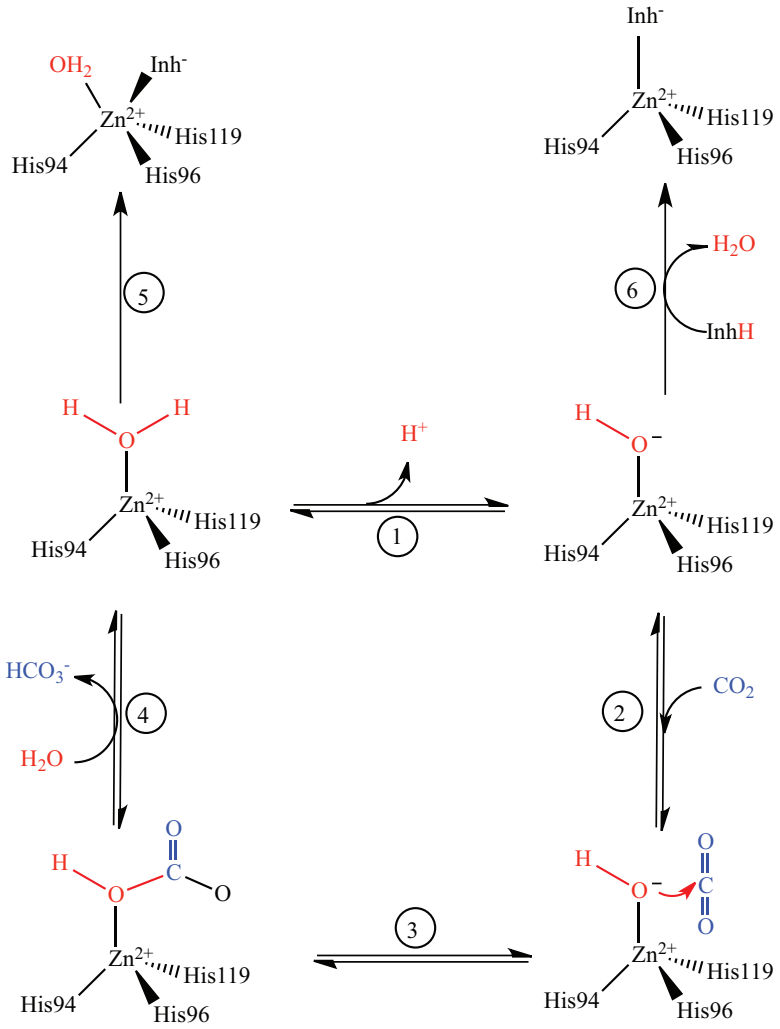
$$\text{Enzyme} \sim Zn^{2+}\text{–}H_2O + B \Longleftrightarrow \text{Enzyme} \sim Zn^{2+}\text{–}OH^{-} + BH^{+}$$

In the hydration direction, primarily, a CO_2 molecule binds to the active site of CA. The second step is the nucleophilic attack of a Zn^{2+}-bound hydroxide ion (OH^-) on CO_2 with the subsequent formation of HCO_3^-. Then HCO_3^- is displaced from the active site by a H_2O molecule. As can be seen in the Scheme, in the next step, Zn^{2+}–OH^- is formed as a result of H^+ transfer from Zn^{2+}–H_2O generated in step one to the bulk solvent.[5] A $\sim Zn^{2+}$–OH^- species of the enzyme is the catalytically active species. It acts as a powerful nucleophile on the CO_2 molecule bound in a hydrophobic pocket nearby.[6–8] Then $\sim Zn^{2+}$–OH^- is formed from H_2O coordinated to the Zn^{2+}, which exists at the bottom of the active site cavity of CA.



Scheme. Catalytic and inhibition mechanisms of carbonic anhydrases.

The molecular characteristics of CA across the plant and animal kingdoms are similar. In the red blood cell, this enzyme is necessary to facilitate the transport of CO_2 out of the body.[9]

The human CA isoenzymes belong to the α-class. So far, 16 CA isozymes have been found and identified. These isoenzymes have distinct molecular properties, oligomeric regulation, and cellular locality; different distribution in living tissues and organs; different expression levels; and varied kinetic features and response to

unlike classes of inhibitor. As a result, in some studies recently, CA isozymes have gained an important role and become an interesting target for designing potent inhibitors or activators with biochemical and biomedical applications.[2]

hCA I is found in the human erythrocyte cell. hCA II is the most studied and analyzed CA isoenzyme. It is purified from human blood when the amount of 2 mg g^{-1} of hemoglobin is calculated and this value is less than that of hCA I.[10] The eye lens, cornea, and ciliary epithelium have high hCA II and IV. hCA II isoenzymes are still in the renal cortex membrane bound and reabsorption of Na^+ and H_2O is provided. When there is deficiency of hCA II, bone calcification, kidney stone formation, and calcification occur.[11]

In skeletal muscle, hCA III isoenzyme was found and this isoenzyme has a very important role in the balance of lactic acid/lactate as studies have shown. hCA VI and hCA IV isoenzymes are 2 signal sequences that enable the signal to reach the target tissues and organs. hCA IV isoenzyme is a membrane-bound enzyme and is bound with the kidney membrane. Moreover, it was found bound with the some epithelial and plasma cells membranes in the lung capillary surfaces. On the other hand, hCA V isoenzyme is located in the mitochondria of some tissues. hCA VI isoenzyme is an enzyme released from the salivary glands. In addition, it has been isolated from human saliva and sputum. hCA VII isoenzyme allows saliva secretion of bicarbonate (HCO_3^-). CA VIII enzyme obtained from rat brain was discovered in a cDNA library. Moreover, human cDNA homologues of the isoenzymes were identified and amino acid sequences determined.[3] In some physiological characteristics of CA, it was demonstrated that the CA isoenzymes had important roles. As a result, CA isozymes have become an interesting scientific field for the design of inhibitors or activators with biomedical applications recently.[2]

Phenols have been studied in detail as inhibitors of CA that contain zinc ion.[12–14] Many natural and synthetic substances can affect the living metabolism by altering enzyme activities and affecting metabolic pathways at low concentrations.[15–20] The inhibitory properties of some antioxidant phenolic and polyphenolic compounds like simple phenol, catechol, resorcinol, hydroquinone, pyrogallol, and quercetin were also studied in detail. In addition, a series of active natural phenolic compounds including catechin, curcumin, dobutamine, silymarin, and resveratrol were investigated for the inhibition of all the catalytically active mammalian CA isozymes (CA I–CA XV). These polyphenolic compounds were effectively inhibited CA isoenzymes, with Ki values in the range of 380 nM–12.02 μM. In addition, the inhibitory effects of some phenolic acids including caffeic acid, ellagic acid, ferulic acid, gallic acid, salicylic acid, syringic acid, tannic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, 3-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, and 3,5-dihydroxybenzoic acid[12,13] on CA isoenzymes were investigated. CA plays an important role in water and ion transport and pH regulation in the kidney, eye, central nervous system, inner ear, and other systems. To date, to the best of our knowledge, there has been no study about the effect of rosmarinic acid on both hCA isozymes.

In the present study, we determined the inhibitory effect of rosmarinic acid as a natural phenolic compound of plant origin on hCA I and hCA II isozymes, which is of vital importance in the living metabolism. CA isoenzymes were purified in only one step via a Sepharose 4B-L-Tyrosine sulfanilamide affinity column from human erythrocytes.

Rosmarinic acid is an ester of caffeic and 3,4-dihydroxyphenyllactic acids. It is generally found in a wide range of species and plants.[21] It is especially present as a secondary metabolite in medicinal and food plants. In addition, it is widely used in Mediterranean and Anatolian folk medicine as a culinary herb. It is also used as a fragrant additive in cosmetic applications[22] and other applications.[23] It was reported that rosmarinic acid had some photoprotective effects against UV and other ionizing radiations.[21] It was also reported that rosmarinic acid had health promoting and beneficial effects. It is supposed to act as a preformed constitutively

accumulated defense compound in plants. Plant extracts that contain rosmarinic acid are a good potential source of antioxidants for food protection and pharmaceutical applications.[23]

2. Results and discussion

Rosmarinic acid is a naturally occurring phenolic compound. This natural phenolic acid is widely dispersed in the Labitae family of herbs, which contains rosemary, basil, and perilla. CA is expressed in almost all mammalian tissues. Known functions of CA comprise pH regulation in red blood cells and tissues, ion exchange in the kidney, oxygen transportation between lungs and tissues, and electrical activity in the retina and nervous system. There are different CA isozymes in many various tissues.[24,25]

CA inhibitors vary according to their affinity of binding to a particular CA isoenzyme, potency for inhibiting that isoenzyme, and physiological/biochemical properties, which can influence their tissue distribution and activity scope.[26]

Recently, it was found that CA isozymes were inhibited by several sulfamate-containing drugs[27] and a larger scale of phenolic compounds.[12,13] Given the physiological importance of CA, the metabolic effects of medically important drugs should receive greater attention, not only erythrocyte hCA I and II, but also the entire array of CA isozymes. For example, in recent studies, total hepatic CA activity was shown to diminish in the streptozotocin-induced diabetic rat. Gluconeogenesis and ureagenesis were also associated with an increase in hepatic CA V activity.[27] There is potent evidence for overexpression of CA IX and XII in cancer, acidifying the extracellular matrix, which is thought to promote tumor growth. Solid tumors are often hypoxic, and expression of CA IX and XII is downregulated under these conditions. CA found within the ciliary epithelium is associated with aqueous humor production and intraocular pressure. It is important for acid–base homeostasis, retina metabolism, and synaptic transmission.[28] In addition, hepatic pH disequilibrium was explained in terms of changes in CA activity. Furthermore, side effects of many drugs may be considered to result from CA isozyme inhibition. For instance, respiratory acidosis is probably the cause of some side effects observed during acetazolamide therapy, such as fatigue, headache, stress, and taste disorders.[5,28]

Measurements of the CO_2 hydratase activity of both CA isoenzymes require specific inhibitors or separation of the isozymes. It is difficult to study the factors and conditions that affect CA activity because standard CA activity assays have serious limitations. Therefore, the estimation of both CA isoenzymes' levels in erythrocytes is complicated by the pronounced differences in enzymatic activity of both CA isoenzymes.[5]

In the present study, given the importance of CA in pH regulation in most tissues, the effects of increasing concentration of rosmarinic acid administration on hCA I and II isozymes were also examined. Thus, both CA isoenzymes were purified by Sepharose 4B-L-Tyrosine sulfanilamide affinity chromatography (Table 1). The purity of both isoenzymes was confirmed by SDS-PAGE (Figure). The Michaelis constant (K_m) and V_{max} were determined using a Lineweaver–Burk plot. All parameters were the mean of triplicate determinations from 3 independent preparations.[29] Both isoenzymes (hCA I and II) were obtained with a yield of 57.86% and 67.17% and a specific activity of 534.88 and 3560.0 EU mg^{-1} proteins. At the same time, hCA I and II were purified 76.5- and 509.3-fold, respectively (Table 1).

The phenyl fragment of phenol was found in the hydrophobic part of the hCA II active site and bound in hCA II as a precatalytic complex.[14] Recently, our groups have analyzed the interactions between some phenols, phenolic acids, polyphenols, antioxidant phenolic compounds, and natural polyphenol products and known mammalian isozymes (CA I–XV). Many of these phenolic compounds have low micromolar or submicromolar inhibition as well as the possibility of modeling isozyme selective CA isoenzyme inhibitors. In fact, the inhibition

background of various CA isozymes with phenolic compounds is very variable, with K_i values ranging from the millimolar to the submicromolar range.[21–23] Above all, most of these natural phenolic compounds are used in clinical, pharmaceutical, and antioxidant applications as food additives. In addition, it was recently reported that some new benzotropone derivatives inhibited both CA isoenzymes at low micromolar levels.[30]

Table 1. Purification scheme of hCA I and hCA II from human erythrocytes by Sepharose-4B-L-Tyrosine sulfanilamide affinity chromatography (hCA: human carbonic anhydrase).

	Activity (EU/mL)		Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity	Specific activity (EU/mg)	Yield (%)	Purification fold
Hemolysate	106		30	15.15	454.15	3180	6.99	100	1
Sepharose-4B-L-Tyrosine sulfanilamide affinity chromatography	CA I	230	8	0.43	3.44	1840	534.88	57.86	76.52
	CA II	534	4	0.15	0.6	2136	3560	67.17	509.29

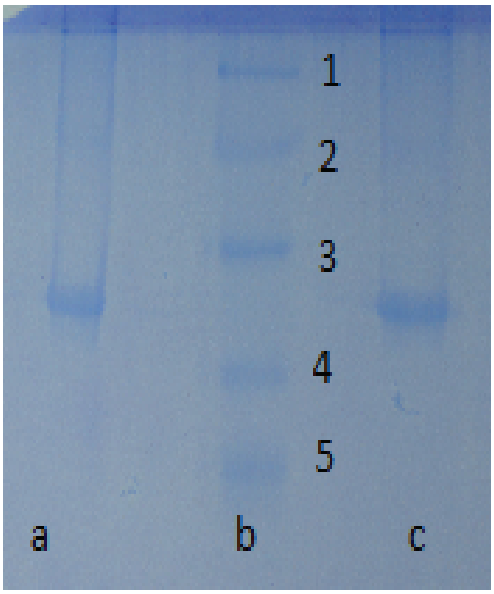


Figure. SDS-polyacrylamide gel electrophoresis of hCA I and II purified by Sepharose 4B-L-Tyrosine sulfanilamide affinity gel chromatography (hCA: human carbonic anhydrase, **a** is hCA isoenzymes, **c** is hCA II isoenzymes, **b** is standard proteins obtained from prestained protein molecular weight marker, 1: 85 kDa, 2: 50 kDa, 3: 35 kDa, 4: 25 kDa, 5: 20 kDa).

Rosmarinic acid was found to be sufficient for inhibition of both isoenzymes. In this respect, it was understood from in vitro studies that both hCA isoenzymes were inhibited by rosmarinic acid (Table 2). Fifty percent inhibitory concentrations (IC_{50}) were 8.0×10^{-5} M for hCA I and 47.0×10^{-5} M for hCA II. Moreover, the K_i value of rosmarinic acid was 86.0×10^{-5} M for hCA I and 57.0×10^{-5} M for hCA II. Recently, in another study, IC_{50} values for dantrolene sodium were 4.09×10^{-5} M for hCA I and $3.24 \times$

10^{-5} M for hCA II.[25] In the same way, both hCA isoenzymes were inhibited by melatonin up to 2 $\times$ 10^{-4} M. After the concentration of 4 $\times$ 10^{-4} M, the inhibitory effect of melatonin decreased with increased melatonin concentration.[9]

Table 2. IC_{50} and K_i values obtained from regression analysis graphs for CA isoenzymes in the presence of different rosmarinic acid concentrations.

Inhibition parameters	hCA I (μM)	hCA II (μM)
IC_{50}	8.00	47.0
K_i	86.0	57.0
Inhibition type	Noncompetitive	Noncompetitive

CA inhibitors are frequently used for the treatment of different diseases including edema, epilepsy, glaucoma, and acute mountain sickness. On the other hand, it is well known that acetazolamide is used in treating these diseases.[29] Recently, it was demonstrated that a simple phenolic compound, such as catechol and resorcinol, acts as a CA inhibitor.[31]

Like rosmarinic acid, good inhibitory activity has been found in food bioactive compounds such as curcumin, catechin, dobutamine, resveratrol, and silymarine for the inhibition of cytosolic hCA II. Structure-activity insight is well known for this small series of phenolic compounds. As with dobutamine, this trend is contained when different groups are present in the *para* position to the phenol moiety (–OH).[14]

CA I and CA II are the 2 major CA isozymes present at high concentrations in the cytosol in erythrocytes, and CA II is the most active of all CAs. The objective of this research was to purify CA I and CA II isozymes from human erythrocytes by Sepharose-4B affinity chromatography column chromatography and determine the in vitro effects of rosmarinic acid on CA I and CA II isozymes.

3. Experimental

3.1. Chemicals

CNBr-activated-Sepharose 4B, rosmarinic acid, protein assay marker, and the other chemicals and reagents for electrophoresis were obtained from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany). The other chemicals were of analytical grade and purchased from Merck.

3.2. Purification of hCA isoenzymes

At the outset, the human blood was obtained from the Atatürk University Research Hospital Blood Center. Erythrocytes were obtained from low-speed centrifugation (1000 $\times g$) for 15 min by elimination of the plasma and buffy coat. The erythrocytes were washed with physiological saline (% w/v: 0.9 NaCl) and hemolyzed with 1.5 volumes of cold water. Then intact cells and ghost were extracted at medium speed centrifugation (13,500 $\times$ g) for 30 min at low temperature (4 °C). The pH of hemolysate medium was adjusted to 8.7 using solid Trisma. Finally, pH-adjusted hemolysate was loaded to the affinity column. The affinity column was packed with SB-L-TS resin and pre-equilibrated with Tris-HCl/Na_2SO_4 (0.25 M/0.1 M) at pH 8.7.[32]

One hundred milliliters of new prepared human erythrocyte hemolysate was applied to the affinity column and washed with Na_2SO_4 (22 mM), in Tris-HCl buffer solution (25 mM, pH 8.7). Both isoenzymes were eluted with NaCl (1.0 M) in sodium phosphate buffer (25 mM, pH 6.3) and $NaClO_4$ (0.5 M) in sodium acetate buffer (0.1 M, pH 5.6), respectively. Column flow rate was 20 mL h^{-1}. The fractions were collected in a fraction

collector at low temperature (4 °C). Absorbance of fractions at 280 nm was recorded for the determination of protein elution by affinity chromatography. Finally, CO_2-hydratase activity in each eluate was determined and the enzymatically active fractions were collected.[33]

3.3. Protein determination

The quantity of protein was calculated by the Bradford method.[34] For this purpose, the absorbance of the sample was measured at 595 nm after the reaction. Bovine serum albumin (BSA) was used as a standard protein, as described previously.[35–37]

3.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous PAGE was used under denaturing conditions after the purification of both hCA isoenzymes, according to Laemmli's procedure,[38] as described previously.[5] The stacking and running gels contained 3% (w/v) and 10% (w/v) acrylamide, respectively, and 0.1% (w/v) SDS. The electrode buffer was Tris (0.25 M)/glycine (2 M), pH 8.3.

The buffer solution was prepared by mixing Tris-HCl (0.65 mL, 1 M, pH 6.8), SDS (3 mL, w/v: 10%), neat glycerol (1 mL), bromphenol blue (1 mL, w/v: 0.1%), β-mercaptoethanol (0.5 mL), and water (3.85 mL). An aliquot of enzyme (20 μg) was added to the sample buffer solution (50 μL) and the mixture was heated in a water bath (100 °C) for 3 min and cooled.

Both isozymes' samples were loaded into each space of the stacking gel. Initially, an electric potential of 80 V was applied until the bromphenol dye reached the running gel. Then it was increased to 200 V over 3–4 h. The gel was kept in Coomassie Brilliant Blue R-250 (w/v: 0.1%) reagent in methyl alcohol (v/v: 50%) and acetic acid (v/v: 10%) and distained with methanol/acetic acid for 90 min. The electrophoretic zymogram was photographed (Figure).

3.5. CA isozymes' inhibition effects

The effects of increasing concentrations of rosmarinic acid on hCA I and hCA II isoenzymes were determined colorimetrically using CO_2 hydration.[39] Briefly, both CA isozymes' samples were added to 4.2 mL (final volume) of incubation mixture containing 1 mL of Veronal buffer (0.025 M, pH 8.2), bromothymol blue (0.1 mL, w/v: 0.04%), and saturated CO_2 solution in water (2.5 mL). Both CA isozymes' activity was measured by colorimetric method considering the required time for changing of pH from 8.2 to 6.3. One enzyme unit (EU) for CO_2-hydratase activity of both CA isozymes was calculated by using the equation $(t_o - t_c/t_o)$, where t_o and t_c are the times for pH change of the nonenzymatic and enzymatic reactions, respectively.

3.6. Statistical analysis

The data are reported as the mean ± SD and were analyzed by SPSS (version 17.0). Significant differences between the means were determined by Duncan's multiple range test. $P < 0.05$ was considered significant. Each experiment was performed in triplicate.

4. Conclusion

Rosmarinic acid is a natural phenol antioxidant carboxylic acid and is found in many plant families. It has 4 phenolic –OH functional groups in their aromatic scaffold. It effectively inhibited both hCA isozymes because

of its 4 functional phenolic –OH groups. These findings clearly indicated that phenolic compounds are another class of possible hCA inhibitors in addition to other putative hCA inhibitors such as sulfonamides, sulfamates, and sulfamides. Rosmarinic acid, as a natural phenolic compound, showed effectiveness in inhibition of both hCA isoenzymes' activities in the low micromolar range. These results indicate that compounds with substituted phenolic may be used as potent hCA isoenzyme inhibitors.

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