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Purification and characterization of carbonic anhydrase from bovine stomach and effects of some known inhibitors on enzyme activity

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Abstract

Carbonic anhydrase (CA) was purified from four different cell localisation (outer peripheral, cytosolic, inner peripheral and integral) in bovine stomach using affinity chromatography with Sepharose-4B-L-tyrosine sulphanilamide. During the purification steps, the activity of the enzyme was measured using *p*-nitrophenyl acetate at pH 7.4. Optimum pH and optimum temperature values for all CA samples were determined, and their K_m and V_{max} values for the same substrate by Lineweaver–Burk graphics. The extent of purification for all CA localizations was controlled by SDS-PAGE. The K_m values at optimum pH and 20°C were 0.625 mM, 0.541 mM, 0.785 mM and 0.862 mM with *p*-nitro phenyl acetate, for all CA localizations. The respective V_{max} values at optimum pH and 20°C were 0.875 μmol/L min, 0.186 μmol/L min, 0.214 μmol/L min and 0.253 μmol/L min with the same substrate. The K_i and I_{50} values for the inhibitors sulphanilamide, KSCN, NaN_3 and acetazolamide were determined for all the CA localizations.

Keywords: *Stomach, carbonic anhydrase, kinetics*

Introduction

Carbonic anhydrase (carbonate hydrolyase, E.C. 4.2.1.1) isozymes are a family of zinc metalloenzymes, which catalyse the interconversion of CO_2 and HCO_3^-. The enzyme, discovered about 70 years ago[1,2], is abundantly present in mammalian red blood cells and to a lesser extent in different types of tissues and secretory organs.[3,4] In addition, carbonic anhydrases have been obtained and characterised from plant, yeast and bacteria.[4–10] The important roles of the enzyme in various cell types have been extensively reviewed.[4,5] At present, 14 isozymes (I–XIV) have been reported and found to be distributed throughout living organisms.[4,5,10] Human and most mammalian red blood cells carbonic anhydrases are known to consist of two isozymes, I and II, however, ruminants and cats have only isozyme II.[4]

Histochemical studies of carbonic anhydrase in the stomach had been reported in many studies by light microscopy.[11] Four approaches have been employed to demonstrate carbonic anhydrase in cell types and in tissue sections: a radioautographics method employing a labelled inhibitor, cytoimmunodiffusion, immunocytochemistry and the much more extensively applied cobalt-bicarbonate method of Haussler and Hansson which was developed from the manganese-based method of Karata.[12–16] Carbonic anhydrase examined immunohistochemically showed, intense staining in the plasma membranes of the gastric epithelium.[12] However, no previous studies have been carried out regarding CA purification and characterisation from bovine stomach.

Here, we describe the purification of carbonic anhydrase from four different cell localisations in bovine stomach and investigation of their kinetic properties.

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Experimental

Purification procedure

The bovine stomach samples were obtained from slaughtered animals at the Erzurum Municipal Slaughterhouse and were rinsed in a solution of 0.9% NaCl. Each piece of stomach was cut into small pieces and washed with physiological saline.

Outer peripheral CA. The washed samples were mixed with 1 M KCl for 2.5–3 h at room temperature in order to remove the outer peripheral proteins. Then, the mixture was centrifuged for 20 min at 20,000 rpm and 4°C, and the precipitate was saved for purification of cytosolic CA at 4°C.

The outer peripheral CA was purified from the supernatant. For this purpose, the supernatant was extracted with CCl_4 to remove lipids and the aqueous phase was dialysed overnight against 0.05 M, pH 7.4 Tris–SO_4. The pH of the dialysed sample was adjusted to pH 8.7 with solid Tris and the solution obtained was used for purification of outer peripheral CA by an affinity column.[17]

Cytosolic CA. The precipitant obtained above was suspended in 0.05 M Tris buffer at pH 7.4. The suspension was frozen using liquid nitrogen to break up the cell membrane. A Sonic Dismembrator was used for 4 h to homogenize the suspension in 0.05 M Tris buffer at pH 7.4. The resultant mixture was filtered and then centrifuged at 20,000 rpm for 20 min. The precipitate was saved for the purification of inner peripheral CA at 4°C (see later), and the supernatant was extracted with CCl_4. The pH of the solution was adjusted to pH 8.7 with solid Tris, and it was applied to an affinity column.[17]

Inner peripheral CA. The precipitant was mixed with 1 M KCl for 2.5–3 h at room temperature, and then centrifuged (20,000 rpm; 10 min). The precipitate was saved for purification of integral CA. The supernatant was extracted with CCl_4 and the inorganic phase was dialyzed against 0.05 M Tris–SO_4 pH 7.4. The pH of the dialysed solution was adjusted to pH 8.7 with solid Tris and it was applied to an affinity column.[17]

Integral CA. The precipitate was suspended in 0.05 M Tris–SO_4, pH 7.4, and TritonX-100 (1%) was added to the suspension to solubilise the integral proteins. The obtained final solution was homogenized using a Sonic Dismembrator for 4 h and then centrifuged (20,000 rpm, 1 h). The supernatant was dialyzed against distilled water to remove TritonX-100 and then against 0.05 M Tris–SO_4, pH 7.4. The pH of the dialysate was adjusted to pH 8.7 with solid Tris before application to an affinity column.[17]

Affinity column chromatography

Outer peripheral, inner peripheral, cytosolic and integral CA from bovine stomach were purified by an affinity column containing Sepharose-4B-L-tyrosine-sulfanilamide, as previously described.[17] The column was equilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7). The enzyme solution was applied and the column was washed with 400 ml of 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7) to remove the other proteins and substances. Then, the enzyme was eluted with 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6) the elution procedure being followed at 280 nm. The column was then re-equilibrated.

Determination of protein content and carbonic anhydrase activity

Specific activity and extent of purification were determined after all subsequent steps. The protein concentration was determined according to the method of Bradford using bovine serum albumine as standard.[18] The absorbance at 280 nm was used to monitor protein in the column effluents.

Carbonic anhydrase activity was assayed by two different methods. Firstly, hydration of CO_2 was measured by the method of Wilbur and Anderson.[19] In this procedure, 2.0 mL of veronal buffer (pH 8.2), 0.4 mL of bromothymol blue (0.004%), 0.8 mL of dilluted enzyme solution and 2 mL of a CO_2 solution (saturated at 0°C) were mixed. The time (tc) interval was determined between addition of CO_2 solution and the occurrence of a yellow–green colour. The same interval was recorded without enzyme solution (to). The activity was calculated from the formula.[19]

$$1\ \text{Wilbur Anderson Unit} = (\text{to} - \text{tc})/\text{tc}$$

Secondly, the principle of this determination is that a substrate of CA (*p*-nitrophenol acetate) was hydrolysed to *p*-nitrophenol and acetic acid, and the product was detected at 348 nm. For this procedure, 1.5 mL of a buffered enzyme solution (0.1 mL enzyme + 1.4 mL 0.05 M Tris–SO_4, pH 7.4) and 1.5 mL of substrate were mixed in a cuvette and 3 min later the absorbance was measured (348 nm, 25°C). A blank measurement was obtained where saline was added to the cuvette instead of enzyme solution[20] and this value was subtracted.

CA specific activity for outer peripheral, inner peripheral, cytosolic and integral CA preparations were calculated by using homogenates and purified enzyme solution. The purification was monitored with

Table I. Purification of carbonic anhydrase enzymes from bovine stomach.

Step	Volume (ml)	Activity (EU/ml)	Total Activity EU	Total Activity %	Protein (μg/ml)	Specific Activity (EU/mg)	Purification (fold)
Outer Peripheral							
Homogenate	690	8.1	5589	100	110	7.3×10^{-2}	–
Purified Enzyme	40	33.5	1340	23.97	0.082	408.5	5596
Cytozolic							
Homogenate	500	4.5	2250	100	127.5	3.5×10^{-2}	–
Purified Enzyme	30	25	750	33.33	0.121	206.6	5903.2
Inner Peripheral							
Homogenate	1000	6.3	6300	100	120	5.25×10^{-2}	–
Purified Enzyme	25	20.6	515	8.2	0.090	228.9	4360
Integral							
Homogenate	410	8	3280	100	128	6.25×10^{-2}	–
Purified Enzyme	40	13.5	540	16.5	0.273	49.45	791.2

SDS-Polyacrylamide gel electrophoresis.[21] The purification data is summarised in Table I.

Kinetic studies

Optimum pH, optimum temperature, K_M and V_{max} and K_i values were determined using *p*-nitrophenyl acetate as substrate; I_{50} value was determined by the CO_2 hydratase activity.

The inhibitors sulfanilamide, KSCN, NaN_3 and acetazolamide were selected for screening of CA activity.

To obtain K_i value at pH 7.4, the enzyme activity was measured for seven different substrates concentration (3.3, 2.0, 1.43, 1.11, 0.9, 0.77 and 0.67 mM) at 20°C by measuring absorbance at 348 nm with or without inhibitor. Inhibitor concentration used are given in Table II.

K_i values were calculated from Linewear–Burk graphs, and an average K_i value was calculated for each inhibitors.

To determine I_{50} values of inhibitors the enzyme activity was measured using hydratase activity at 20°C. Percent activity values were obtained from six different inhibitor concentration. CA activity without inhibitor was taken as 100%. The inhibitor concentration causing 50% inhibition (I_{50}) was calculated from the activity-inhibitor concentration graph.

Table II. K_m and V_{max} values obtained from Linewear–Burk graphs for five different concentrations of *p*-nitrophenyl acetate.

Position of Carbonic Anhydrase in cell	K_m (mM)	V_{max} (μmol/L min)
Outer Peripheral CA	0.625	0.875
Cytosolic CA	0.541	0.186
Inner Peripheral CA	0.785	0.214
Integral CA	0.862	0.253

Molecular weight determination by gel filtration

The molecular weight of the purified outer peripheral, inner peripheral, cytosolic and integral carbonic anhydrase from bovine stomach was determined on a Sephadex G-150 column. A mixture of standard proteins, each at a concentration of 0.2 mg/mL, was applied to the column. Purified CAs were added to the equilibrated column and eluted with 0.05 M NaP_i/1 M dithioerythritol.[22]

Zinc determination of the CAs

The zinc content of the CAs obtained from bovine stomach was determined. In order to obtain ApoCA, the zinc ion bound to CA was removed by chelating with dipicolinic acid as used by Demir et al.[23] In this procedure, the enzyme solution was placed in a dialysis sack and dialyzed against phosphate buffer (0.2 M; pH 7.0) containing dipicolinic acid (0.075 M) for 5 h followed by dialysis against deionised water. The resultant apoenzyme showing no enzymatic activity because of the lack of Zn^{2+}. For reactivation of ApoCA using standard Zn^{2+} solution, we added the 2.4×10^{-5} M Zn^{2+} solution to the ApoCA solution and reactivation of the CA was observed 3 min later.[23]

Results and discussion

Carbonic anhydrase is well known as a pH-regulating enzyme in most tissues, including humans and 14 different CA isoenzymes or CA-related proteins have been described with very different subcelluler localisation and tissue distribution.[24] Mann[25,26] discovered CA inhibition by sulphanilamide and sulphanilamide has been used as an antiglaucoma agent.[27–29] Many chemicals at relatively low

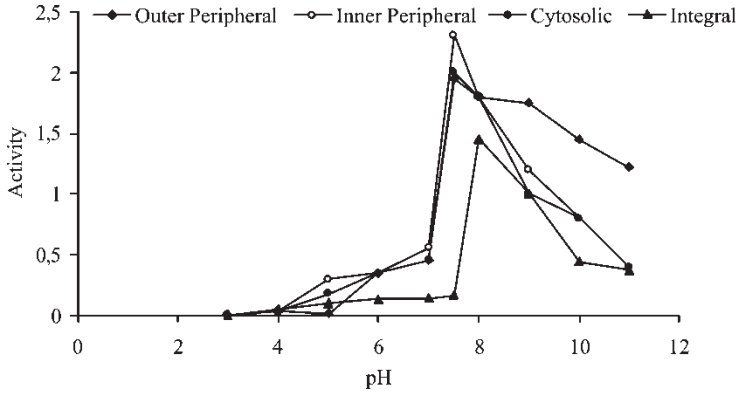

Figure 1. The effect of pH on activity of carbonic anhydrase from bovine stomach (outer peripheral, cytozolic, inner peripheral, and integral).

dosage affect the metabolism of biota by altering normal enzyme activity, particularly inhibition of a specific enzyme.[30] Indeed, CA isoenzymes are important enzymes for body metabolism because they regulate pH in most tissue. CA isoenzymes have been purified from different tissues such as erythrocytes, skeletal muscle, salivary, human lung etc[31–35] but there is no detailed study concerning purification of CA from bovine stomach. Therefore, in the present study, we firstly purified carbonic anhydrase enzymes from outer peripheral, cytosolic, inner peripheral and integral of bovine stomach.

Outer peripheral, cytosolic, inner peripheral and integral stomach carbonic anhydrase isoenzymes from bovine stomach were separated and then purified using Sepharose-4B-L-tyrosine-sulfanylamide affinity chromatography. Specific activities for carbonic anhydrase were calculated in both crude extract and purified enzyme solution for all samples. A purification of 5596-fold for outer peripheral, 4360-fold for cytosolic, 5903-fold for inner peripheral and 791.2-fold for integral position was obtained (Table I).

Kinetics parameters such as optimum pH, optimum temperature K_m, and V_{max} were calculated from graphs using *p*-nitro phenyl acetate as substrate on the stomach CA samples. As shown in Figure 1, optimum pH values for outer peripheral, cytosolic, inner peripheral and integral stomach carbonic anhydrase was found by means of activity-pH graphs and was determined to be pH 7.5 for outer peripheral, cytosolic and inner peripheral and pH 8 for integral CA.

Optimum temperature values were found at the optimum pH for outer peripheral, cytosolic, inner peripheral and integral stomach carbonic anhydrase and was determined to be 40°C for cytosolic and 35°C for the other CAs as shown in Figure 2.

As shown in Table II, K_m and V_{max} values at optimum pH were determined at 20°C by means of Lineweaver–Burk graphs. K_m and V_{max} values at optimum pH and 25°C were 6.7×10^{-3} mM and 2.58×10^{-4} mM min^{-1} using *p*-nitro phenyl acetate, for bovine CA, respectively.[36] However, here K_m values at optimum pH and 20°C were 0.625 mM, 0.541 mM, 0.785 mM and 0.862 mM, using *p*-nitro phenyl acetate, for outer peripheral, cytosolic, inner peripheral and integral CA, respectively. V_{max} values at optimum pH and 20°C were 0.875 μmol/L min, 0.186 μmol/L min,

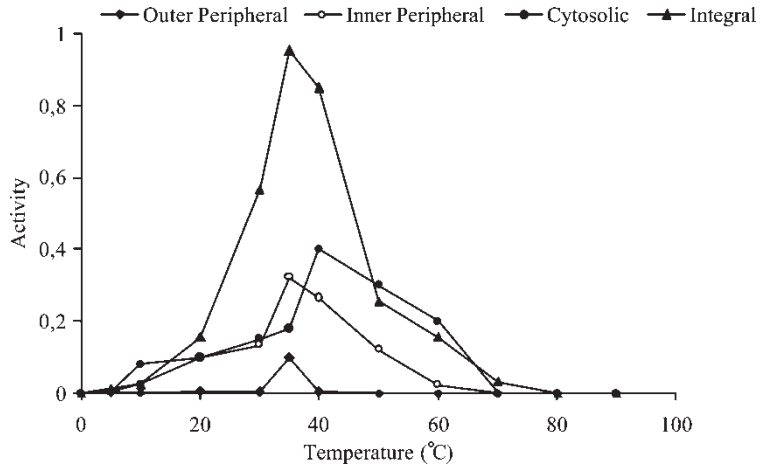

Figure 2. Effect of temperature on the purified carbonic anhydrase enzyme from bovine stomach (outer peripheral, cytosolic, inner peripheral, integral).

0.214 μmol/L min and 0.253 μmol/L min with the same substrate, respectively.

The K_i values for acetazolamide, sulfanilamide, and NaN_3 were 2×10^{-7} mM, 1.45×10^{-6} mM, 2.6×10^{-6} mM, 5.9×10^{-4} mM for bovine CA, respectively.[36]

As shown Table III, the K_i values for sulfanilamide, KSCN, NaN_3 and acetazolamide were 1.19×10^{-4} mM, 1.52×10^{-4} mM, 6.68×10^{-4} mM and 1.79×10^{-4} mM for outer peripheral CA; 1.30×10^{-4} mM, 1.34×10^{-4} mM, 3.30×10^{-4} mM and 1.03×10^{-4} mM for in inner peripheral CA; 2.30×10^{-4} mM, 0.94×10^{-4} mM and 5.96×10^{-4} mM and 1.41×10^{-4} mM for cytosolic CA; 2.85×10^{-4} mM, 1.65×10^{-4} mM, 1.5×10^{-4} mM and 1.87×10^{-4} mM for integral CA, respectively.

The I_{50} values for sulfanilamide, KSCN, NaN_3 and acetazolamide were 0.860 mM, 1212 mM and 1.161 mM and 0.64 mM for outer peripheral CA; 1.364 mM, 1.340 mM, 1.397 and 1.373 mM for inner peripheral CA; 0.476 mM, 0.5 mM, 0.770 mM and 0.89 for cytocolic CA; 0.890 mM, 1.354 mM, 0.373 mM and 0.64 mM for integral CA, respectively.

All purification steps were controlled by SDS-PAGE. Bovine erythrocyte CA was used as a standard as shown in Figure 3. The molecular weight of bovine stomach CAs was similar to that of the CA of bovine erythrocyte (30 kDa) for outer peripheral, cytosolic, inner peripheral and integral CA.

Table III. K_i values obtained from Linewear–Burk graphs for outer peripheral, cytosolic, inner peripheral and integral in the presence of three inhibitors and six substrate concentrations. I_{50} values were obtained from regression analysis graphs in the presence of 3 mM substrate concentration.

Inhibitor	K_i mM*	Mean Value	Inhibition	I_{50} mM
Outer pheriperal				
Sulphanilamide	0.9×10^{-5} 1.46×10^{-5} 1.2×10^{-5}	1.19×10^{-4}	Uncompetitive	0.860
KSCN	1.56×10^{-4} 1.48×10^{-4} 1.52×10^{-4}	1.52×10^{-4}	Uncompetitive	1.212
NaN_3	5.06×10^{-4} 8.06×10^{-4} 6.93×10^{-4}	6.68×10^{-4}	Uncompetitive	1.161
Cytosolic				
Sulphanilamide	1.17×10^{-4} 1.37×10^{-4} 1.35×10^{-4}	1.30×10^{-4}	Uncompetitive	0.476
KSCN	1.82×10^{-4} 1.19×10^{-4} 1.0×10^{-4}	1.34×10^{-4}	Uncompetitive	0.500
NaN_3	5.8×10^{-4} 2.11×10^{-4} 2.0×10^{-4}	3.3×10^{-4}	Uncompetitive	0.770
Inner Peripheral				
Sulphanilamide	2.63×10^{-4} 2.97×10^{-4} 1.30×10^{-4}	2.3×10^{-4}	Uncompetitive	1.364
KSCN	1.04×10^{-4} 1.08×10^{-4} 0.7×10^{-5}	0.94×10^{-4}	Uncompetitive	1.340
NaN_3	5.12×10^{-4} 5.49×10^{-4} 7.29×10^{-4}	5.96×10^{-4}	Uncompetitive	1.397
Integral				
Sulphanilamide	2.27×10^{-4} 3.41×10^{-4} 2.86×10^{-4}	2.85×10^{-4}	Uncompetitive	0.890
KSCN	1.46×10^{-4} 1.99×10^{-4} 1.49×10^{-4}	1.65×10^{-4}	Uncompetitive	1.354
NaN_3	2.36×10^{-4} 1.0×10^{-4} 1.03×10^{-4}	1.5×10^{-4}	Uncompetitive	1.023

* Inhibitor concentration was 1×10^{-2} M, 1×10^{-4} M and 1×10^{-6} M, respectively.

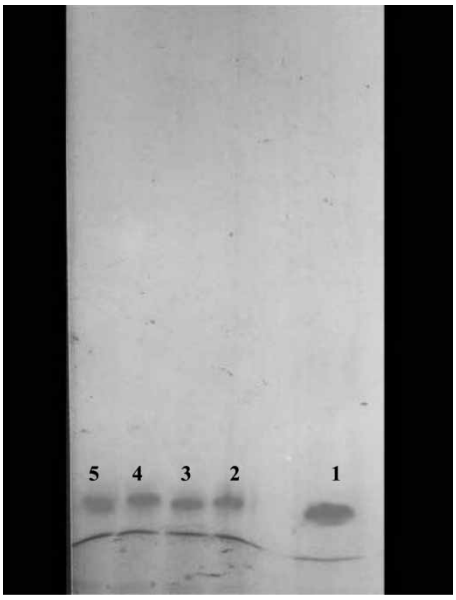

Figure 3. SDS-polyacrilamide gel electrophoresis of CA isoenzymes purified by affinity gel (lane I: bovine CA, lane II: outer peripheral, lane III: inner peripheral, lane IV: cytosolic CA, lane V: integral CA).

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