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Purification and Properties of Carbonic Anhydrase from Bone Marrow

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In this work, the carbonic anhydrase was purified from bovine bone marrow and investigated its kinetic properties. Carbonic anhydrase was purified from bovine bone marrow using affinity chromatography by sepharose 4B-L-tyrosine sulphanilamide. During purification steps, the activity of enzyme was measured using *p*-nitrophenyl acetate at pH: 7.4. Optimum pH and optimum temperature values for bovine bone marrow carbonic anhydrase were determined and then K_M and V_{max} values for the same substrate were obtained by means of Linewearver-Burk graphics. The purification degree for bovine bone marrow was calculated. The V_{max} and K_M values at optimum pH and at 20 ºC for the substrate (*p*-nitrophenyl acetate) were 120.418 μmol/L min and 2.409×10^{-3} mM, respectively. The K_i and I_{50} values for sulfanilamide, KSCN, NaN_3 and acetazolamide were determined in bovine bone marrow carbonic anhydrase.

Key Words: Bone marrow, Carbonic anhydrase, Kinetics.

INTRODUCTION

Carbonic anhydrase (carbonate hydrolyase, E.C. 4.2.1.1) isozymes are a family of zinc metalloenzymes that catalyze the interconversion[1,2] of CO_2 and HCO_3^-. The enzyme is abundantly present in mammalian red blood cells and to a lesser extent in different types of tissues and secretory organ[3,4]. In addition, carbonic anhydrase have been obtained and characterized from plant, yeast and bacteria[4-10]. The important roles of the enzyme in various cell types have been extensively reviewed[4,5,10]. Human and most mammalian red blood cell carbonic anhydrases are known to comprise two isozymes, I and II, however, ruminants and cats have only one isozyme II[4].

The first membrane-associated carbonic anhydrase purified to homogeneity was obtained from bovine lung[5]. Several years later, a different type of purification of a membrane-bound carbonic anhydrase from human kidney membranes was

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reported[6,7]. The apparent molar mass was initially reported to be 68.000[7], but more recent purification by this method yielded an inactive polypeptide with a molar mass of 34.400 on SDS-PAGE[6]. Carbonic anhydrase was purified and characterization from bovine bone[8].

Bone marrow is the soft, sponge-like material found inside bones. It contains immature cells known as hematopoietic or blood-forming stem cells. Hematopoietic stem cells divide to form more blood-forming stem cells or they mature into one of three types of blood cells: white blood cells, which fight infection; red blood cells, which carry oxygen and platelets, which help the blood to clot. Bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT) are procedures that restore stem cells that have been destroyed by high doses of chemotherapy[9].

A lot of cells are produced in the bone marrow and it has the most important role as physiologic. It was deficiency that wasn't defined carbonic anhydrase that was determined to be in a lot of tissues, in the bone marrow. However, no previous study has been carried out purification and characterization from bovine bone marrow and its characteristics features were determined.

EXPERIMENTAL

Preparation of bone marrow: The bone was first broken into parts and then bone marrows were taken out from bones. The last washing was made with physiologic serum in order to remove the blood.

The bone marrow was suspended in 0.05 M *Tris*-SO_4 (pH: 7.4) and Triton X-100 (1 %) was added to the suspension to solve the integral proteins. The resulting solution was put into ultrasonic homogenizer and lasted for 4 h. It was then centrifuged (20,000 rpm, 1 h). The supernatant was subjected to extraction with CCl_4 and the watery phase was dialyzed against distilled water to remove Triton X-100 and then against *Tris*-SO_4 (0.05 M, pH: 7.4). After the dialysis process, the pH of the solution was adjusted to 8.7 with solid *Tris*[10].

Affinity column: The enzyme was purified with a sepharose-4B-L-tyrosine-sulphanilamide affinity column. The column was balanced with a solution of 25 mM *Tris*-HCl/0.1 M Na_2SO_4, pH: 8.7. The homogenate was applied to the column. The column was then washed with 400 mL of a solution of 25 mM *Tris*-HCl/22 mM Na_2SO_4, pH: 8.7, resulting in a significant amount of adsorption of carbonic anhydrase on affinity gel. Using a buffer, we carried out the elution of carbonic anhydrase from the column (0.1 CH_3COONa/0.5 M $NaClO_4$, 0.01 mM EDTA, pH: 5.6). The elution was stopped at the point where no further absorbance was obtained at 280 nm. The column was then rebalanced[11].

Determination of protein content and carbonic anhydrase activity: To determine the specific activity and purification rate after all subsequent steps, the protein concentration was determined according to the method given by Bradford using bovine serum albumin as a standard[12]. The absorbance 280 nm was used to monitor protein in the column effluents. Carbonic anhydrase activity was assayed by two

different method of Wilbur and Anderson[13] and the hydrolysis of *p*-nitrophenyl acetate as described by Verpoorte *et al.*[14]. CO_2-hydratase activity as enzyme unit (EU) was calculated by the equation $\left(\frac{t_o - t_c}{t_c}\right)$ where t_o and t_c are the times for pH change of the non-enzymatic (buffer) and the enzymatic reaction, respectively. Specific activity for carbonic anhydrase was calculated by using homogenates and purified enzyme solution.

SDS Poliacrylamide gel electrophoresis: SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme. It was carried out in 10 and 3 % acrylamide concentrations for the running and the stacking gel, respectively, containing 0.1 % SDS according to Laemmli[15]. 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. The electrophoretic pattern was monitored.

Kinetic studies: While, optimum pH, optimum temperature, K_m, V_{max} and K_i values were found for *p*-nitrophenyl acetate substrate, I_{50} value determined by CO_2 hydratase activity. Inhibitors such as sulfanilamide, KSCN, NaN_3 were selected for screening of carbonic anhydrase activity. To obtain K_i value at pH 7.4, the enzyme activity was measured for 7 different substrate concentrations at 20 ºC by measuring absorbance at 348 nm. In the media with or without inhibitor the substrate concentration were 3.3, 2.0, 1.43, 1.11, 0.9, 0.77, 0.67 mM. Inhibitor concentrations in the reaction medium are given in the Table-2. K_i values were calculated from Lineweaver-Burk graphs and average K_i values were calculate for each inhibitors.

To determine the I_{50} values of inhibitors the enzyme activity was measured by using hydratase activity at 20 ºC. Per cent activity values were obtained from six different inhibitor concentrations. Carbonic anhydrase activity without inhibitor was taken as 100 %. The inhibitor concentrations causing 50 % inhibition (I_{50}) by the inhibitors were calculated from the activity-inhibitor concentration graphs.

Molecular weight determination with gel filtration: The molecular weight the purified carbonic anhydrase from bovine bone marrow was determined bu using Sephadex G-150. A mixture of standard proteins, each of which having a concentration of 0.2 mg/mL, was applied on the column. Purified carbonic anhydrases were added to the equilibrated column and eluted with 0.05 M Na_3PO_4/1 M dithioerythritol[16].

RESULTS AND DISCUSSION

Carbonic anhydrase is well known as a pH regulating enzyme in most tissues, including humans, 14 different carbonic anhydrase isoenzyme or carbonic anhydrase-related proteins were described with very different subcellulare localization and tissue distribution[17]. Carbonic anhydrase inhibition with sulphanilamide was determined by Mann[1,18,19]. Many chemicals at relatively low dosage affect the metabolism of biota by altering normal enzyme activity, particularly inhibition of a specific enzyme[20].

Indeed, carbonic anhydrase isoenzymes are important enzymes for body metabolism because they regulate pH in most tissue. Carbonic anhydrase isoenzymes were purified different tissue such as erythrocytes, kidney membrane, salivary, human lung[6,21-25].

There is no study about purification of bone marrow carbonic anhydrase. The purpose of this study was to determine the presence of carbonic anhydrase in the bovine bone marrow.

Carbonic anhydrase from bovine bone marrow was purified by sepharose 4B-L-tyrosine-sulfanilamide affinity chromatography. It was detected that the bone marrow bone carbonic anhydrase had a high hydratase activity. As shown in Table-1, specific activity for bovine bone marrow carbonic anhydrase was calculated for crude extract and purified enzyme solution, yielding a purification of 150.82-fold. Kinetics parameters such as optimum pH, optimum temparature, K_m and V_{max} were calculated from graphs for *p*-nitrophenyl acetate substrate on bovine bone marrow carbonic anhydrase.

TABLE-1
CARBONIC ANHYDRASE FROM BOVINE BONE MARROW

Steps	Volume (mL)	Activity (EU/mL)	Total activity		Protein (µg/mL)	Specific activity (EU/mg)	Purification (fold)
			EU	%			
Homogenate	500	1.048	524	100.00	20.150	0.0520	–
Enzyme	55	4.00	220	41.98	0.510	7.8430	150.82

As shown Fig. 1, optimum pH value of carbonic anhydrase from bovine bone marrow was 7. Carbonic anhydrase with activity in the pH range 3-10 is different from bovine erythrocyte plasma membrane[24], which has an optimum pH of 7.5. This data in harmony with other carbonic anhydrase's pH. But it was shown that enzyme was resistance against pH variations (Fig. 1). The optimum temperature for bovine bone marrow carbonic anhydrase was 35 ºC (Fig. 2). Other mammalian carbonic

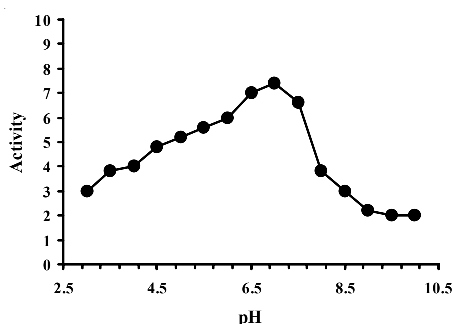

Fig. 1. Effect of pH on activity of carbonic anhydrase from bovine bone marrow in *Tris*-acetate buffer of pH: 3-10

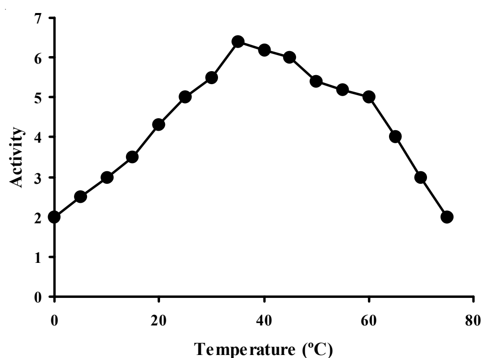

Fig. 2. Effect of temperature on purified carbonic anhydrase from bovine bone marrow

anhydrase optimum temperatures are *ca.* 37 ºC. Enzyme had activity in a wide temperature interval and this result was very interesting[24]. K_m and V_{max} values at optimum pH were determined at 20 ºC by means of Linewaver-Burk graphics using 1/v-1/s values. V_{max} and K_m values at optimum pH and 20 ºC were 120.418 µmol/L min and 2.409×10^{-3} mM. The molar mass of the carbonic anhydrase from bovine bone marrow, determined by gel filtration chromatography was *ca.* 36.000. This is higher than the molar mass of the erythrocyte carbonic anhydrase, but bovine bone marrow's carbonic anhydrase was harmony with bovine leukocyte and bovine bone's carbonic anhydrase[8,26]. Purified bovine bone marrow carbonic anhydrase was controlled by SDS electrophoresis. Bovine was used as a standard, the molar mass of each subunit of carbonic anhydrase was 36,000.

As shown in Table-2, K_i values for acetazolamide, sulfanilamide, KSCN and NaN_3 were 5.21×10^{-5}, 7.18×10^{-6}, 3.43×10^{-5} and 2.12×10^{-5} mM, respectively. The I_{50} values for acetazolamide, sulfanilamide, KSCN and NaN_3 were 0.824, 1.151, 1.529 and 1.145 mM, respectively.

TABLE-2
K_i VALUES OBTAINED FROM LINEWEAR-BURK GRAPHS FOR BOVINE BONE MARROW CARBONIC ANHYDRASE IN THE PRESENCE OF THREE INHIBITORS AND SIX SUBSTRATE CONCENTRATIONS. I_{50} VALUES OBTAINED FROM REGRESSION ANALYSIS GRAPHS FOR BOVINE BONE MARROW CARBONIC ANHYDRASE IN THE PRESENCE OF THREE INHIBITORS AND 3 mM SUBSTRATE CONCENTRATIONS

Inhibitor	[Solvent] (M)	K_i (mM)	Mean value	Inhibition	I_{50} (mM)
Acetazolamide	1×10^{-2} 1×10^{-4} 1×10^{-6}	4.32×10^{-5} 5.04×10^{-5} 6.27×10^{-5}	5.21×10^{-5}	Uncompetitive	0.824
Sulfanilamide	1×10^{-2} 1×10^{-4} 1×10^{-6}	7.11×10^{-6} 6.87×10^{-6} 7.56×10^{-6}	7.18×10^{-6}	Uncompetitive	1.151
KSCN	1×10^{-2} 1×10^{-4} 1×10^{-6}	3.56×10^{-5} 3.13×10^{-5} 3.60×10^{-5}	3.43×10^{-5}	Uncompetitive	1.529
NaN_3	1×10^{-2} 1×10^{-4} 1×10^{-6}	3.05×10^{-5} 1.89×10^{-5} 1.42×10^{-5}	2.12×10^{-5}	Uncompetitive	1.145

There is no detailed study about purification and kinetic properties of carbonic anhydrases from bovine bone marrow. Because of produced of erythrocyte from bone, tissue of bovine bone marrow's carbonic anhydrase was important. In present studies, it is aimed to investigate the kinetic properties for the reason given above. In this work, therefore, the carbonic anhydrase is purified from bovine bone marrow and then determined its kinetic properties.

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