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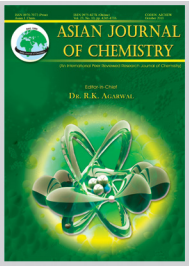

Expression of Cytosolic and Noncytosolic Carbonic Anhydrase Enzymes from Bovine Brain Membrane

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Carbonic anhydrase is an enzyme that takes responsibility in inhalation function but, until today, carbonic anhydrase is not examined if it is present in the bovine brain membrane or not. The enzyme carbonic anhydrase was purified and separately characterized according to the bonding forms in 4 steps such as outer peripheral, cytosolic, inner peripheral and integral. Affinity chromatography was used for purification of the enzyme in all four steps. The affinity column was prepared with sepharose-4B-L-tyrosine-sulphanilamide. Purified carbonic anhydrase was obtained at each step. Enzyme activity was measured by CO_2 hydratase activity and esterase activity methods. Optimum pH and optimum temperature were defined for purified enzymes at each step. Morover molecular weight and purity were detected by gel filtration and SDS-PAGE electrophorose. In addition, the enzyme's Km and ν_{max} values were found with the Lineweaver-Burk method. The obtained results are discussed in comparison with other mammalian carbonic anhydrases. Carbonic anhydrase was shown to be exist in bovine brain membrane and this enzyme was optimized.

Key Words: Brain membrane, Carbonic anhydrase.

INTRODUCTION

Carbonic anhydrase (CA; E.C.4.2.1.1), containing Zn^{2+}, is a ubiquitous metalloenzyme known for catalyzing the reversible hydration/dehydration reactions of carbon dioxide, which are crucial for many homeostatic processes such as acid-base balance and gas transport[1].

The enzyme was first isolated from mammalian erythrocytes[2] and then carbonic anhydrase have been purified and characterized from plants, bacteria[3-9], human most mammalian red blood cells[3], bovine bone[10], *Escherichia coli*[10,11] many tissues and other biological materials[10]. It's localization is largely cytosol and partly cell membrane. The molecular weights differ from cell to cell of the same species and from one organism to another and were reported as 30.000, 36.000, 180.000, 66.000, 54.000 for human erythrocyte, for human erythrocyte membranes, for parsley, for human kidney and for rabbit erythrocytes respectively[3,12,13]. The apparent molar mass was initially reported to be 68.000[6], but more recent purification by this method yielded an inactive polypeptide with a molar mass of 34.400 on SDS-PAGE[6]. So far, eleven isozymes of carbonic anhydrase and carbonic anhydrase-related proteins have been identified in mammals[12-15]. Some are expressed in almost all tissues, while others are tissue or organ specific. Four of them are cytosolic isozymes (I, II, III and VII), four of them are membrane bound (IV, IX, XII and XIV), two of them are present in mitochondria (VA and VB) and one of them is a secretory isozyme (VI)[16].

The first membrane-associated carbonic anhydrase purified to homogeneity was obtained from bovine lung[4]. After several years, a different type of purification of a membrane-bound carbonic anhydrase from human kidney membranes was reported[5,6]. It has also been expressed that membrane-bound carbonic anhydrases is in osteoclasts[1]. Membrane-bound carbonic anhydrase IV isozyme is widely expressed in most species[1] and has been thought to play a role in the regulation of acid-base balance in the kidney. The other membrane-bound carbonic anhydrase IX isozyme, in turn, is expressed in many cancers but also in normal gastrointestinal tract tissues[1].

In brain, carbonic anhydrase has an important role in the neuron-glia metabolic relationships because it regulates anion and acid-base balance of brain cells and extracellular cerebrospinal fluids[4-6].

It has been thought that enzyme is bonded weakly (peripheral) to brain membrane, dissolved to cytoplasma and

could be bonded hardly (integral) to membrane. Consequently, the purification of carbonic anhydrase enzyme from brain membrane in four steps that are outer peripheral, cytosolic, inner peripheral and integral was aimed. There is defining conditions in which these enzymes could be shown maximum activity and determination of molecular weights in this investigation.

EXPERIMENTAL

Homogenate of brain membrane: Brain membrane from bovine brain tissue was kept in physiological serum and then washed with 0.09 % NaCl solvent. This procedure was applied until erythrocyte was completly removed from the medium.

Homogenate for outer peripheral carbonic anhydrase enzyme: Bovine brain membrane was added to 50 mL of 1 M KCl solution and mixed by vortexing. It was then centrifuged by cooling centrifuge in 12.186 × g for 5 min. Precipitate and supernatant were separated. Separated supernatant was washed with 25 mL CCl_4 by this way lipids were extracted. After that pH of homogenate was adjusted to 8,7 with solid *Tris*. By this way homogenate was suited in situation for applying column[11].

Homogenate for cytosolic carbonic anhydrase enzyme: First remaining precipitate from the former step of centrifuge was washed thoroughly with 1 M KCl. It is frozen, it was taken in 30 mL of 0.05 M *Tris*-SO_4 (pH = 7.4) buffer in manner, which will have 2 mL for each gram after solution procedure and it was experienced to ultrasonic sound wave in ultrasonic dismembrator for 4 h. After that it was centrifuged by cooling centrifuge in 12.186 × g for minutes.

Lipids were extracted by washing the supernatant with CCl_4 while precipitate is being separated for inner peripheral proteins and it was prepared for affinity column by bringing pH to 8.7 with solid Tris buffer[11].

Homogenate for inner peripheral carbonic anhydrase enzyme: Remaining precipitate from former step of centrifuge was washed by 0.05 M *Tris*-SO_4 (pH = 7.4) buffer after that it was taken into solvent of 50 mL 1 M KCl and mixed by vortexing. It was centrifuged after mixing in low velocity for 2 h and then supernatant and precipitate was separated each other. Precipitate was kept for the separation of integral proteins. First supernatant was washed with CCl_4 after pH was calibrated to 8.7[11].

Homogenate for integral carbonic anhydrase enzyme: Remaining precipitate from the former step was added in 0.05 M *Tris*-SO_4 (pH = 7.4) which is containing 30 mL 1 % Triton X-100. The sample was experienced to ultrasonic sound wave in ultrasonic dismembrator for 4 h. Then precipitate was thrown away by centrifuging. However supernatant was tried to clean from all detergent excess by making dialysis to pure water for 2 days and than to 0.05 M *Tris*-SO_4 (pH = 7.4) for one day. Later probable lipids were removed by washing 10 mL CCl_4. pH of homogenate was brought to 8.7 by solid *Tris*-SO_4 homogenate was brought in lading statute to column.

Application of homogenates, which is prepared of bovine brain membrane and purifying of carbonic anhydrase enzyme: Affinity gel was prepared on Sepharose-4B matrix. Tyrosine was picked on as covalent after activated sepharose-4B by CNBr. Then sulphanilamide was clamped to tyrosine by diazotization. In this point tyrosine was formed to stretch out of affinity gel however sulphanilamide was formed the part which is bonded to enzyme specificaly. This affinity column has been successfuly used in purifiying carbonic anhydrase enzyme in high rate. Same column has been used respectively in purifiying inner peripheral, cytosolic, outer peripheral and integral proteins. It has been checked that the column is balanced completely before application of homogenate. The carbonic anhydrase has also been purified from bovine erythrocyte by affinity chromatography[9] for using and comparing as protein in electrophore.

Protein determination: After scanning at 280 nm, the tubes with have absorbance were pooled and a quantitative protein determination was done by the Coomassie Brillant Blue G-250 method[9].

Enzyme activity determination: Esterase and hydratase activities were determined the isoenzyme.

CO_2-Hydratase activity determination: Two mL of veronal buffer (pH: 8.2), 0.2 mL of Brome Thymol Blue (0.004 %), 0.8 mL of diluted enzyme and 2 mL of a CO_2 solution (saturated at 0 ºC) 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 formul[17].

1 Wilbur-Anderson Unit = (to-tc)/tc

Determination of esterase activity: The principle of this determination is that the substrate of carbonic anhydrase (*p*-nitrophenylacetate) is hydrolyzed to *p*-nitrophenol plus acetic acid. The reaction is detected at 348 nm[14]. V_{max}, K_M and optimal pH were determined by this method. V_{max} and K_M values were determined from Lineweaver-Burk graph.

SDS-PAGE electrophoresis: The enzymes eluted from affinity column were controlled the purity by SDS gel electrophoresis[15]. Bovine erythrocyte carbonic anhydrase was purified by affinity chromatography and used as standard[18].

Molecular weight determination with gel filtration: For this purpose, Sephadex G-150 was incubated with distilled water at 90 ºC for 5 h and was poured into column (3 cm × 70 cm). The column was balanced for 24 h with the buffer (0.05 M Na_3PO_4, 1 mM dithiothreitol, pH = 7) until no absorbance at 280 nm was obtained. A standard solution of protein was added to the column and the standard graphics were obtained. The concentration of protein solution was 0.2 mg/mL. The standard proteins and carbonic anhydrase-IV were eluted under the same conditions in separate steps. The flow rate through the column was 20 mL/h[15].

RESULTS AND DISCUSSION

In this study carbonic anhydrase enzyme of bovine brain membrane has been prufied separetely as bonding weakly (peripheral) solved in cytoplasma (cytosolic) and bonded to membrane (integral) and it has not been characterized separetely yet. During purifiying of carbonic anhydrase enzyme, the technique of affinity chromatography has been used. Protein contents in eluents have been followed by measuring of absorbances in 280 nm. However determination of

TABLE- 1
CARBONIC ANHYDRASE ENZYMES FROM BOVINE BRAIN MEMBRANE

Outer Peripheral	Volume (mL)	Activity (EU/mL)	Total Activity		Protein (mg/mL)	Specific activity (EU/mg)	Purification (fold)
			EU	%			
Homogenate	65	11,88	772	100	15	$7,9*10^{-1}$	-
Purified Enzyme	30	22,35	671	86	0,28	80	101
Cytozolic							
Homogenate	65	2,6	169	100	43	$6,06*10^{-2}$	-
Purified Enzyme	25	5,8	145	86	0,23	25,35	418
Inner Peripheral							
Homogenate	50	5,9	295	100	18	$3,2*10^{-1}$	-
Purified Enzyme	30	8,3	249	84	0,17	49,1	153,4
Integral							
Homogenate	40	2	80	100	20	0,1	-
Purified Enzyme	20	3,75	75	94	0,044	86,1	861

protein in solutions was defined by Coomassie Blue method. This method has more sensitivity, requires less time and is less reactive.

It was detected that the bovine brain membrane carbonic anhydrase had a high hydratase activity. As shown in Table-1, specific activity for carbonic anhydrase was calculated for crude extract and purified enzyme solution and yielding a purification was determinated 101 fold for outer peripheral, 418 fold for cytozolic,153.4 fold for inner peripheral, 861 fold for Integral.

Optimum pH values and pH intervals with activity have been detected for brain membrane enzymes, which are subtained purely. It seems that outer peripheral, cytosolic and inner peripheral carbonic anhydrase enzymes' optimum pH is 6.5 and pH intervals with activity is 5-9.5. However integral carbonic anhydrase enzyme's optimum pH is 7 and intervals of pH with activity is 5-9 (Fig. 1). Similarly, carbonic anhydrase in bovine muscle, erythrocyte plasma membrane and bone showed activity[10,19].

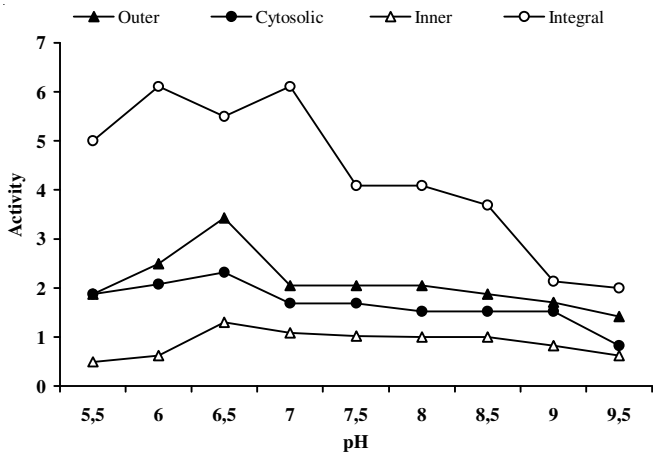

Fig. 1. Effect of pH on activity of carbonic anhydrase from bovine brain membrane

Outer peripheral and integral carbonic anhydrase enzymes of brain membrane have optimal temperature of 37 ºC. However this value was 35 ºC for cytosolic and inner peripheral carbonic anhydrase (Fig. 2). Range of temperature with activity has been detected as 0-70 ºC for these 4 enzymes. Optimum temperature values found the same or nearly to living as body temperature. The optimum temperature for bovine bone marrow and other mammalian carbonic anhydrase are between 37 ºC and 35 ºC[10].

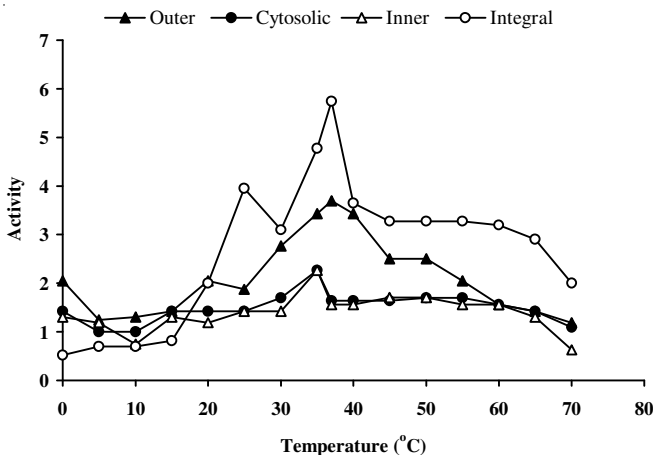

Fig. 2. Effect of temparature on the purified carbonic anhydrase enzymes from bovine brain membrane

SDS-polyacrylamide gel electrophresis has been performed for determining subunits of enzymes that are purified from brain membrane. Carbonic anhydrase enzyme that is purified from bovine erythrocyte has been used for comparison (Fig. 3). As seen in photograph when it has been observed cytosolic, inner peripheral and integral carbonic anhydrase enzymes have been formed from one subunit, outer peripheral enzyme has been formed of 3 subunits. It has been thought that band, which is 29.000 dalton equality has been welded of erythrocyte carbonic anhydrase II but could be taken away for testing, which recured several times. In each time brain membrane has been washed until erythrocyte cells are left completely. In microscopic cells have been looked for in order to be sure that there weren't any erythrocyte. As a result, it has been decided that two bands belonging to carbonic anhydrase enzyme appear similar to purified from bovine erythrocyte membrane[20].

Molecular weights of purified carbonic anhydrase enzymes from brain membrane were detected by using gel filtration chromatography. Purified outer peripheral carbonic anhydrase enzyme of brain membrane was formed of 3 subunits and molecular weights of these subunits have been detected as 27.830, 39.652, 41.023 dalton in similar way. Molecular weights of cytosolic, inner peripheral and integral carbonic anhydrase enzymes have been found 37.412, 36.610 and

34.402 dalton, respectively (Fig. 4). It is observed that present results are similar to molecular weights of the same species and organism [3,12,13,20]. Especially, human kidney membrane carbonic anhydrase has a molar mass of 34.400 like integral carbonic anhydrase enzymes[10].

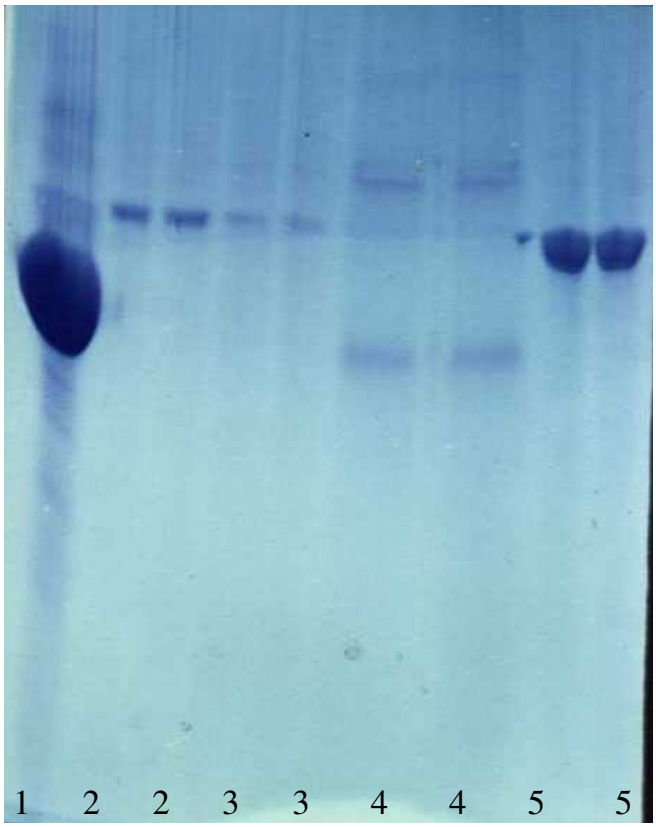

Fig. 3. SDS-PAGE electrophoretic pattern of brain membrane carbonic anhydrase [human erythrocyte CA I (1), carbonic anhydrase of brain membrane (inner peripheral) (2), cytosolic (3), outer peripheral (4) and integral (5)].

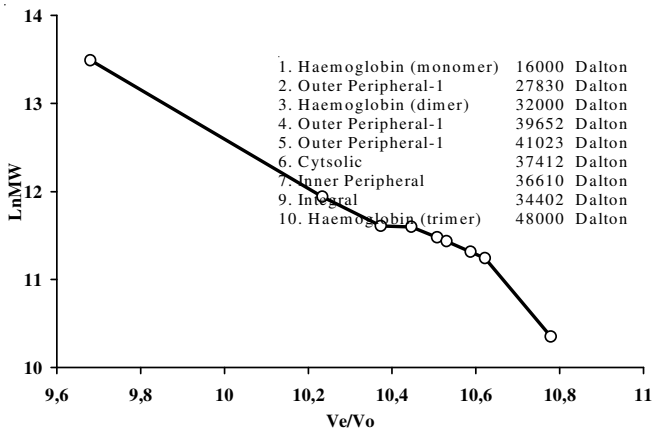

Fig. 4. Gel-filtration analysis of carbonic anhydrases from brain membrane. The chromatography was on a Sephadex G-150 column in 0.05 M sodium phosphate, 1 mM dithiothreitol, pH 7.0 (V_e elution volume, V_o column void volume)

While V_{max} values of outer peripheral, cytosolic, inner peripheral and integral carbonic anhydrase enzymes which are purified from brain membrane, are 2.98×10^{-2}, 7.99×10^{-2}, 4.2×10^{-2} and 2.25×10^{-2} mmol/L min, K_M values are found 0.457, 1.886, 0.618 and 0.00996 mM, respectively. It is observed that values were different from each other.

Membrane-bound isoenzymes have been thought to play an important role in the regulation of acid-base balance, in expression in many cancer types, in buffering the extracellular space. Recent studies have shown that membrane-bound carbonic anhydrases are both able to bind to anion exchangers (AE proteins) forming metabolons and that such an interaction may facilitate anion transport activity[1].

It is understood that the enzyme exists in a lot of bovine tissues and in the brain. It is known that brain has the most important role as physiologic[4,5,6,10]. Its high efficiency catalysis is fundamental to many biological processes, such as photosynthesis, respiration; pH homeostasis and ion transport[21]. However, no previous study has been carried out on purification and characterization from bovine brain membrane. Therefore in this studies, we determined carbonic anhydrase enzyme that is bonded weakly (peripheral), dissolved to cytoplasma and hardly (integral) to brain membrane and its characteristics features in all of the bovine membrane. Defining brain membrane carbonic anhydrase to bond forms seperately was important form increasing the knowledge to meninge especially peripheral and integral carbonic anhydrase is interested in membrane transport, this could be important in solution of about brain disease.

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