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Expression of Cytosolic and Non-Cytosolic Carbonic Anhydrase Enzymes from Bovine Large Intestine

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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 large intestine 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 K_m and V_{max} values were found with the Lineweaver-Burk method. The results are discussed in comparison with other mammalian carbonic anhydrases. Carbonic anhydrase was shown to be exist in bovine large intestine and this enzyme was optimized.

Keywords: Large intestine, 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 anhydraserelated 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]. Immunohistochemically, carbonic anhydrase IV was found to localize in the distal small and the large intestine to the apical (luminal) side of the epithelial plasma membranes. The regional, cellular and subcelluler localizations of carbonic anhydrase IV in the gut epithelium are consistent with proposed physiologic roles for gastrointestinal carbonic anhydrase.

Carbonic anhydrase activity participates in ion and water transport in both the ileum and large intestine [17]. It has been thought that enzyme is bonded weakly (peripheral) to large intestine, dissolved to cytoplasma and could be bonded hardly (integral) to membrane. Consequently, the purification of carbonic anhydrase enzyme from large intestine 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 large intestine: Large intestine 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 large intestine was added to 300 mL of 1 M KCl solution and mixed by vortexing. It was then centrifuged by cooling centrifuge in 12.186 × g for 30 min. Precipitate and supernatant were separated. Separated supernatant was washed with 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 was taken in 250 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 30 min. 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 itwas taken into solvent of 300 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 300 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 1 day. Later probable lipids were removed by washing 100 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 large intestine 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 °C) were mixed. The time (t_c) 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 (t_o). The activity was calculated from the equation [18].

$$\text{Wilbur-Anderson Unit} = (t_o - t_c)/t_c$$

Determination of esterase activity: The principle of this determination is that the substrate of carbonic anhydrase (*p*-nitrophenyl acetate) 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 [19].

Molecular weight determination with gel filtration: For this purpose, Sephadex G-150 was incubated with distilled water at 90 °C 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 anhydrases 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 large intestine was prufied separetely as bonding weakly (peripheral) solved in cytoplasma (cytosolic) and bonded to membrane (integral) since it has not been characterized separetely yet. During purifiying of carbonic anhydrase enzyme, the technique

of affinity chromatography was used. Protein contents in eluents were determined by measuring of absorbances in 280 nm. However, determination of 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 large intestine 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. Purification was determinated 2865 fold for outer peripheral, 1490 fold for cytozolic, 2622 fold for inner peripheral, 2262 fold for integral.

Optimum pH values and pH intervals with activity were detected for large intestine enzymes, which are subtained purely. It seems that outer peripheral carbonic anhydrase enzyme's optimum pH was 6 and pH intervals with activity was 5.5-8. Cytosolic carbonic anhydrase enzyme'soptimum pH was 7.5 and pH intervals with activity was 5.5-8. However, inner peripheral and integral carbonic anhydrase enzymes' optimum pH was 7 and pH intervals with activity was 5.5-8 (Fig. 1). Similarly, carbonic anhydrase in bovine muscle, erythrocyte plasma membrane and bone showed optimum pH and pH interval activities [10,20].

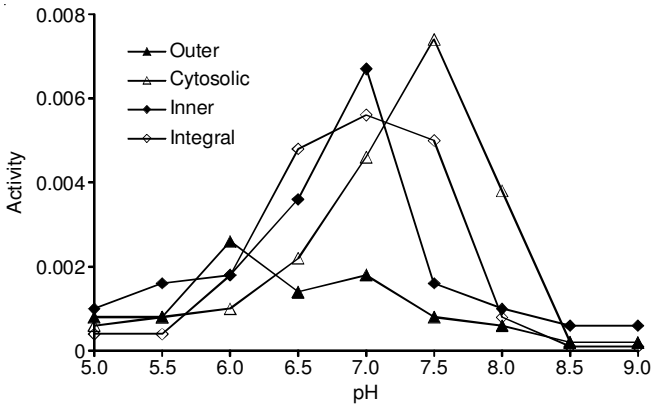

Fig. 1. Effect of pH on activity of carbonic anhydrase from bovine large intestine

Outer peripheral carbonic anhydrase enzyme's of large intestine had optimal temperature of 40 °C and cytosolic carbonic anhydrase enzyme's optimal temperature was 37 °C, However, this value was 35 °C for inner peripheral and 25 °C for integral carbonic anhydrase (Fig. 2). Range of temperature

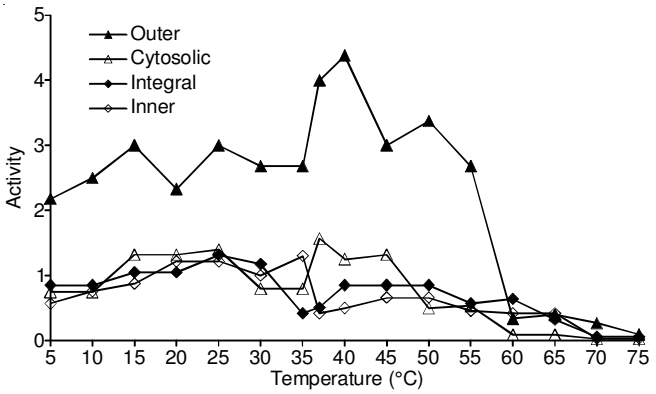

Fig. 2. Effect of temperature on the purified carbonic anhydrase enzymes from bovine large intestine

with activity was detected between 5-65 °C for these 4 enzymes. Optimum temperature values were found the same or close to live body temperature. The optimum temperature for bovine bone marrow and other mammalian carbonic anhydrase are between 37 °C and 35 °C [10].

SDS-polyacrylamide gel electrophresis was performed for determining subunits of enzymes that are purified from large intestine. Carbonic anhydrase enzyme that was purified from bovine erythrocyte for comparison (Fig. 3). Molecular weights of purified carbonic anhydrase enzymes from large intestine were detected by using gel filtration chromatography. Purified outer peripheral, cytosolic, inner peripheral and integral carbonic anhydrase enzymes of large intestine were formed of 2 subunits. Molecular weights of carbonic anhydrase enzymes were found 62.541 and 30.739 dalton for outer peripheral carbonic anhydrase, 53.606 and 30.739 dalton for cytosolic carbonic anhydrase, 60.221 and 28.086 dalton for inner peripheral carbonic anhydrase and 53.606 and 29394 dalton for integral carbonic anhydrase (Fig. 4). It is observed that present results are similar to molecular weights of the same species and organism [3,12,13,21].

To conclude, the present results showed that carbonic anhydrase enzymes obtained from bovine large intestine were formed from monomer and dimer. While V_{max} values of outer peripheral, cytosolic, inner peripheral and integral carbonic anhydrase enzymes which were purified from large intestine, were 1.06×10^{-2}, 4.49×10^{-3}, 5.45×10^{-3} and 9.27×10^{-3} µmol/L min. K_M values were found 1.901, 0.51, 0.55 and 1.866 mM,

TABLE-1
CARBONIC ANHYDRASE ENZYMES FROM BOVINE LARGE INTESTINE

	Volume (mL)	Activity (EU/mL)	Total activity EU	Total activity %	Protein (mg/mL)	Specific activity (EU/mg)	Purification (fold)
Outer peripheral							
Homogenate	300	9,4	2820	100	102	0.092	–
Purified enzyme	30	33,75	1012.5	35.9	0.128	263.6	2865
Cytosolic							
Homogenate	250	6	1500	100	93.7	0.064	–
Purified enzyme	25	11.3	282.5	18.8	0.1185	95.35	1490
Inner Peripheral							
Homogenate	300	3,6	1080	100	87.6	0.041	–
Purified enzyme	25	10	250	23.1	0,093	107.5	2622
Integral							
Homogenate	300	3.9	1170	100	81.7	0.0477	–
Purified enzyme	30	9.5	285	24.3	0.088	107.9	2262

Fig. 3. SDS-PAGE electrophoretic pattern of large intestine carbonic anhydrase [BCA (1), carbonic anhydrase of large intestine (outer peripheral (2), cytosolic (3), inner peripheral (4) and integral (5), BCA (6)]

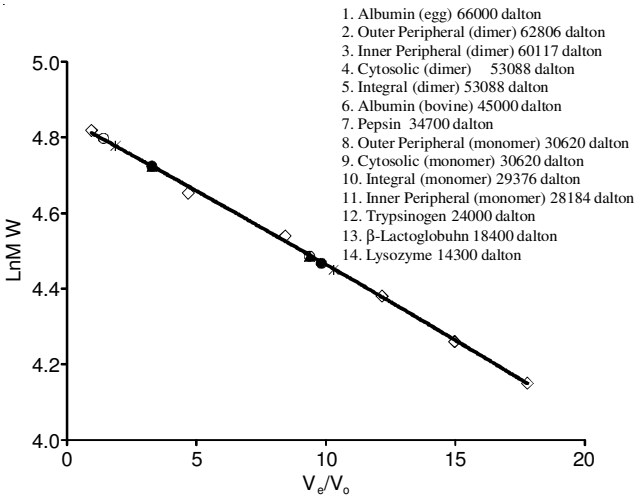

Fig. 4. Gel-filtration analysis of carbonic anhydrases from large intestine. The chromatography was on a Sephadex G-150 column in 0.05 M sodium phosphate, 1 Mm dithiothreitol, pH 7.0 (V_e = elution volume, V_o = column void volume)

respectively. It is observed that these 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 has been understood that the enzyme exists in a number of bovine tissues. Its high efficiency catalysis is fundamental to many biological processes, such as photosynthesis, respiration, pH homeostasis and ion transport [17]. However, there has been no study carried out on purification and characterization from bovine large intestine.

Defining large intestine carbonic anhydrase to bond forms seperately would be important for increasing the knowledge on meninge, especially with respect to peripheral and integral carbonic anhydrase which are related to membrane transport, being important in medicating gastrointestinal diseases.

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