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RESEARCH PAPER

Identification of Cytosolic and Noncytosolic Carbonic Anhydrases in Brain

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Abstract
Carbonic anhydrase is an enzyme found in many mammalian tissues, but it has not previously been determined whether it is present in the bovine brain. In this work, carbonic anhydrase was purified and characterized according to localizations: outer peripheral, cytosolic, inner peripheral and integral in four steps. Affinity chromatography was used for purification of the enzyme from the four different cell regions. The affinity column was prepared with Sepharose-4B-L-tyrosine-sulfanilamide. Purified enzymes obtained at each step activity were determined by hydratase activity and esterase activity methods. Optimum pH and optimum temperature values were defined for the purified enzymes. The behavior of carbonic anhydrase with specific inhibitors, sulfanilamide, KSCN and NaN_3, was investigated. Molecular weights of enzymes were determined by gel filtration, and its purity controlled by SDS-PAGE electrophoresis. In addition, the enzyme's K_M and V_{max} values were determined with the Lineweaver–Burk method. The results obtained are discussed in comparison with other mammalian carbonic anhydrases.

Keywords Brain · Carbonic anhydrase · Sulfanilamide · KSCN · NaN_3

1 Introduction

Carbonic anhydrase (CA: carbonate hydrolase, E.C.4.2.1.1) is an enzyme that assists rapid inter-conversion of carbon dioxide and water into carbonic acid, protons and bicarbonate ions. This enzyme is found in excess quantity in erythrocytes, and it is thought that CA plays a role in transferring of CO_2, H^+, HCO_3^- and Cl^- ions.

This enzyme was first isolated from mammalian erythrocytes (Maren 1967) and from many tissues and other biological materials. Its localization is largely in the cytosol and partly in the cell membrane. Molecular weights differ from cell to cell in the same species and from one organism to another and have been reported to be 30.000, 36.000, 180.000, 66.000 and 54.000 for human erythrocytes, human erythrocyte membranes, parsley, human kidney and rabbit erythrocytes, respectively (Dodgson 1991; Fujikawa et al. 1999; Tobin 1970). In the brain, carbonic anhydrase plays an important role in neuron–glia metabolic relationships because it regulates the anion and acid–base balance of brain cells and extracellular cerebrospinal fluids (Coulson and Herbert 1984; Guillaume et al. 1991; Maren 1984).

It is thought that this enzyme is bound weakly (peripheral) to the brain, is dissolved in the cytoplasm and is bonded with strong (integral) to the membrane. Therefore, the purpose of this study was to purify CA from the brain in four steps, namely outer peripheral, cytosolic, inner peripheral and integral. Also, it was aimed to define the conditions under which the enzymes exhibit maximum activity, determine molecular weights and V_{max} and K_M values and investigate some chemicals affecting carbonic anhydrase enzyme activity.

2 Materials and Methods

2.1 Preparing Brain Homogenate

Brain tissue separated from the bovine brain membrane was kept in physiological saline and then washed with

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0.9% NaCl until erythrocytes were completely removed from the medium.

2.2 Obtaining Homogenate for Outer Peripheral CA

Bovine brain (150 g) was added to 200 mL of 1 M KCl solution and mixed by vortexing. Then it was centrifuged in a cooling centrifuge at 12.000×g for 30 min. The precipitate and supernatant were separated. The supernatant was washed with CCl_4, and in this way, lipids were extracted. Then, the pH of the homogenate was adjusted to 8.7 with solid Tris. Thus, the homogenate was suitable for applying to the column (Demir et al. 1996).

2.3 Obtaining Homogenate for Cytosolic CA

In the first step, the remaining precipitate from the previous step of centrifugation was washed with 1 M KCl. It was frozen and put in 250 mL 0.05 M Tris-SO_4 (pH 7.4) buffer so as to give 2 mL for each gram after the solution procedure. Then, it was exposed to an ultrasonic wave in an ultrasonic dismembrator for 4 h. After that, it was centrifuged in a cooling centrifuge at 12.000×g for 30 min. Lipids were extracted by washing the supernatant with CCl_4, while the precipitate was separated for inner peripheral proteins and was prepared for the affinity column by adjusting the pH to 8.7 with solid Tris buffer (Demir et al. 1996).

2.4 Obtaining Homogenate for Inner Peripheral CA

The remaining precipitate from the previous step of centrifugation was washed with 0.05 M Tris-SO_4 (pH 7.4) buffer. It was then transferred to 200 mL 1 M KCl and mixed by vortexing. It was centrifuged after mixing at low velocity for 2 h, and then the supernatant and precipitate were separated. The precipitate was kept for the separation of integral proteins. The supernatant was washed with CCl_4, after the pH was calibrated to 8.7 (Demir et al. 1996).

2.5 Obtaining Homogenate for Integral CA Enzyme

The remaining precipitate from the previous step was added to 0.05 M Tris-SO_4 (pH 7.4) containing 200 mL 1% Triton X-100. The sample was exposed to an ultrasonic wave in an ultrasonic dismembrator for 4 h. Then, the precipitate was removed by centrifuging. We tried to clean the supernatant of all excess detergent by performing dialysis against pure water for 2 days and then to 0.05 M Tris-SO_4 (pH 7.4) for 1 day. Later on, probable lipids were removed by washing with CCl_4. The pH of the homogenate was adjusted to 8.7 with solid Tris, and thus, it was suitable for loading onto the column.

2.6 Application of Homogenates Prepared from Brain and Purifying CA

The affinity gel was prepared on a Sepharose-4B matrix. Tyrosine was reacted with Sepharose-4B activated by CNBr. Then sulfanilamide was coupled to tyrosine by diazotization. Tyrosine functioned as a spacer arm; sulfanilamide was the part, which is bound to enzyme specifically. This affinity column has been used successfully in purifying CA at a high rate (Arslan et al. 1996).

The same column was used in purifying inner peripheral, cytosolic, outer peripheral and integral proteins. It was ensured that the column was equilibrated completely before the homogenate was applied.

CA has also been purified from human erythrocyte by affinity chromatography (Arslan et al. 1996) to be used as an electrophoretic standard.

2.7 Protein Determination

After scanning at 280 nm, the tubes with absorbance were pooled and quantitative protein was determined by the Coomassie Brilliant Blue G-250 method (Bradford 1976).

2.8 Enzyme Activity Determination

Esterase and hydratase activities were determined in the isoenzymes.

2.8.1 CO_2-Hydratase Activity Determination

Two milliliters of veronal buffer (pH 8.2, 0.025 M), 0.2 mL of bromothymol 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 the addition of CO_2 solution and the appearance of a yellow–green color. The same interval was recorded without enzyme solution (t_o). The activity was calculated from the formula (Rickli et al. 1964):

$$\text{Wilbur-Anderson unit} = (t_o - t_c)/t_c \quad (1)$$

2.8.2 Esterase Activity Determination

The principle of this determination is that the substrate of CA (p-nitrophenyl acetate) is hydrolyzed to p-nitrophenol and acetic acid. The reaction is detected at 348 nm (Verpoorte et al. 1967). V_{max} and K_M were determined by this

Table 1 Carbonic anhydrase enzymes from bovine brain

	Volume (mL)	Activity (EU/mL)	Total activity		Protein (mg/mL)	Specific activity (EU/mg)	Purification (fold)
			EU	%			
Outer peripheral							
Homogenate	160	8,8	1408	100	106	$8,3 \times 10^{-2}$	–
Purified enzyme	40	13,5	540	38,3	0,2112	63,9	770
Cytozolic							
Homogenate	155	6,8	1054	100	120	$5,6 \times 10^{-2}$	–
Purified enzyme	40	19,9	763,6	72,4	0,1625	117,5	2098,2
Inner peripheral							
Homogenate	145	3,03	439,35	100	137,5	$2,2 \times 10^{-2}$	–
Purified enzyme	50	8,3	415	94,45	0,257	32,29	467,7
Integral							
Homogenate	65	9	585	100	129	$6,9 \times 10^{-2}$	–
Purified enzyme	40	13,07	522,8	89,3	0,3783	34,55	500,7

method. V_{max} and K_M values were determined from the Lineweaver–Burk graph.

2.9 Enzyme Activity Determination in Inhibitor

For this purpose, sulfanilamide, NaN_3 and KSCN were used as inhibitors. The Rickli method was used for the determination of the hydrolysis activity of the enzyme (Rickli et al. 1964).

2.10 SDS-PAGE Electrophoresis

The purity of the enzymes eluted from the affinity column was determined by SDS gel electrophoresis (Laemmli 1970). Human erythrocyte carbonic anhydrase was purified by affinity chromatography and used as standard (Demir et al. 1993).

2.11 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 the column (3 cm × 70 cm). The column was balanced for 24 h with buffer (0.05 M Na_3PO_4, 1 mM dithiothreitol, pH 7) until no absorbance at 280 nm was obtained. A protein standard solution was added to the column, and the standard graphics were obtained. The concentration of the protein solution was 0.2 mg/mL. The standard proteins and CAs were eluted under the same conditions in separate steps. The flow rate through the column was 20 mL/h (Whitaker 1963).

3 Results and Discussion

In this study, CA of bovine brain was purified separately as bonding weakly (peripheral) solved in cytoplasma (cytosolic) and bonded to the membrane (integral). It has not been characterized separately yet. During the purification of CA, the technique of affinity chromatography was used. Protein contents in eluents were determined by measuring absorbances at 280 nm. However, determination of protein in solutions was defined by the Coomassie brilliant blue method. This method has more sensitivity, requires less time and is less reactive.

It was detected that the bovine brain 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 determined 770-fold for outer peripheral, 2098-fold for cytozolic, 467-fold for inner peripheral and 500-fold for integral.

Optimum pH values and pH intervals with activity were detected for brain enzymes, which are sustained purely. It seems that outer peripheral and cytosolic CA enzymes' optimum pH was 6 and the pH interval with activity was 5–8.5. Inner peripheral CA enzyme's optimum pH was 7–7.5 and the pH interval with activity was 5–9. However, integral CA enzyme's optimum pH was 6.5 and the pH interval with activity was 5–8 (Fig. 1). Similarly, carbonic anhydrase in bovine muscle, erythrocyte plasma membrane, brain membrane and bone showed optimum pH and pH interval activities (Demir et al. 2000, 2012; Tasgın et al. 2009).

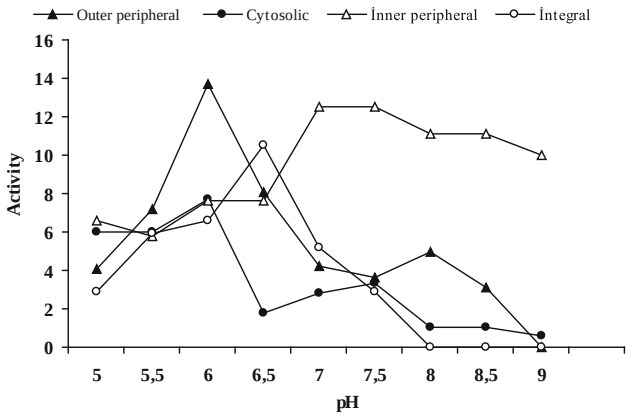

Fig. 1 Effect of pH on activity of carbonic anhydrase from bovine brain

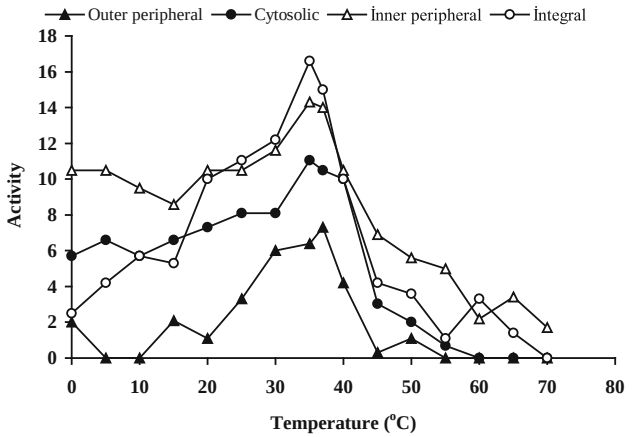

Fig. 2 Effect of temperature on the purified carbonic anhydrase enzymes from bovine brain

Outer peripheral CA enzymes of brain had optimal temperature of 37 °C; however, this value was 35 °C for cytosolic, inner peripheral and integral CA (Fig. 2). Range of temperature with activity was detected between 0 and 70 °C for inner peripheral, cytosolic and integral CA enzymes. However, the temperature range of outer peripheral CA was detected between 15 and 55 °C. Optimum temperature values were found to be same as or close to live body temperature.

SDS-polyacrylamide gel electrophoresis was performed to determine subunits of enzymes purified from the brain. Carbonic anhydrase enzyme was purified from human erythrocyte for comparison (Fig. 3). As seen in the photograph, outer peripheral, cytosolic, inner peripheral, integral CA enzymes were formed from one subunit.

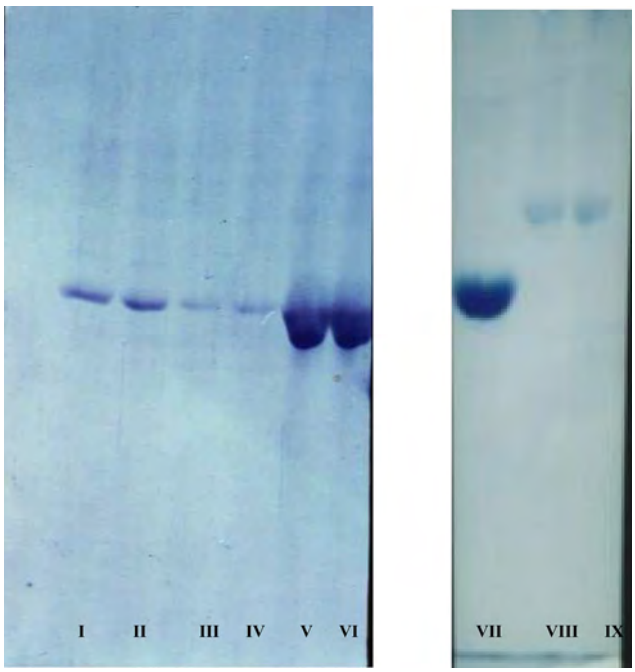

Fig. 3 SDS-PAGE electrophoretic pattern of brain carbonic anhydrase [carbonic anhydrase of brain cytosolic (I, II), inner peripheral (III, IV), integral (V, VI), outer peripheral (VIII, IX) and human erythrocyte CA I (VII)]

Molecular weights of purified CA enzymes from brain were determined by using gel filtration chromatography. Molecular weights of outer peripheral, cytosolic, inner peripheral and integral CA were, respectively, 34,448, 39,387, 38,667 and 34,739 Da (Fig. 4).

The V_{max} values of outer peripheral, cytosolic, inner peripheral and integral carbonic anhydrase enzymes were 3.62×10^{-2}, 2.66×10^{-2}, 3.03×10^{-2} and 5.18×10^{-2} μmol/Lmin., respectively. K_M values were 0.794, 0.361, 0.666 and 0.623 mM, respectively. Values were different from each other.

We examined the effects of sodium azide, sulfanilamide and thiocyanate, in three different concentrations (10^{-2}, 10^{-4} and 10^{-6} M), on CA of outer peripheral, cytosolic, inner peripheral and integral.

Purified outer peripheral CA was inhibited in all three concentrations of NaN_3, and it was detected that the activity of outer peripheral CA was increased by KSCN and sulfanilamide (Figs. 5, 6, 7). In the same way, the activity of cytosolic CA was inhibited by sulfanilamide, NaN_3 and KSCN (Figs. 8, 9, 10). However, it was detected that the activity of brain inner peripheral CA was increased by three matters (Figs. 11, 12, 13). In similar way, the

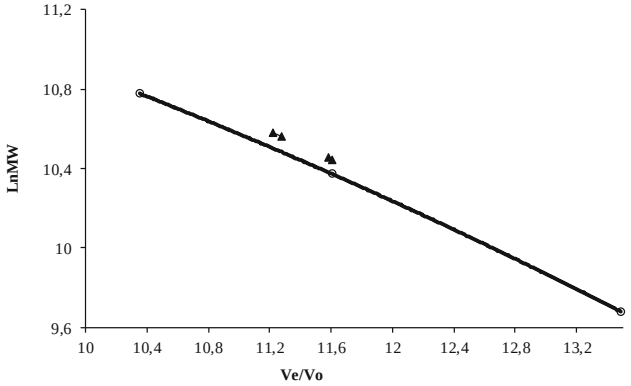

Fig. 4 Gel filtration analysis of carbonic anhydrases from brain. The chromatography was on a Sephadex G-150 column in 0.05 M sodium phosphate, 1 mM dithiothreitol, pH 7.0 (*Ve* elution volume, *Vo* column void volume)

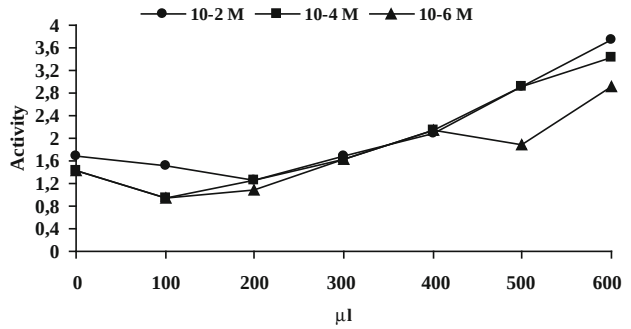

Fig. 5 Effect of sulfanilamide on purified carbonic anhydrase from brain (outer peripheral)

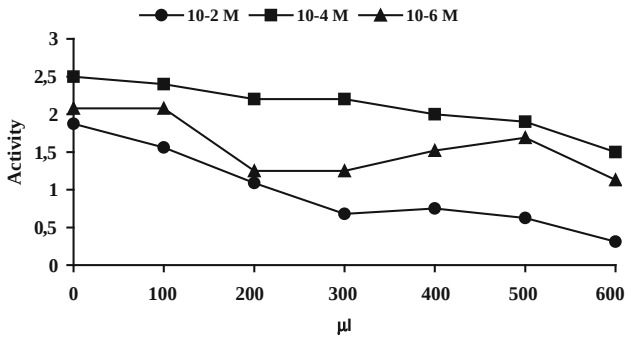

Fig. 6 Effect of NaN_3 on purified carbonic anhydrase from brain (outer peripheral)

activity of integral CA increased in all three concentrations of sulfanilamide, NaN_3 and KSCN (Figs. 14, 15, 16).

In result, defining brain CA, according to bonding forms separately, would be important for increasing the knowledge on meninge, especially with respect to peripheral and

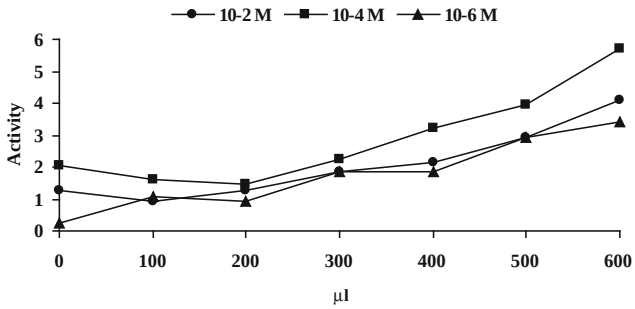

Fig. 7 Effect of KSCN on purified carbonic anhydrase from brain (outer peripheral)

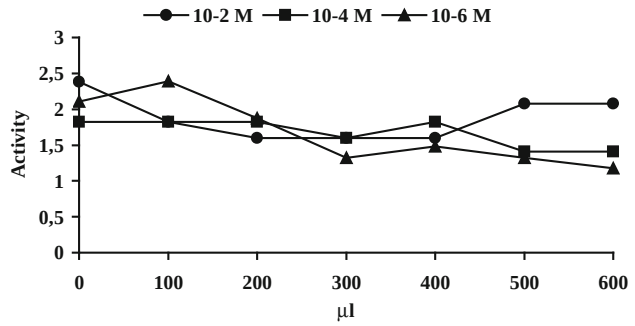

Fig. 8 Effect of sulfanilamide on purified carbonic anhydrase from brain (cytosolic)

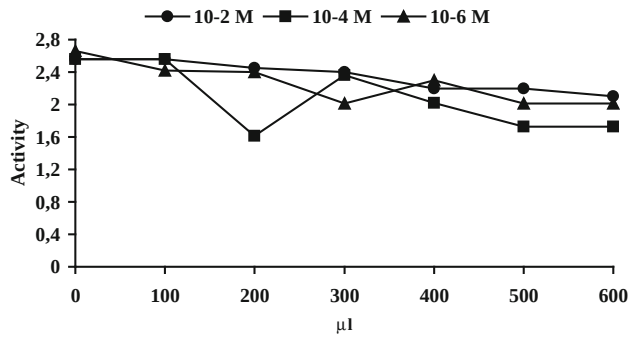

Fig. 9 Effect of NaN_3 on purified carbonic anhydrase from brain (cytosolic)

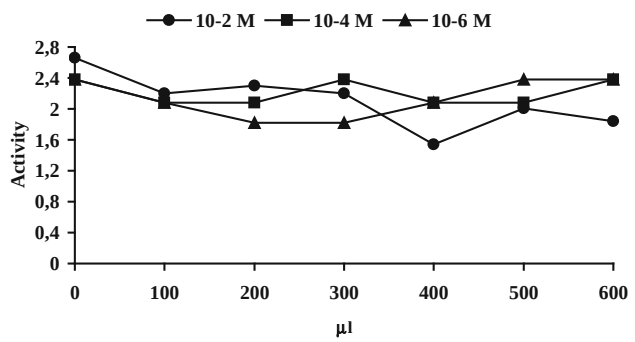

Fig. 10 Effect of KSCN on purified carbonic anhydrase from brain (cytosolic)

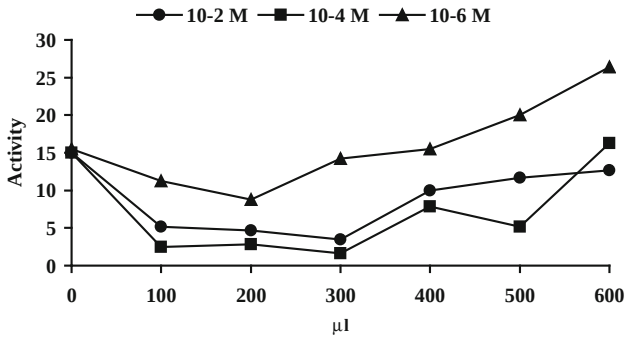

Fig. 11 Effect of sulfanilamide on purified carbonic anhydrase from brain (inner peripheral)

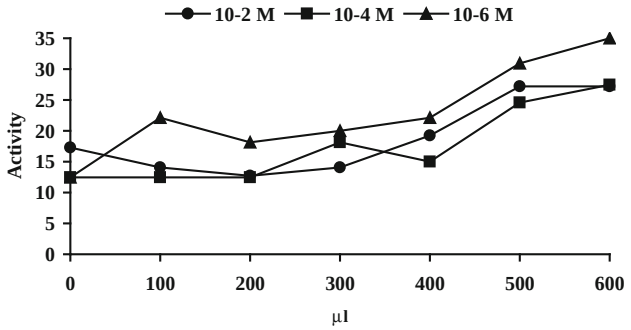

Fig. 12 Effect of NaN_3 on purified carbonic anhydrase from brain (inner peripheral)

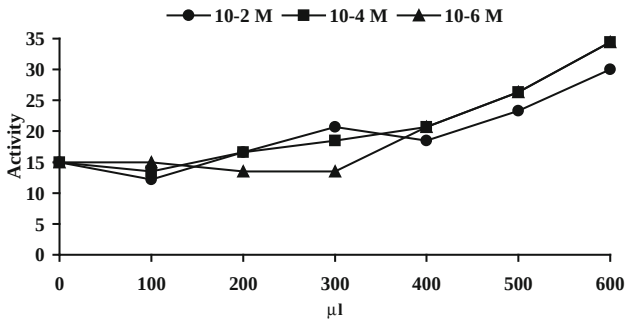

Fig. 13 Effect of KSCN on purified carbonic anhydrase from brain (inner peripheral)

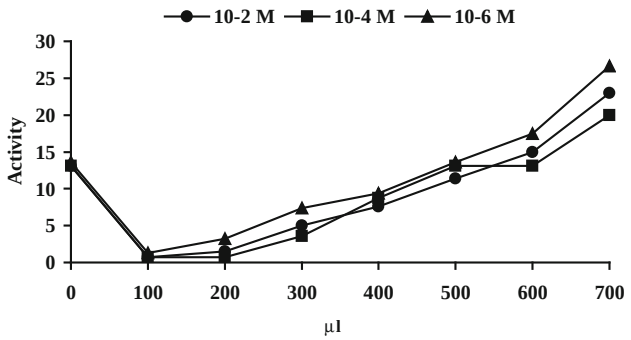

Fig. 14 Effect of sulfanilamide on purified carbonic anhydrase from brain (integral)

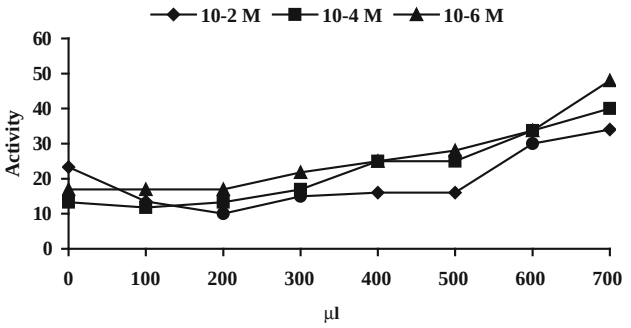

Fig. 15 Effect of NaN_3 on purified carbonic anhydrase from brain (integral)

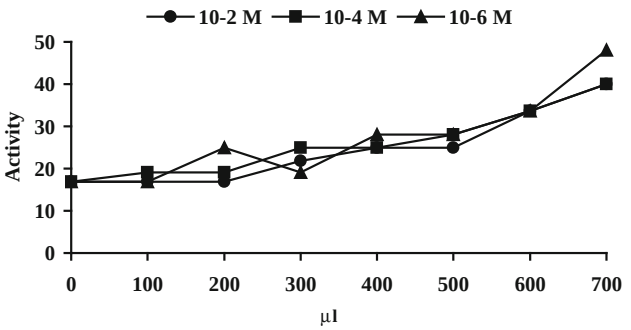

Fig. 16 Effect of KSCN on purified carbonic anhydrase from brain (integral)

integral CA which are related to membrane transport, being important in medicating brain diseases. Studies on this subject are continuing.

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