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Immunohistochemical and Partial Characterization Studies of Carbonic Anhydrase in the Brain of Teleost Fish *Cyprinus carpio*

S. M. Rahim[1,5,*], S. J. Abdurrahman[2], E. M. Taha[3], H. F. Hassan[4], K. D. Simon[5], A. G. Mazlan[5]

[1] Dept. of Biology, Faculty of Education, University of Tikrit, 34001 Tikrit, Salah Al Deen, Iraq.
[2] Dept. of Biology, Faculty of Science, University of Tikrit, 34001 Tikrit, Salah Al Deen, Iraq.
[3] Dept. of Chemistry,Faculty of Science for Women,University of Baghdad, Baghdad, Iraq.
[4] Dept. of Biology, Faculty of Science, University of Kirkuk, Kirkuk, Iraq..
[5] School of Environmental and Natural Resource Sciences, Faculty of Science and Technology,Universiti Kebangsaan Malaysia,43600 UKM Bangi Selangor, D.E., Malaysia

* Corresponding Author: Tel.: +60.123 328443; Fax: +60.123 328443;
E-mail: saleh_mr57@yahoo.com

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Abstract

Carbonic anhydrase (CA) activity is related to a variety of intermingled biological processes, including respiration, ionic transport, acid–base balance, and calcification. Carps *Cyprinus carpio* were investigated with respect to purification, partial characterization, and immunohistochemical localization of brain CA. The enzyme was purified up to 72 fold by affinity chromatography on sulfanilamide sepharose gel. The result is a yield of 59.6% and a specific activity of 1143 EU/mg protein with an apparent molecular weight of 28.6 kDa. The optimum pH and temperature were observed at 8.0 and 35 ºC, respectively. Ions of Na^+, K^+, Mg^{2+}, and Ca^{2+} at concentrations of 1, 5, and 10 mM exhibited up to 25% to 30% inhibition on brain CA activity, whereas Cl^- exhibited the most inhibition on enzyme activity. Acetazolamide also displayed significant inhibition on brain CA activity (54%, 83%, and 92% at respective concentrations of 1, 5, and 10 mM). Antibodies against purified gill CA from trout *Oncorhynchus mykiss* reacted positively only to one band corresponding to CA in carp brain supernatant. Immunohistochemical staining was observed in oligodendrocytes, its processes, and in myelinated fibers. Our findings are similar to those of previous studies on higher vertebrates CA II.

Keywords: Teleost fish, enzyme purification, immunohistochemistry, metal ions, oligodendrocytes.

Introduction

Carbonic anhydrase (CA; EC4.2.1.l.) was first identified by Meldrum and Roughton (1933) in mammalian erythrocytes as a zinc metalloenzyme involved in the reversible hydration of CO_2 to produce H^+ and HCO_3^-. The enzyme is found in many tissues, wherein it plays key roles in a number of physiological and pathological processes, such as pH regulation, ions and gas exchanges, calcification, photosynthesis, tonic modulation of brain excitability through modulation of amino acid receptors, and biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis); prominent pathological effects include the acceleration of plaque deposition in Alzheimer's disease and the exacerbation of excitotoxic neuron injury (Ozensoy *et al*., 2006; Henry, 1996; Maren, 1967). CA has evolved independently at least five times with five genetically distinct unrelated enzyme families (Supuran, 2010). Sixteen distinct isoenzymes of CA are known to exist in mammals (Aspatwar *et al*., 2010; Gilmour, 2010; Hilvo *et al*., 2008; Parkkila and Parkkila, 1996), and most of these isoenzymes have been extensively investigated in terms of their biochemical properties, tissue distribution, and genetic control (Supuran, 2008). These isoenzymes differ in their subcellular localization, catalytic activity, and susceptibility to different classes of inhibitors (Ekinci *et al*., 2011; Aspatwar *et al*., 2010). The presence of these enzymes in many different isoforms and tissue organs represent an important subject in the design of inhibitors for biomedical applications. CA inhibitors are clinically used as drugs against many diseases and as anti-bacterial/anti-fungal treatment (Supuran, 2007).

Genetic databases reveal over 16 isoenzymes of CA reported and/or predicted in fish species (Georgalis *et al*., 2006; Lin *et al*., 2008; Gilmour and Perry, 2009). Rahim *et al*. (1988) first purified two distinct branchial and blood CA isoenzymes in rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*). This finding has been confirmed by the cloning of *O. mykiss* blood and cytosolic CA isoenzymes (Esbaugh *et al*., 2005). Sender *et al*. (1999) found that CA isoenzymes purified from red

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blood cells and flounder (*Platichthys flesus*) gills are identical. Georgalis *et al*. (2006) cloned an additional CA isoenzyme from *O. mykiss* kidney that most closely resembled the mammalian membrane-bound isoenzyme CA IV.

Extensive information on the localization of CA II in vertebrate central nervous system is widely available (Cammer and Tansey, 1987; Delaunoy *et al*., 1986; Kumpulainen and Korhonen, 1978). According to some researchers, CA II is mainly localized in oligodendrocytes and is implicated in the regulation of tissue pH by facilitating CO_2 transport. Purification, characterization, as well as histochemical and immunohistochemical studies on CA have been carried out on various tissue organs, gills (Ekinci *et al*., 2011; Sender *et al*., 1999; Rahim *et al*., 1988), pseudobranch (Rahim *et al*., 2013), liver (Ceyhun *et al*., 2011), nervous tissue (Soyut *et al*., 2008; Parthe, 1981), kidney (Soyut and Beydemir, 2008), ear (Tohse *et al*., 2006), and eye (Linser *et al*., 1985; Beydemir *et al*., 2006) of fish. However, no studies have reported the purification, characterization, and localization of CA in the brain of *C. carpio*. The animal brain is actively involved in the control of the physiological functions of bodily organs, such as the reproductive and respiratory organs. For example, the gills perform numerous vital functions, including gas exchanges, acid–base regulation, osmoregulation, and clearance of waste products from nitrogenous metabolism. However, these functions require control and coordination by a complex web of neural and endocrine pathways that are expressed in functionally analogous system in mammals (Evans *et al*., 2005). A variety of environmental problems have emerged because of the contamination of water and soil by toxic materials and organic pollutants from industrial and agricultural activities. Such conditions may adversely affect the physiology of whole organisms by interfering with the physiological functions of enzymes like CA in fish brain. Fresh water *C. carpio* is economically beneficial because of its meat quality. In addition, *C. carpio* is one of the most easily cultured animals among other fresh water fish in Iraq. For the first time, the brain CA of *C. carpio* is purified and partially characterized in the present work. The localization of the enzyme is also investigated immunochemically.

Materials and Methods

Purification of Brain CA from *C. carpio*

Fresh water carp *Cyprinus carpio* weighing 250 g to 350 g were obtained from Baiji station for river fish (Iraq). The fish were freed of blood by intracardiac perfusion (10 $ml.min^{-1}$) with 40 ml phosphate buffer saline (PBS; 0.92% NaCl, 0.16% $Na_2PO_4.2H_2O$, 0.02% $NaH_2PO_4.H_2O$). Brains were excised, weighed, and homogenized using a Potter-Elvehjem homogenizer (Bioblock Scientific-France) at 4 °C in 10 ml PBS containing 1% Triton X-100 (Sigma) per g of fresh tissue. The homogenate was centrifuged at 5000 xg (Hitachi-Japan) for 30 min at 4 °C. The purification of CA from the supernatant was carried out by affinity chromatography on sulfanilamide (Sigma) coupled to a CH-sepharose 4B gel (Pharmacia) by the intermediate of the dicyclohexylcarbodiimide (Filippi *et al*., 1978). Gel affinity preparation was performed in accordance with the description of Marriq *et al*. (1974). The gel column (0.9 cm x 15 cm) was equilibrated and washed with 0.1 M Tris-SO4 (pH 7.5). The elution was realized by 0.1 M Tris-SO_4 (0.2 M NaI, pH 7.5) and 0.1 M Tris-SO_4 (0.2 M KCNO, pH 6.6). The purification was carried out at room temperature (Rahim *et al*., 1988).

CA Activity

CA activity was assayed following the CO_2 hydration method described by Wilbur and Anderson (1948). CO_2 hydratase activity as an enzyme unit (EU) was calculated as $EU= t_0 - t_c/t_c$, where t_0 and t_c are the times for pH change of the non-enzymatic and enzymatic reactions, respectively. CO_2 hydration activity was used in the routine monitoring of the various stages of the preparation of fish brain CA and in the study of the effect of various physical and chemical factors on CA activity.

Protein Concentration

Protein concentration was determined by the Lowry technique (Lowry, 1951) using a standard curve prepared with bovine serum albumin.

Determination of Molecular Weight

The molecular weight (MW) of the enzyme was determined according to Andrews's method (Andrews, 1962). Sephadex G-100 gel filtration column (34 cm x 1.6 cm) was used to establish the void volume. Blue dextran (2000 kDa) was passed through the column; urease (480 kDa), lactate dehydrogenase (140 kDa), bovine serum albumin (67 kDa), ovalbumine (43 kDa), and cytochrome C (12 kDa) were used as standard proteins. The column was pre-equilibrated with 50 mM Tris-HCI buffer (pH 7.5) and eluted with the same buffer.

Optimum Temperature Determination

To determine the optimum temperature, enzyme activity was assayed at temperatures ranging from 5 °C to 50 °C. The desired temperature was obtained using a water bath.

Optimum pH Determination

To determine the optimum pH, a range of pH

buffers, namely, 1 M Tris-SO_4 (pH 7.0 to 9.5) and 1 M phosphate (pH 5.0 to 7.5), was used.

In vitro Effect of Ions and Acetazolamide on Brain CA Activity of Carp

To determine the effect of ions and acetazolamide inhibitors on the brain CA activity of carp, different concentrations of these ions and inhibitors were added to the reaction medium. The enzymatic activity was measured, and an experiment in the absence of these ions and inhibitors was used as control (100% activity).

Purification of Gill CA and Production of Antibodies

Fresh water rainbow trout *Oncorhynchus mykiss* (200 g to 250 g) from a fish farm were anesthetized with MS 222 (Sandoz, Switzerland). The gills were freed of blood by intracardiac perfusion (10 $ml.min^{-1}$) with 50 ml PBS. Pure antigens (CA) were purified by affinity chromatography on sulfanilamide coupled to a CH-sepharose 4B gel (Filippi *et al*., 1978) and injected into rabbits to obtain specific antibodies. The method was in accordance with the technique that has been used by our research group to purify CA enzyme from different sources of vertebrate tissues and to produce antibodies (Rahim, 1988).

Polyacrylamide Gel Electrophoresis

One-dimensional polyacrylamide (10%) gel electrophoresis (Merck Germany) was performed under denaturing condition (sodium dodecyl sulfate, SDS) (Laemmli, 1970) to test the purity of the antigens. The following markers were used: phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), and bovine CA (30 kDa). Gels were stained with coomassie brilliant blue R250.

Immunoblotting Experiment

This technique was used to determine the monospecificity of the antibodies against gill and brain homogenates (Towbin *et al*., 1979). The gills and CNS homogenates were transferred from (10%) gel electrophoresis to nitrocellulose membrane. They were then incubated with gill CA antibodies (1/100 dilution) and then with sheep anti-rabbit IgG (1/500 dilution) peroxidase conjugate (Biosys-France) for 2 h. Nitrocellulose membranes were washed thrice for 10 min in PBS. Antigen-antibody complexes were detected with 4-chloro-1-napthol. The reaction was carried out in a dark place at laboratory temperature for 15 min (Rahim, 1988).

Immunohistochemical Technique

Fresh water *C. carpio* (250 g to 350 g) were perfused by intracardiac perfusion with 15 ml of 2% paraformaldehyde in PBS. Then, the brains were excised and immersed immediately in a cold mixture of 4% paraformaldehyde + 0.2% picric acid + 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h. All treatments were carried out at 4 °C. The dehydration of brain samples as well as the paraffin embedding and deparaffination of sections were performed according to Kumpulainen (1981). Thick paraffin sections (7 µm) were cut and placed on a slide for indirect immunoperoxidase staining. The inhibition of endogenous activity was performed by incubating the sections with 3% H_2O_2. The sections were then incubated with gill CA antibodies (1/200 dilution) for 1 h. After washing thrice for 10 min in PBS, the sections were treated with sheep anti-rabbit serum IgG-peroxidase conjugate (1/150 dilution) for 1 h and washed again thrice for 10 min in PBS. A 10 min incubation with 3,3' diaminobenzidine for immunoperoxidase detection followed. Control sections were treated as above except that of non-immune rabbit serum replaced gill CA antibodies (Rahim, 1988).

Results and Discussion

The physiological function of CA is to facilitate the interconversion of CO_2 and HCO_3^-. Therefore, CA plays key roles in diverse vital processes, such as physiological pH regulation, gas exchanges, calcification, and photosynthesis. In addition, CA plays an important role in ion transport and in pH regulation in the eye, kidney, and the central nervous system (Ceyhun *et al*., 2011; Gilmour, 2012; Randall *et al*., 2000; Heisler, 1984).

In this study, brain CA enzyme from carp *Cyprinus carpio* was purified and partially characterized for the first time. The purification procedure was carried out by affinity chromatography on sulfanilamide coupled to a CH-sepharose 4B gel. The enzyme was purified up to 72 fold with a recovery ratio of 59.6% compared to the homogenate and specific activity was approximately 1143 EU/mg protein (Table 1). At the end of this step, a single band of purified enzyme was obtained through SDS-gel electrophoresis (Figure 1d), thereby confirming the purity of the antigen. One-step affinity chromatography was employed on sulfanilamide coupled to a CH-sepharose gel, which strongly binds with CA. These two elutions were used to determine the probable existence of different isoenzymes. The active CA fractions were eluted as a single peak with CNO^- inhibitor only. A small protein peak with no CA activity was absorbed onto the column and was eluted with the I^- inhibitor. The procedure used in this study, which has been applied in experiments on mammalian and fish gill erythrocytes CA (Rahim *et al*., 1988; Delanuoy, 1983), may be suitable for further research, particularly because it requires only a short experimental period. The result is similar to

the CA obtained from various tissues (brain, liver, gill, and kidney) of rainbow trout *Oncorhynchus mykiss* using one-step affinity chromatography (Ekinci and Beydemir, 2010; Soyut *et al*., 2008). The purified enzyme exhibited the highest activity at 35 °C and pH 8.0. Gel filtration was carried out to determine the molecular weight of CA. This process involved the calculation of k_{av} values for the enzyme and marker proteins as well as the generation of a k_{av} - Log MW graph. The MW was 28.6 kDa, which is almost similar to that obtained for the enzyme from different sources. For example, *O. mykiss* CA in gill, erythrocytes and the brain are 30.0 kDa and 29.0 kDa (Rahim *et al*., 1988; Hall and Schraer, 1983; Soyut *et al*., 2008), respectively; CA isoenzymes in human erythrocytes are 29.0 kDa (CA I, CA II) (Rickli *et al*., 1964), and those in bovine erythrocytes are 29.0 kDa (Bayram *et al*., 2008).

The effect of metal ions of different concentrations on brain CA activity was studied. Generally, metals affecting the different environments of organisms are highly important, especially in light of recent industrial developments that cause harmful residues that are difficult to control. Fish in the aquatic environment serves as an important food source for humans. Hence, fish exposure to toxic metals is equated with human exposure, the harmful effects of which may be passed on to the next generations. Metals affect the metabolism of organisms through the inhibition of enzyme activity

Table 1. Summary of purification steps for carp brain carbonic anhydrase enzyme by affinity chromatography on sulfanilamide sepharose gel

Purification steps	Total volume (ml)	Total protein (mg)	Protein (mg/ml)	Activity (EU/ml)	Total activity (EU)	Specific activity (EU/mg)	Purification fold	Yield (%)
Homogenate	37	352	8.78	150.3	5560	15.8	1	100
Sulfanilamide coupled to a CH-sepharose 4B get affinity chromatography	5.2	2.9	0.56	684.6	3315	1143	72.4	59.6

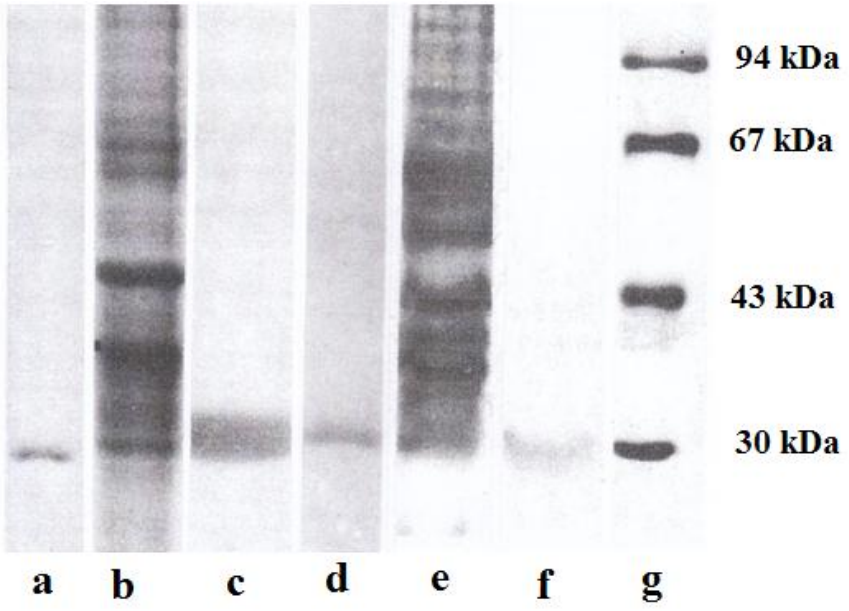

Figure 1. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis analyses and immunoblot of purified carbonic anhydrases.
a-SDS-gel electrophoresis (10%) of purified gill CA. The amount of sample was 25 µg, only single stained band of proteins with commassie brilliant blue which correspond to the molecular weight of 30 kDa when blotted against MW markers (g). This confirm purified enzyme purity.
b-Stained SDS-gel electrophoresis (10%) with commassie brilliant blue of gill homogenate, the amount of sample was 50 µg.
c-Nitrocellulose membrane from the corresponding SDS-gel electrophoresis (b) immunostained with gill CA antibodies (1/100 dilution). Only single band from gill homogenate was detected by gill CA antibodies. This confirms antibodies specificity for gill CA.
d-Commassie brilliant blue stained SDS-gel electrophoresis (10%) of purified brain CA, the amount of sample was 30 µg. Only single stained band of proteins
which correspond to the MW of 30 kDa when blotted against MW markers (g). This confirm purified enzyme purity.
e-Commassie brilliant blue Stained SDS-gel electrophoresis (10%) of brain homogenate, the amount of sample was 50 µg.
f-Nitrocellulose membrane from the corresponding SDS-gel electrophoresis (e) immunostained with gill CA antiserum (1/100 dilution). Only single band from brain homogenate was detected by gill CA antiserum. This confirm antiserum specificity for brain CA.
g-Stained SDS-gel electrophoresis of markers stained with Commassie brilliant blue (Bovine CA 30 kDa;Ovalbumine 43 kDa:Albumin 67 kDa; Phosphprylase b 94 kDa).

and the transport system in the cell membrane, thereby influencing the basic functions of organisms (Ekinci *et al*., 2011; Ceyhun *et al*., 2011; Soyut and Beydemir, 2008; Soyut *et al*., 2008). The results of the current study were expressed in EU/ml and in the percentage of activity if the initial activity was 100%. Ions of Na^+, K^+, Mg^{2+}, and Ca^{2+} at different concentrations (1, 5, and 10 mM) were found to display no more than 25% to 30% inhibition on the enzymatic activity of brain CA (Table 2). The metal anion Cl^- at various concentrations (1, 5, and 10 mM) was found to exhibit the most inhibition on the enzymatic activity of brain CA (Table 2). These results are in agreement with those of several studies (Li *et al*., 2012; Bond *et al*., 2001). Acetazolamide was found to exhibit a strong inhibition of 54%, 83%, and 92% at respective concentrations of 1, 5, and 10 mM on the enzymatic activity of brain CA (Table 2). The result is the same for CA purified from *C. carpio* erythrocytes and gill tissues (Rahim *et al*., 2001a; Rahim *et al*., 2001b) and for mammalian CA II (Rickli *et al*., 1964). Therefore, brain CA enzyme has a number of biochemical properties that are comparable to those of high vertebrate CA II, which is highly distributed in mammalian tissue (Filippi *et al*., 1978).

The immunoblotting technique revealed that gill CA antiserum stained only one protein band with approximately 30 kDa, with the gill and brain homogenates transferred onto nitrocellulose membranes. These immunostained bands correspond to the MW of the gill and brain CA in the homogenates (Figures 1c and 1f), which confirm the monospecificity of gill CA antiserum (Figure 1a, 1b and 1c). The immunolocalization of CA in paraffin sections of the brain of *C. carpio* demonstrates that CA was essentially localized in the cytoplasm of oligodendrocytes and their processes (Figure 2a, 2b,

Table 2. Effect of metal ions and acetazolamide inhibitor on brain carbonic anhydrase activity

Metal ions and acetazolamide	Enzyme activity EU/ml			Enzyme activity %		
	1mM	5mM	10mM	1mM	5mM	10mM
control		11250± 32			100%	
Na^+	10463±27.78	9450±21.00	8325±12.75	93	84	74
K^+	10238±14.20	9338±19.80	8538±14.70	93	83	76
Mg^{2+}	10800±16.70	10013±14.41	9225±12.96	96	89	82
Ca^{2+}	10800±27.39	10125±24.68	9563±15.82	96	90	85
Cl^-	8775±15.24	7988±11.38	6750±13.12	78	71	60
Acetazolamide	6075±15.40	9338±14.36	10350±17.71	54	83	92

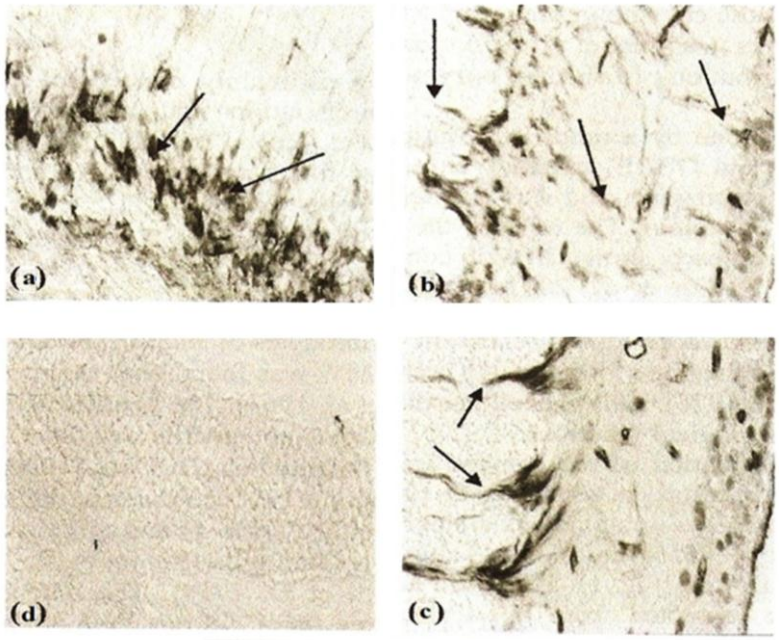

Figure 2. Paraffin sections of brain carp incubated with gill CA antiserum.
a : Immunostaining of CA in paraffin sections of brain tissues incubated with gill CA antiserum. Note the immunoreactivity of CA in oligodendrocytes (arrows). 400x.
b-c : Immunostaining of paraffin sections in brain tissues incubated with gill CA antiserum. Myelinated fibers and oligodendrocytes were positively stained . (arrows) 400x
d : Paraffin section from brain tissue incubated with non-immune rabbit serum. No staining is visible. 400x.

and 2c). compared to control section (Figure 2d). The results also indicate that CA was localized along the myelinated fiber tract (Figure 2a and 2b). The findings are in agreement with the data of several studies on the nervous system of rats and humans (Delaunoy *et al*., 1986; Ghandour *et al*., 1980; Kida *et al*., 2006; Cammer, 1984). Using the Häusler histochemical method, Parthe (1981) found that CA activity in the nervous tissue of teleost fish was positive in the myelin of the axon, the perineuronal oligodendroglial, and in the protoplasmic astrocytes. The specificity of the histochemical technique used by Parthe (1981) has been questioned on several occasions (Churg, 1973; Muther, 1977). The localization of CA in the brain of teleost fish, which was first determined by this investigation, suggests that the enzyme plays a major role in the regulation of cerberospinal fluid pressure and its ionic exchanges as well as in lipid synthesis as a HCO_3^- source during active myelination in oligodendrocytes (Cammer, 1984; Sapirstein *et al.*, 1984).

In the present study, brain CA from *C. carpio* was purified and munohistochemically localized for the first time. The inhibitory effects of acetazolamide and several ions on the enzyme activity were reported. Our results are in good agreement with the literature, which shows that CA is an important enzyme that is vulnerable to the effect of metal ions in the environment. This work provides useful information for physiological and genetic studies.

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