Purification of Ca Isoenzymes from Human Cancerous Colon Tissue and Inhibitory Effects of Some Analgesics on Enzyme Activity

İnsan Kanserli Kolon Dokusundan CA İzoenzimlerinin Saflaştırılması ve Enzim Aktivitesi Üzerine Bazı Analjezik İlaçların İnhibitör Etkisi

Research Article

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ABSTRACT

Carbonic Anhydrase (CA) is an enzyme which is responsible for the hydration of carbon dioxide to carbonic acid and it also takes places in many biological processes in the living organisms. In this study, CA isoenzymes (CA II and CA IX) together were purified 78.4 fold with a yield of 54.86 and specific activity of 106.67 by using Sepharose 4B-L-tyrosine sulfanilamide affinity chromatography. In SDS-PAGE molecular weights of CA II and CA IX were calculated as 29 kDa and 56 kDa respectively. Besides inhibitory effects of some analgesics on purified total enzyme was investigated. IC50 values were found as 0.0077, 0.025, 0.011 and 0.04 mM for dexketoprofen, pethidine, phenyramidol and tramadol respectively.

Key Words
Purification, Cancer, Colon, Carbonic Anhydrase, Inhibitor

ÖZET

Karbonik anhidraz (CA) karbon dioksitin karbonik aside hidrasyonundan sorumlu bir enzimdir ve aynı zamanda canlı organizmalarda birçok biyolojik proseslerde yer almaktadır. Bu çalışmada, CA izoenzimleri (CA II ve CA IX) birlikte sepharose 4B-L-tirozin sülfanilamid afinite kromatografisi kullanılarak 106.67 spesifik aktivite ile % 54.86 verimle 78.4 kat saflaştırıldı. SDS-PAGE'de CA II ve CA IX izoenzimlerin molekül ağırlıkları sırası ile 29 kDa ve 56 kDa olarak hesaplandı. Ayrıca saflaştırılan total enzim üzerine bazı analjezik ilaçların inhibisyon etkisi araştırıldı. Deksketoprofen, pethidin, pheniramidol, tramadol'ün IC50 değerleri sırasıyla 0.0077, 0.025, 0.011, ve 0.04 mM olarak bulundu.

Anahtar Kelimeler
Saflaştırma, Kanser, Kolon, Karbonik Anhidraz, İnhibitör

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INTRODUCTION

Carbonic anhydrase is a metal enzyme which contains zinc ion in its active site and it is found in all living organisms. CA was firstly discovered at bovine erythrocytes. It is responsible for hydration of CO_2 and dehydration of HCO_3^- reactions and an important enzyme catalyzing these reactions reversibly [1].

$$CO_2 + H_2O \xrightleftharpoons{\text{Carbonic Anhydrase}} HCO_3^- + H^+$$

CA was firstly isolated from mammalian erythrocytes, and then it has been purified and characterized from various sources like human erythrocytes, rat erythrocytes, rat saliva, bovine osteoporoses, bovine leucocytes, different kinds of bacteria and plant sources. It has been determined that in mammals carbonic anhydrase enzyme has a molecular weight of 30 kDa approximately [2-6].

CA has been characterized as a pH regulator in many tissues involving erythrocytes. It plays an important role in metabolic reactions such as respiration of bicarbonate between tissues/organs and lung, homeostasis of pH and CO_2, biosynthetic reactions like gluconeogenesis, lypogenesis, and synthesis of urea, calcification, tumor formation and so many in physiological and pathological events [7-9].

In the animal kingdom, there are 16 isozymes of carbonic anhydrase. Five of them (CA I, II, III, VII and XIII) are cytoplasmic and two of them are mitochondrial (CA VA, VB), one of them (CA VI) is the secretory, four (CA IV, IX, XII and XIV) of which are membrane-bound, three of them (VIII, X, XI) are non catalytic [10,11].

It has been determined that CA-XV has a low catalytic activity and it has similar properties with CA-IV. CA VIII, IX and XII are detected to be tumourigenic isozymes [12,13].

Carbonic anhydrase IX is a transmembrane glycoprotein that catalyzes carbondioxide (CO_2) to carbonic acid (HCO_3^-). It makes contribution to tumor growth and invasion by asidificating the tumor medium. CA IX, one of the four transmembrane isoenzymes, is also related with the control of the cell proliferation and transformation of the cells [14,15]. Compared with healthy tissues, CA IX expression shows an increase in a wide tumor spectrum [18]. In solid tumors, there is an important relationship between CA IX expression and hypoxia areas and this enzyme also takes part in adaptation of tumor cells and tumor cell growth in hypoxia case [16,17]. Many clinical studies shows clearly this relationship between CA IX expression and poor prognosis and metastases of tumor cells [18,19]. However it hasn't been determined yet whether CA IX expression is a marker of hypoxia or not and function of CA IX in cells can't be illuminated [20].

Analgesics are used to relieve pain as a medicine. They commonly used for all sorts of pains like headaches, backaches, sore muscles etc. In response to injury or inflammation, cells release chemical messengers. Analgesics work by either blocking the signals that go to the brain or by interfering with the brain's interpretation of the signals [21]. Analgesics are mainly divided into three groups: opioids, non-steroidal anti-inflammatory drugs (NSAIDs) and local anesthetics. Dexketoprofen is a non-steroidal anti-inflammatory drug. It shows its effect in the body by blocking the action of a substance called cyclo-oxygenase which is involved in the production

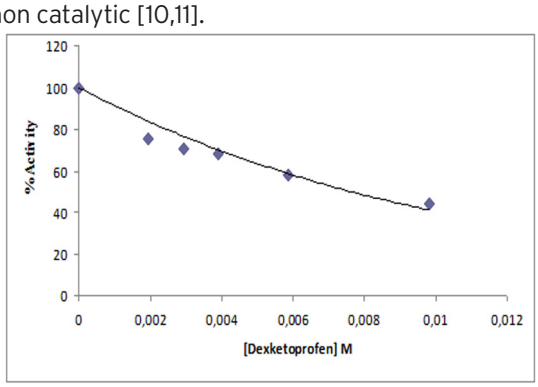

Figure 1. Effect of Dexketoprofen concentrations on human cancerous colon CA activity.

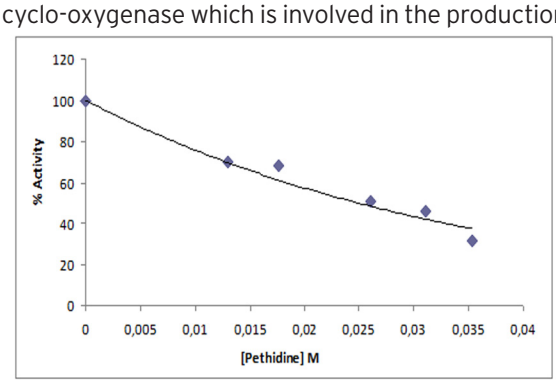

Figure 2. Effect of Pethidine concentrations on human cancerous colon CA activity

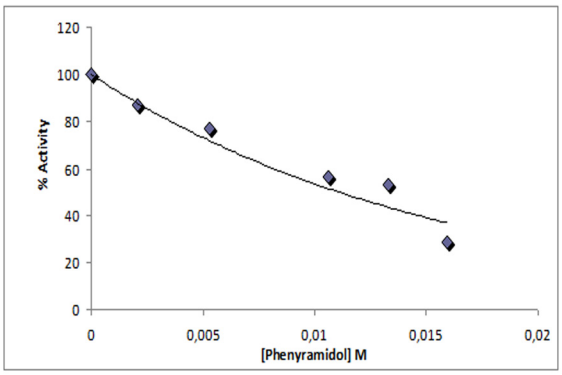

Figure 3. Effect of Phenyramidaol concentrations on human cancerous colon CA activity.

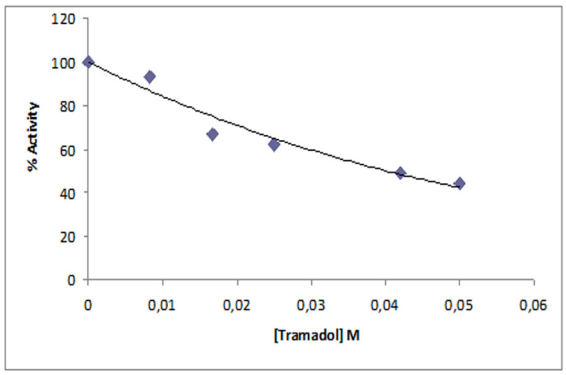

Figure 4. Effect of Tramadol concentrations on human cancerous colon CA activity

of prostaglandins (causes pain in injury or certain diseases). The first synthetic opioid, pethidine, was synthesized as a potential anti-spasmodic agent by Otto Eislib. It is used for the treatment of moderate to severe pain Phenyramidol is a chemical substance has a property of muscle relaxant. Tramadol hydrochloride, another opioid analgesic, is used in treating moderate to severe pain and has a wide range of applications [22].

As a result, recent studies suggest that CA IX is a potential marker for the tumor cells in the cancer treatment. It is highly expressed in human cancerous colon according to healthy tissues. Therefore we purified CA isozymes from human cancerous colon and investigated inhibitory effects of some analgesics on purified CAs.

MATERIAL AND METHOD

Chemicals

Sepharose 4B, protein assay reagents, 4-nitro-phenylacetate were obtained from Sigma-Aldrich Co. All other chemicals were of analytical grade and obtained from Merck. Medical drugs used in the experiments were obtained from the local pharmacy.

Purification of carbonic anhydrase from cancerous human column by affinity chromatography

Cancerous human column was obtained from the Research Hospital of Atatürk University after operation and stored at -80ºC until usage. 30 grams of thawed column was chopped into small pieces with a knife. The fragments were homogenized with liquid nitrogen. The homogenate was taken to 1-1.5 volumes of buffer solution (50mM Tris-HCl, pH: 7.4) and 30 ml hexane was added to solution to solve the lipids. The homogenate was filtered through 4 layers of cheesecloth. Then lipid fraction has been removed from the homogenate by using a Separatory Funnel. Then 0.1 % Triton X-100 was added to homogenate and then centrifuged at 18.000 x g for 1 hour. The pellet (mitochondria and cell debris) was discarded. Supernatant dialyzed with 50mM Tris-SO4 (pH: 7.4). The pH of the homogenate was adjusted to 8.7 with solid Tris. The homogenate was applied to the prepared Sepharose 4B L-tyrosine sulfanilamide affinity column equilibrated with 25 mM Tris-HCl/0.1M Na_2SO_4 (pH: 8.7). The affinity gel was washed with 25 mM Tris-HCl / 22 mM Na_2SO_4 (pH: 8.7). The cancerous human column carbonic anhydrase (sCA) enzymes were eluted with 0.1 M $NaCH_3COO$ / 0.5 M $NaClO_4$ (pH: 5.6).

Table 1. Purification of CA isoenzymes

Purification Steps	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity	Specific activity(EU/mg)	% yield	Purificaiton fold
Homogenate	16.5	20	12.09	241.8	330	1.36	100.0	1.0
Affinity Chrom.	16	12	0.15	1.8	192	106.67	54.86	78.4

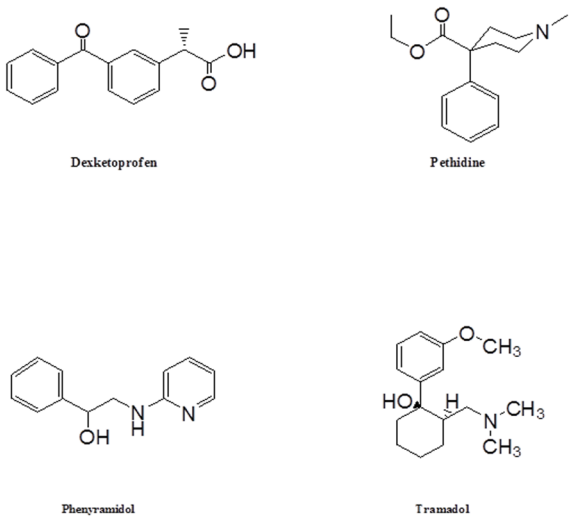

Figure 5. Chemical compounds used as analgesics.

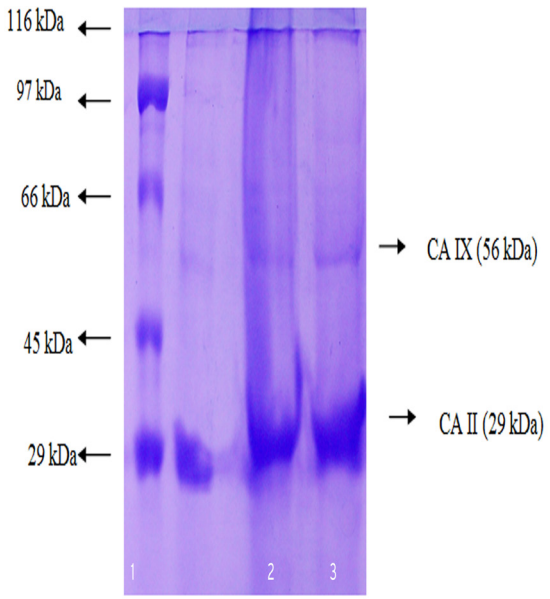

Figure 6. Lane (1) standard proteins (E.coli b-galactosidase (116 kDa), rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa) and bovine carbonic anhydrase (29 kDa) SDS-PAGE analysis of purified. Lane (2, 3) human cancerous colon CA

Table 2. IC_{50} values for some analgesics

Compound	IC_{50} value (mM)
Dexketoprofen	0.0077
Pethidine	0.025
Phenyramidol	0.011
Tramadol	0.04

Hydratase activity assay

Carbonic anhydrase hydratase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson [23]. CO_2-hydratase activity was calculated as an enzyme unit (EU) by using the equation $(t_o\text{-}t_c/t_c)$ where t_0 and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

Esterase activity assay

Carbonic anhydrase esterase activity was assayed by following the change in absorbance of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion at 348 nm over a period of 3 min at 25ºC using a spectrophotometer (BECKMAN COULTER UV-VIS) according to the method described by Verpoorte et al. [24]. The enzymatic reaction, in a total volume of 1.0 mL, contained 0.55mL 0.05M Tris-SO_4 buffer (pH 7.4), 0.35 mL 3 mM 4-nitrophenylacetate, and 0.1mL enzyme solution.

Protein determination

During for each purification steps, protein determination was performed spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard [25].

SDS polyacrylamide gel electrophoresis

After the purification steps, SDS polyacrylamide gel electrophoresis was performed to verify enzyme purity. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli procedure. A 20 μg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coommassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without dye [26].

In Vitro Effects of Compounds

Inhibitory effects of some analgesic drugs were investigated at different inhibitor concentrations. Compounds showing inhibitor effects were tested in triplicate at each concentration. We measured CA activities in the presence of different drug concentrations. Control activity in the absence of inhibitor was taken as 100%. For each drug, activity (%) vs Inhibitor concentration graphs were drawn [27].

RESULTS AND DISCUSSION

Swinson et al. stated that CA IX is a marker of hypoxia and after immunoblotting studies with non-small-cell lung cancer tumors they found that CA IX has a doublet appearent of molecular weight as 54 kDa and 58 kDa in these cells [28]. In a similar study, Vermylen and colleagues studied in lung carcinoma cells and by using western blot analysis they found molecular weight of 54-58 kDa bands showing MN/CA IX genes [29].

In our study, we tried to purify human cancerous colon CA IX by using Sepharose 4B-L-tyrosine sulfanilamide affinity chromatography. However we couldn't isolate it from CA II isoenzyme. So we purified total CA enzymes (CA II and CA IX) with a yield of 54.86 and specific activity of 106.67. In SDS-PAGE we observed two bands. Their molecular weights were calculated approximately as 29 kDa and 56 kDa respectively. It can be seen clearly that our findings go well together studies above and our band showing molecular weight of CA IX is suitable with literature.

Colorectal cancer is the third most common form of cancer observed in the world. Colon and rectal cancer constitutes 9% in men and 10% in women of all cancers. Each year, 395.000 person's life is resulting in death due to colorectal cancer in the world [30, 31]. In colerectal tumors, evindences related with proliferation and dysplasia of tumor cell and CA IX expression have been introduced by Saarnio and his colleagues [32].

CA IX expression in some tumors increases excessively depending upon metastases and poor prognosis. It has been reported that asetozolamidin one of CA inhibitors inhibits the invasion of tumor cells. Robertson and colleagues showed that CA IX, an important enzyme in tumor growth and survival of tumor cells in normal and hypoxia conditions, is a potential target for cancer therapy but not effective for tumor invasion [33].

While CA IX is a few expressed in human colon cells, it has a high proportion of expression in cancer cells of the same tissue.

Since CA IX has an important role in the development and spread of tumor cells under hypoxic conditions in the metabolism, CA IX inhibitors have an important place in recent studies. In our study, we purified CA enzymes from human colon cancer tissue having a high percentage of CA IX expression, and investigated inhibitory effects of analgesic drugs, dexketoprofen, pethidine, phenyramidol and tramadol hydrochloride, on human cancerous colon CA isoenzymes. For these drugs we calculated IC_{50}. We determined IC_{50} values as 0.0077, 0.025, 0.011 and 0.04 mM for dexketoprofen, pethidine, phenyramidol and tramadol respectively.

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