Turk J Vet Anim Sci
26 (2002) 227-233

Research Article

Membrane Specific Carbonic Anhydrase (CA-IV) Expression in Bovine Lung: The Effects of Alcoholic and Non-Alcoholic Drinks

Nazan DEMİR, Hayrunnisa NADAROĞLU
Department of Biochemistry, Faculty of Science, Atatürk University, Erzurum - TURKEY
Yaşar DEMİR
Department of Chemistry, Kazım Karabekir Education Faculty, Atatürk University, Erzurum - TURKEY

Received: 24.05.2000

Abstract: Carbonic anhydrase (CA) (carbonate hydrolyase: E. C. 4.2.1.1) from bovine lung was purified by a new method and characterized. The purification level was 4306-fold.

The optimum temperature for maximum enzyme activity was 37.5°C.

The optimum pH was 7.4, varying between 3.5 and 10.0. SDS-polyacryamide gel electrophoresis (3-10% discontinuous SDS-PAGE) showed two distinct bands for CA-IV. The molecular weights of the enzymes were found to be approximately 54.000 and 29.000, respectively.

V_{max} and K_M values were calculated with p-nitrophenyl acetate as substrate (166.7 µmol/L*min, $5.59*10^{-2}$ M, respectively).

Changes in enzyme activity were determined in the presence of some chemicals: sulfanilamide, NaN_3, KSCN, glucose, nicotine, nicotinamide, caffeine, $CuSO_4$ and $MnCl_2$; and some drinks: brandy, vodka, raki, whisky, cognac, beer, martini, apple liqueur, red wine, Pepsi, Yedigün, 7UP, Vimto and Coca-Cola (carbonated and non-carbonated).

Key Words: Carbonic anhydrase (IV), Alcohol, Nicotine, Nicotinamide, Caffeine

Membrana Spesifik Bağlı Karbonik Anhidrazın (CA-IV)'nın Sığır Akciğerinden Tanımlanması: Alkollü ve Alkolsüz İçeceklerin Etkisi

Özet: Karbonik anhidraz (CA) (Karbonat Hidroliyaz: E.C.4.2.1.1) yeni bir metot ile sığır akciğerinden saflaştırıldı ve karakterize edildi. Enzimin 4306 kat saflaştırıldığı tespit edildi.

Enzimin maksimum aktivite gösterdiği optimum sıcaklığın 37.5°C olduğu tespit edildi. pH: 3.5-10 arası taranarak enzimin optimum pH'nın 7.4 olduğu bulundu ve SDS-PAGE (3-10% kesikli) ile saflık kontrolü yapıldığında da iki farklı bant gözlendi. Enzimlerin molekül ağırlıkları Jel Filtrasyon kromatografisi yardımıyla yaklaşık olarak 54.000 Dalton ve 29.000 Dalton olarak bulundu.

Substrat olarak p-Nitrofenil asetat kullanılarak ölçüm yapıldığında V_{max} ve K_m değerleri sırasıyla (166.7 µmol/L*dak., $5.59x10^{-2}$ M) olarak tespit edildi.

Enzim aktivitesi üzerine Sülfanilamid, NaN_3, KSCN, Glikoz, Nikotin, Nikotinamid, Kafein, $CuSO_4$, $MnCl_2$ gibi bazı kimyasalların ve Brendi, Votka, Rakı, Viski, Konyak, Bira, Martini, Elma Likörü, Kırmızı Şarap, Pepsi, Yedigün, 7UP, Vimto, Coca Cola, (gazlı ve gazsız) bazı içeceklerin etkileri de araştırıldı.

Anahtar Sözcükler: Karbonik Anhidraz (IV), Alkol, Nikotin, Nikotinamid, Kafein.

Introduction

Carbonic anhydrase (E. C. 4. 2. 1. 1) is an efficient catalyze of the following reaction:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

It is present at high levels in erythrocytes and electrolyte-transporting epithelia, where it is believed to mediate the transfer of CO_2, H^+, HCO_3^- and Cl^- (1).

A variety of loci encode the isoenzymes identified in mammalian tissues. These include CA-II, which is ubiquitously expressed in mammalian cells, and CA-I and CA-III, where expression is largely restricted to erythrocytes and skeletal muscle, respectively (2,3).

In humans, CA-IV has been identified in the microvilli and basal infoldings of renal tubular cells and this isoform has recently been purified and characterized (4). An apparently homologous enzyme (5) has also been purified from human lung. It is proposed that the CA-IV isoenzyme is primarily responsible for the re-absorption of HCO_3^- in the proximal tubule and is involved in the formation of lung liquid during fetal life.

The carbonic anhydrase enzyme taken from bovine lung has already been partially purified (6). The process of purification carried out at our laboratory produced some new and significant results. The present study yielded new data about the CA-IV enzyme, which is of vital importance for respiration. A new definition for this enzyme is presented.

Materials and Methods

Obtaining Bovine Lung Samples

Bovine lung samples about 250 g in weight were obtained during the slaughter of bovine animals at the Erzurum Municipal Slaughterhouse and were rinsed in a solution of 9% NaCl. Each piece of lung was cut into small pieces and washed with physiological saline and then chopped in a blender. Approximately 60 liter of physiologic serum was used in order to eliminate erythrocytes from the medium. Washing was continued until the precipitation was clear enough.

The lung sample, entirely free of erythrocytes, was washed in 500 ml buffer of 0.05 M Tris-SO_4 (pH: 7.4) containing 1% TritonX-100 (2 ml per gram) and dismembered by an ultrasonic dismemberator for 4 h and then centrifuged for 30 min at 3000 x g. The material was filtered through fiber glass to remove the precipitation. The supernatant was treated with distilled water for 2 days and with 0.05 M Tris-SO_4 (pH: 7.4) buffer for dialysis. Then the homogenate was extracted by CCl_4 to eliminate the lipids from the medium and this was followed by the removal of organic media. The pH of the homogenate was decreased to 8.7 by using solid Tris, until it was prepared for application to a column.

Enzyme Purification

The enzyme was purified with a Sepharose-4B-L-tyrosine-sulfanilamide affinity column. The column was balanced with the solution of 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH: 8.7). The hemolysate was applied to the column. The column was then washed with 400 ml of the solution of 25 mM Tris-HCl/22 mM Na_2SO_4 (pH: 8.7), resulting in a significant amount of adsorption of CA-IV on affinity gel. The elution of CA-IV on affinity gel was carried out. The elution was stopped at the point at which no further absorbency was obtained at 280 nm. The column was then rebalanced (7).

Protein Determination

After scanning at 280 nm, the tubes with significant absorbency were pooled and a quantitative protein determination was performed with Coomassie Brilliant Blue G-250 (8).

Enzyme Activity Determination

Esterase and hydratase activities were determined for both isoenzymes.

CO_2 -Hydratase Activity Determination

Two mililiters of veronal buffer (pH: 8.2), 0.4 ml of bromothymol blue (0.004 %), 0.8 ml of diluted enzyme solution 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 occurrence of a yellow-green color. The same interval was recorded without enzyme solution (t_o). The activity was calculated from the following formula (9):

1 Wilbur-Anderson Unit = $(t_o-t_c)/ t_c$

Esterase Activity Determination

The principle of this determination is that the substrate of CA (p-nitrophenyl acetate) is hydrolyzed to p-nitrophenol plus acetic acid. The reaction is detected at 348 nm. For this procedure, 1.5 mL of a buffered enzyme solution (0.1 mL enzyme, 1.4 mL 0.05 M Tris-SO_4, pH: 7.4) and 1.5 mL substrate were mixed in a measurement cuvette and three minutes later the absorbency was measured (348 nm, 25°C). A blank measurement was obtained by preparing the same cuvette without the enzyme solution (10). V_{max}, K_M and optimal pH were determined by this method. While substrate volume was increased as follows: 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.5 ml, the volume of enzyme was fixed

at 1 ml and buffer was added to make the total volume 3 ml in the determining of V_{max} and K_M values. V_{max} and K_M values were determined from the Lineweaver-Burk graph.

Enzyme Activity Determination in Some Chemicals and Drinks Containing Media

For this purpose, sulfanilamide, NaN_3, KSCN, glucose, nicotine, nicotinamide, caffeine, $CuSO_4$, $MnCl_2$, brandy, vodka, raki, whisky, cognac, beer, martini, apple liqueur, red wine, Pepsi, Yedigün, 7UP, Vimto and Coca-Cola (carbonated and non-carbonated) were used as inhibitors. The method described by Rickli et al. was used to determine the hydrolysis activity of the enzyme (9).

SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out on SDS-PAGE gel as described by Laemmli (11). Human CA-I and CA-II were purified by affinity chromatography and used as standards (12).

Molecular Weight Determination with Gel Filtration

Sephadex G-150 was incubated with distilled water at 90°C for 5 h and was put into a column (3 cm x 70 cm). The column was balanced for 24 h with the buffer (0.05 M Na_3PO_4/1 mM dithiothreitol pH: 7.0) until no absorbency at 280 nm was obtained. A protein standard solution was added to the column and standard graphs were obtained. The concentration of the protein solution was 0.2 mg/ml. The standard proteins, CA-IV, were eluted under the same conditions in separate steps. The flow rate through the column was 120-ml/h (13).

Results and Discussion

As seen in the Table, the CA-IV enzyme was purified 4306-fold. The purification of the CA-IV enzyme from bovine lung was performed in 1982 for the first time. This article contained no electrophoretic photographs and reported that a polypeptide chain with a molecular weight of 54,000 was formed.

Using a Sepharose-4B-L-tyrosine sulfanilamide affinity column, CA-IV isoenzyme obtained from the lungs of 20 young male bovine animals was purified. The amount of protein was measured at 280 nm for each fraction during elution and CO_2 -hydrate activity was measured for each tube that exhibited absorption (Figure 1).

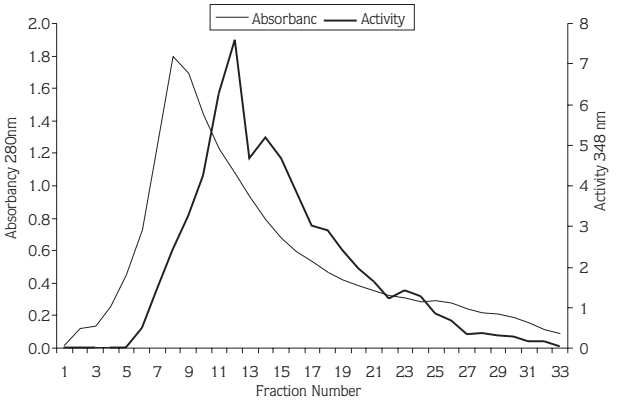

Figure 1. Activity-absorbency graph of carbonic anhydrase enzymes from Sepharose-4B-L-tyrosine–sulfanilamide affinity chromatography elution with 0.1 M $NaCH_3COO$/0.5 mM $NaClO_4$/0.01 mM EDTA (pH: 5.6)

As seen on the activity-absorption graph of CA-IV, there are two different maximum values. Further measurements were taken in order to determine to what extent the enzyme was purified by consolidating the fractions that showed protein absorption. The CO_2 -hydratase activity was also used to measure the degree of activity.

When this enzyme was purified for use in further experiments, the electrophoresis showed two separate bands, one of which was about 54,000 Daltons while the other was approximately 29,000. The level of the latter

Table. The purification of CA-IV from bovine lung

Steps	Volume ml	Activity EU/ml	Total Activity EU	%	Protein mg/ml	Specific Activity EU/mg	Purification Times
Before Dialysis	500	1.09	545	100	153	7.10*10-3	-
After Dialysis	500	2.06	1030	189	151	1.36*10-2	1.915
Pure Enzyme CA-IV	30	6.42	192.6	18.69	0.21	30.371	4305.8

suggests that it may belong to carbonic anhydrase from which the erythrocytes were not completely eliminated. This made us take utmost care in washing the erythrocytes membrane and so the samples were cleaned with 40-50 liters of physiological saline until the water was completely clear and limpid, with no protein absorption.

Having been purified as such, the enzyme continued to exhibit two different bands, both in activity absorption and in electrophoresis. This might mean that the carbonic anhydrase of the lung is not composed of only one polypeptide, as is generally thought, or the sub-unit weighing 29,000 Daltons forms a dimer. This assumption is supported by the fact that the molecular weight doubles the unit (Figure 2). These values are consistent with bovine erythrocyte plasma membrane carbonic anhydrase (14).

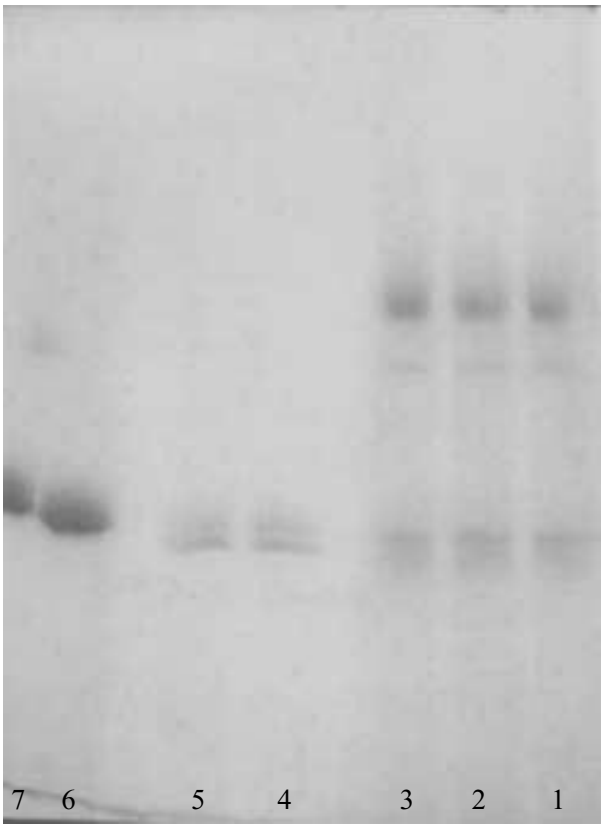

Figure 2. SDS-PAGE electrophoretic pattern of bovine lung carbonic anhydrase IV (1,2,3), human erythrocyte carbonic anhydrase II (4,5) and I (6,7).

Furthermore, the finding of gel filtration chromatography agree well with the values given above. With these results, the question arises as to why the second band of 29,000 Daltons was not observed during the first purification in 1982. This may be attributed to the fact that it might have been seen in the electrophoresis but vanished afterwards because of the absence of erythrocytes that had not been eliminated from the medium. This is why the study report did not include a photograph of the electrophoresis. In addition, the purification technique employed may have produced such results. The technique used in our experiment was that of affinity chromatography with a new type of affinity gel capable of achieving 4306-fold purification. The type of gel used in the experiment in 1982 was reported to be CM-Biogel and they obtained a band of 54,000 Daltons.

To conclude, it may be argued that the literature about lung carbonic anhydrase enzyme is inadequate and incomplete. The present study attempts to contribute new techniques and material to those already known, such as re-optimization of the enzyme following purification, optimum pH and temperatures, together with V_{max} (166.7 µmol/L*min.), K_M (5.59*10^{-2} M) values. The pH range was 3.5-10.0 when it continued to be active and the maximum activity was observed at 7.4 (Figure 3). The enzyme proved to be active between 10 and 55°C (Figure 4) and the maximum activity was observed at 37.5°C.

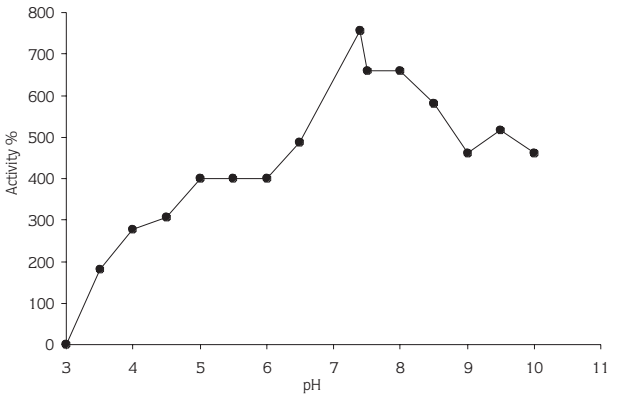

Figure 3. Activity of carbonic anhydrase from bovine lung in Tris-SO_4 buffer of pH: 3-11

The second phase of this study investigated the effects of some chemicals on purified CA-IV enzyme. The chemicals tested in the early phase were KSCN, sulfanilamide and sodium azide, which are known to be specific inhibitors of the enzyme. These chemicals

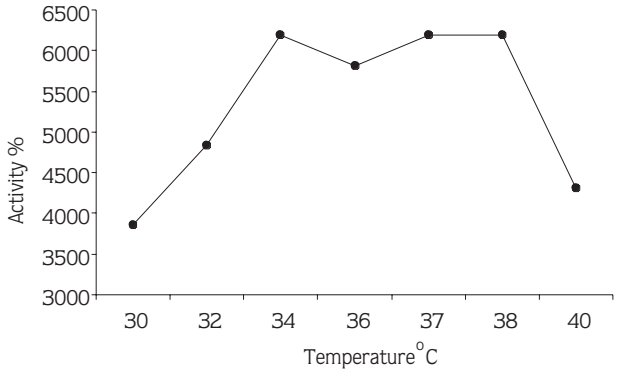

Figure 4. Effect of temperature on purified carbonic anhydrase from bovine lung.

appeared to cause inhibition (Figure 5). $CaSO_4$ and $MnCl_2$ seemed to produce inhibition (Figure 6). Glucose, with the metabolism of basic carbohydrates, appeared to cause activation (Figure 7). Caffeine, nicotine and nicotinamide were proved to activate the enzyme (Figure 8).

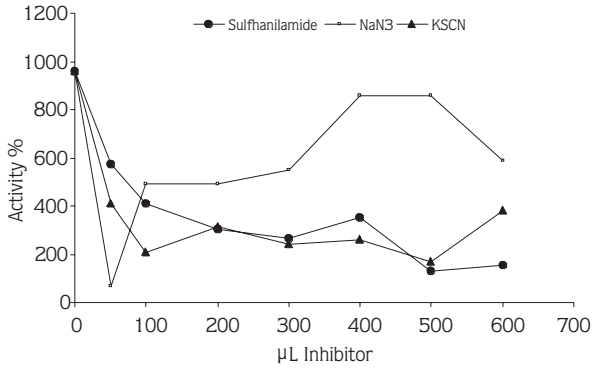

Figure 5. Effect of 10^{-4} M Sulfanilamide, 10^{-4} M NaN_3 and 10^{-4} M KSCN on the activity of purified carbonic anhydrase enzyme from bovine lung.

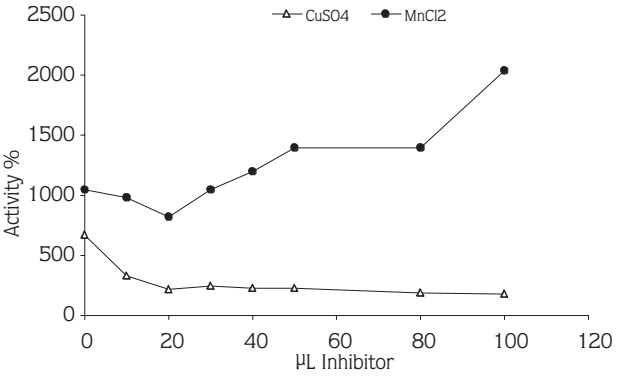

Figure 6. Effect of 10^{-4} M $CuSO_4$ and 10^{-4} M $MnCl_2$ on the activity of purified carbonic anhydrase enzyme from bovine lung.

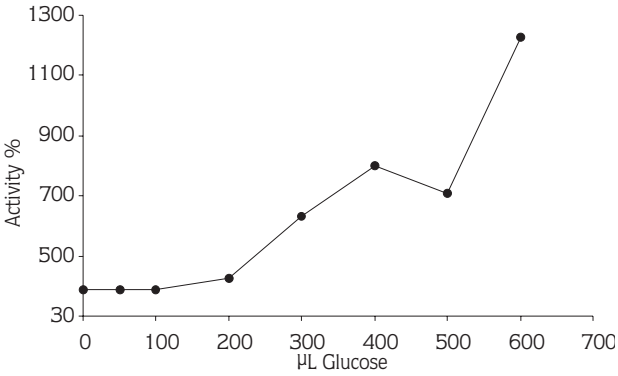

Figure 7. Effect of 10^{-4} M glucose on the activity of purified carbonic anhydrase enzyme from bovine lung.

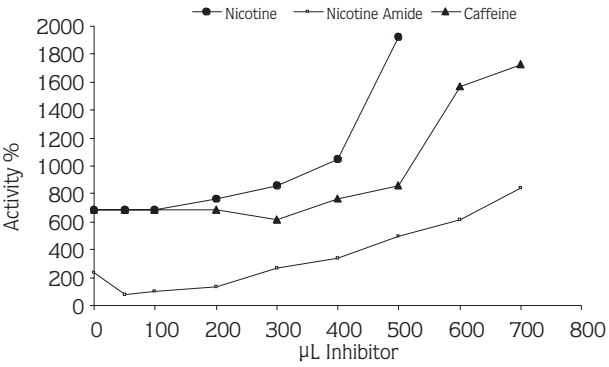

Figure 8. Effect of 10^{-4} M nicotine, 10^{-4} M nicotinamide and 10^{-4} M caffeine on the activity of purified carbonic anhydrase enzyme from bovine lung.

The last groups of chemicals tested on the enzyme were alcoholic beverages and beverages containing carbonic acid. The 10 different alcoholic beverages activated the enzyme (Figures 9, 10). The carbonated

Figure 9. Effect of brandy, vodka, raki, whisky and cognac on the activity of purified carbonic anhydrase enzyme from bovine lung.

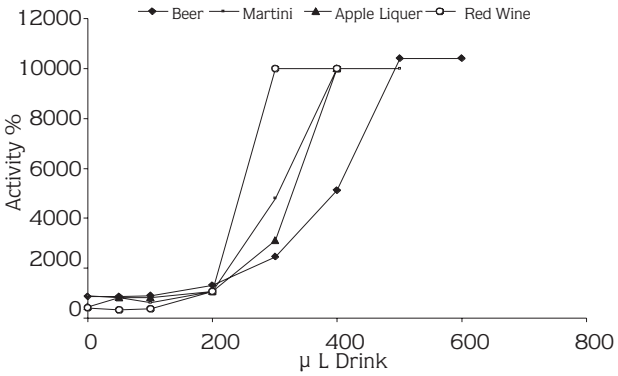

Figure 10. Effect of beer, martini, apple liqueur and red wine on the activity of purified carbonic anhydrase enzyme from bovine lung.

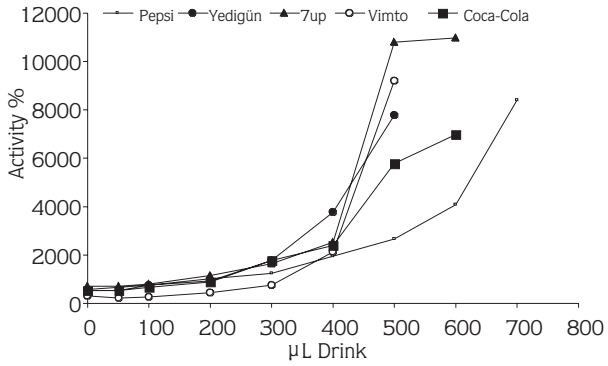

Figure 11. Effect of Pepsi, Yedigün, 7UP, Vimto and Coca-Cola on the activity of purified carbonic anhydrase enzyme from bovine lung (carbonated)

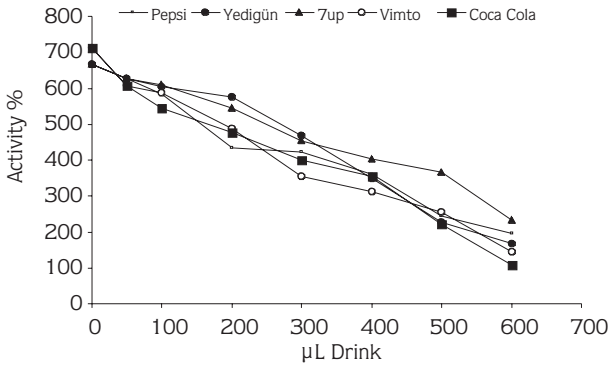

Figure 12. Effect of Pepsi, Yedigün, 7UP, Vimto and Coca-Cola on the activity of purified carbonic anhydrase enzyme from bovine lung (non-carbonated).

beverages were kept at room temperature for a certain period to let the gass escape. Then they were applied to the enzyme to produce inhibition. They caused activation when used before the gass was allowed to escape (Figures 11,12).

It is clear that alcoholic beverages and carbonated beverages have direct effects on respiration.

References

1. Carter M J, Carbonic Anhydrase: Isoenzymes, Properties, Distribution and Functional Significance, Biol. Rev. (Cambridge), 1972, 47, 465-513.
2. Endberg P, Millquist E, Pohl G and Lindskog S, Purification and Some Properties of Carbonic Anhydrase from Bovine Skeletal Muscle., Arch. Biochem., 1985, 241, 628-38.
3. Carlson U, Kjellström B and Antonsson B, Purification and Properties of Cyclostome Carbonic Anhydrase from Erythrocytes of Hagfish, Biochem. Biophys. Acta., 1980, 612, 60-70.
4. Carter N D, Fryer A, Grand A G, Hume R, Strange R G and Wistrand P J, Membrane Specific Carbonic Anhydrase (CA-IV) Expression in Human Tissues, Biochem. Biophys. Acta., 1990, 1026, 113-16.
5. Zhu X L and Sly W S, Carbonic Anhydrase IV from Human Lung. Purification, Characterization and Comparison with Membrane Carbonic Anhydrase from Human Kidney, J. Biol. Chem., 1990, 265, 8795-801.
6. Whitney P L and Briggle T V, Membrane Associated Carbonic Anhydrase Purified from Bovine Lung, J. Biol. Chem., 1982, 257, 12056-59.
7. Whitney P L, Affinity Chromatography of Carbonic Anhydrase, Anal. Biochem., 1974, 57, 467-76.
8. Bradford H P, A Rapid and Sensitive Method for the Quantitation of Microgram Quantites of Protein Utilizing the Principle of Protein-Dye Binding, Anal. Biolchem., 1976, 72, 248-54.
9. Rickli E E, Ghanzanfar S A, Gibbons B H and Edsal J T, Carbonic Anhydrase from Human Erythrocytes, J. Biol. Chem., 1964, 239, 1065-78.
10. Verpoorte J A, Mehta S and Edsall J T, Esterase Activities of Human Aarbonic Anhydrase, J. Biol. Chem., 1967, 242, 4221-29.
11. Laemmli D K, Cleavage of Structural Proteins During in Assembly of the Heat of Bacterophage T4, Nature (London), 1970, 227, 680-85.

12. Demir N, Demir Y, Bakan E, Küfrevioğlu Ö İ, A Different Structural Feature for Carbonic Anhydrase in Human Erythrocytes., Prep. Biochem. & Biotech., 1997, 27 (4), 279-87.

13. Demir N, Demir Y, Yıldırım A, Carbonic Anhydrase from Leaves and Trunks of Daucus Carota., Phtochem., 1997, 44, 1247-50.

14. Demir Y, Demir N, Nadaroğlu, H, Bakan E, Purification and Characterization of Carbonic Anhydrase from Bovine Erythrocytes Plasma Membrane, Prep. Biochem. & Biotech., 2000, 30 (1), 49-59.