Purification and Characterization of α-Carbonic Anhydrase II from Sheep Liver and Examining the Inhibition Effect of Kanamycin on Enzyme Activity

Koyun Karaciğerinden α-Karbonik Anhidraz II' nin Saflaştırılması ve Karakterizasyonu ve Enzim Aktivitesi Üzerine Kanamisinin İnhibisyon Etkisinin İncelenmesi

Research Article

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ABSTRACT

Sheep carbonic anhydrase - II (SCA-II) (E.C: 4.2.1.1) was purified from sheep liver and some characteristic properties were investigated. The enzyme was purified approximate 43.1-fold with a yield of 38.6%, and a specific activity of 4000 EU/mg proteins. For the enzyme, optimum pH, optimum temperature, optimum ionic strength and stable pH were determined to be 7.5, 40 ºC, 10 mM and 8.5, respectively. The molecular weight was found 29 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Kanamycin exhibited *in vitro* inhibitory effect on the enzyme activity.

Key Words
Characterization, Carbonic anhydrase (CA), Sheep, Liver, Inhibition.

ÖZET

Koyun karbonik anhidraz-II (E.C: 4.2.1.1) enzimi koyun karaciğerinden saflaştırıldı ve bazı karakteristik özellikleri araştırıldı. Enzim 4000 EU/mg protein spesifik aktivitesi ve % 38.6 verim ile yaklaşık olarak 43,1 kat saflaştırıldı. Enzim için optimum pH, optimum sıcaklık, optimum iyonik şiddet ve stabil pH sırasıyla 7.5, 40ºC, 10 mM ve 8.5 olarak belirlendi. Moleküler ağırlığı sodyum dodesil sülfat poliakrilamid jel elektroforezi ile 29 kDa olarak bulundu. Kanamisinin enzim aktivitesi üzerine *in vitro* inhibitör etkisi incelendi.

Anahtar Kelimeler
Karakterizasyon, Karbonik Anhidraz, Koyun, Karaciğer, İnhibisyon.

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INTRODUCTION

A very simple but essential physiological reaction, carbon dioxide hydration to bicarbonate and protons is catalyzed by the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1).This reaction also occurs without a catalyst but it is too slow [1-4].

In all life kingdoms (Bacteria, Archaea, and Eukarya); as CO_2, bicarbonate, and protons are essential molecules/ions in many important physiologic processes, throughout the phylogenetic tree, and relatively high amounts of them are present in different tissues/cell compartments of all such organisms. It is no wonder that CAs evolved freely at least five times, with five genetically distinct enzyme families known to date: the α–, β–, γ–, δ– and ζ CAs. All of them are metalloenzymes, but whereas α–, β– and γ–CAs use Zn(II) ions at the active site, the γ–CAs are possibly Fe(II) enzymes (but they are active also with bound Zn(II) or Co(II) ions), while the ζ – class uses Cd(II) or Zn(II) to perform the physiologic reaction catalysis.The 3D-fold of the five enzyme classes is very different from each other, as it is their oligomerization state: α–CAs are normally monomers and barely dimmers; β–CAs are dimers, tetramers, or octamers; γ–CAs are trimers, whereas the δ– and ζ–CAs are possibly monomers but in the case of the last family, on the same protein backbone which is in fact a pseudotrimer, three simply different active sites are present. Many representatives of all these enzyme classes have been crystallized and characterized in detail, except the δ–CAs [1-4].

CA, firstly, has been isolated from mammal erythrocytes,at the later years enzyme was purificated from human erythrocytes, fish erythrocytes, rat erythrocytes, rat spit, cow bone, cow leukocytes, various bacteries and plant sources and characterized from many sources.It has been found that the molecule weight of enzyme at the mammals is about 30 kDa [5-7].

16 different CA isoenzymes placed at the different tissues and cells of vertebrate have been found. These isoenzymes have some important differences at catalitic activities and inhibitor bounding specialities. Five of these isoenzymes, CA- I, -II, -III, -VII and –XIII, are cytosolic. CA-IV, CA-IX, CA-XII and CA-XIV are also bound to membrane. CA-VA and CA-VB are mitochondrial, CA-VI is salivated in the spit. And CA-XV which has been clarified recently, doesn't express at the human beings and other primates but it expresses at the rodent and high vertebrate [8]. Besides, CA isoenzymes have three uncatalitical forms.These are called as CA related proteins (CARPs): CARP-VIII, CARP-X ve CARP-XI [9].

Why so many CA isoforms must be present in an organism is still a mystery. In fact, in many tissues isoforms are abundantly, concomitantly present, but there are also several tissues, or organs in which just one of them predominates [1].

Mammalian CAs were the first enzymes of this type to be isolated and studied in detail, and many of them are established therapeutic targets with the potential to be inhibited or activated to treat a wide range of disorders [1-3].

Taking into account above information, the current study aims in purification and characterization of carbonic anhydrase enzyme from sheep liver for the first time, and investigation of impact of Kanamycin on the enzyme activity.

METHODS AND MATERIALS

Chemicals

Sepharose 4B, protein assay reagents, 4-nitrophenylacetate were obtained from Sigma-Aldrich Co. All other chemicals were of analytical grade and obtained from Merck. And Kanamycin was provided from a commercial company selling the vet drugs.

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 [10]. CO_2-hydratase activity was calculated as an enzyme unit (EU) by using the equation (t_0-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

The 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. [11]. The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05 M Tris-SO_4 buffer (pH 7.4), 1.0 mL 3 mM 4-nitrophenylacetate, 0.5 mL H_2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution.

Protein determination

During each purification steps, protein determination was performed spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard [12].

Purification of carbonic anhydrase II from sheep liver by affinity chromatography

Sheep liver was obtained from Erzurum Ozbeyli Municipal Slaughterhouse and stored at -80 ºC until usage. 25 grams of thawed liver was chopped into small pieces with a knife. The fragments were homogenized with liquid nitrogen and taken into 1.5-2 volumes of buffer solution (50mM Tris-HCl, pH 7.4). The homogenate was filtered through 4 layers of cheesecloth and then centrifuged at 18.000 x g for 1 hour. The pellet (mitochondria and cell debris) was discarded. The pH of the Supernatant 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 SCA-II enzyme was eluted with 0,1 M $NaCH_3COO$/0,5 M $NaClO_4$ (pH 5.6). Then purified SCA-II enzyme was dialyzed for 3 hr against 0.05 M Tris-SO_4/1 mM 2-mercaptoethanol (pH 7.4). All procedures were performed at 4ºC.

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 [13].

Stable pH Determination

For this aim, equal volumes of the buffers Na-phosphate at pH of 6.0, 6.5, 7.0, 7.5 and 8.0, Tris-HCl at pH of 7.0,7.5, 8.0, 8.5 and 9.0, and purified enzyme were mixed and kept in a refrigerator (+4°C). The enzyme activity was assayed during a week at twenty-four hours intervals.

Optimum pH Determination

In order to determine the optimum pH, Tris-HCl and Na-phosphate buffers were used in the pH range of 5.5 to 8.0 and 7.0 to 9.0, respectively.

Optimum Temperature Determination

For determination of the optimum temperature, enzyme activity was assayed at different temperatures in the range from 5°C to 70°C. The desired temperature was provided by using a Grant bath.

Optimum Ionic Strength Determination

For determination of optimum ionic strength, enzyme activity was determined using different concentrations of Na-phosphate buffer, pH: 7.5, in the range from 10mM to 1000mM.

In Vitro **Inhibition Effect of Kanamycin**

Kanamycin is in a group of drugs called aminoglycosides. It fights bacteria in the body. Kanamycin is used to treat serious infections caused by bacteria [14].

In order to determine the effect of Kanamycin on sheep liver CA II, different concentrations of Kanamycin were added to the reaction medium. The enzyme activity was measured, and an experiment in the absence of inhibitor was used as control (100% activity). The IC_{50} values were obtained from activity (%) vs. Kanamycin concentration plots.In order to determine K_i constants in the media with inhibitor, the substrate (NPA) concentrations were 0.3, 0.45, 0.6, 0.75, and 0.9 mM. Inhibitor solutions were added to the reaction medium, resulting in 3 different fixed concentrations of inhibitors in 1 mL of total reaction volume. Lineweaver-Burk graphs

[15] were drawn by using 1/V vs. 1/[S] values and Ki constant were calculated from these graphs.

RESULTS AND DISCUSSION

In this study, sheep liver CA II enzyme was first isolated and characterized. Purification procedure was carried out by the preparation of the homogenate and affinity chromatography on Sepharose 4B tyrosine-sulfanilamide. As a result of the two consecutive steps, the enzyme was purified up to 43.1-fold with a recovery ratio of 38.6% compared to homogenate (Table 1). After the sample had completely passed through, the column was washed with 10mM Tris-HCl/0.1M Na_2SO_4 buffer whose pH was 7.5. While washing was being done, absorbencies of fractions were spectrophotometrically measured at 280 nm and 348 nm. These values of the absorbance showed that some proteins, bound to the affinity material, have been removed from the column by the washing solutions. Then, the enzyme was eluted with 1.2 M NaCl/25 mM Na_2HPO_4 (pH 6.3). At the end of the last step, purified enzyme was obtained exhibiting a single band on SDS-PAGE (Figure 1). We used only two chromatographic techniques, Sepharose 4B tyrosine-sulfanilamide affinity chromatography by modification of washing and elution conditions. These results mean that the procedure used in the purification is good enough to be used in further studies, and also has an advantage of an experimental period as short as a day.

The optimum pH, optimum temperature, optimum ionic strength and stable pH were determined to be 7.5, 40ºC, 10 mM and 8.5 for the enzyme, respectively. The stable pH profile of the enzyme was determined at five different pHs in 10 mM Tris-HCl and five different pHs in 10 mM Na-phosphate buffer.

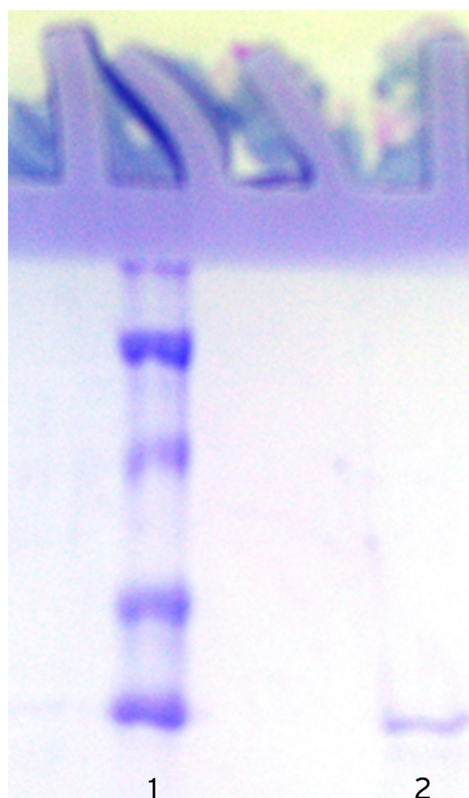

Figure 1. SDS-PAGE analysis of purified sheep liver CA II . Lane (1) standard proteins (E.coli b-galactosidase (116 kDa), rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa) and bovine carbonic anhydrase (29 kDa)).Lane (2) sheep liver CA II.

The enzyme was able to protect 86% of maximum activity at the end of 7 days in 10 mM Tris-HCl buffer (pH: 8.5). The stable pH was found to be 8.5. And optimum pH was found to be 7.5 The result is similar to CAs obtained from human erythrocytes [16], bovine erythrocyte [17], bovine stomach [18], European sebass liver [19] and rainbow trout liver [20].

The optimum ionic strength of the enzyme was estimated to be 10 mM Na-phosphate buffer. The result is similar to CAs obtained from European sebass liver [19]. To determine molecular weight of

Table 1. Summary of purification procedure for sheep liver CA II

Purification Steps	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity	Specific activity(EU/mg)	% yield	Purificaiton fold
Homogenate	290	3.122	30	93.66	8700	92.88	100.0	1.0
Affinity Chromatography After Dialyzed	280	0.07	12	0.84	3360	4000	38.6	43.1

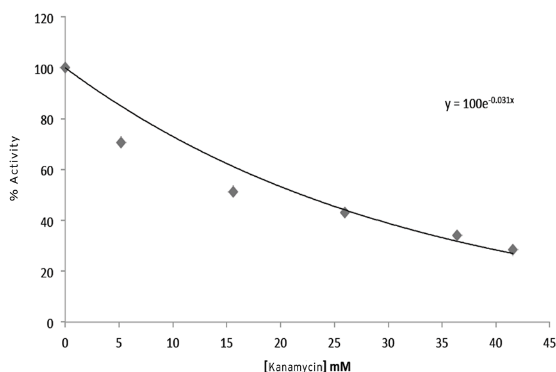

Figure 2. Activity %-[Kanamycin] graph.

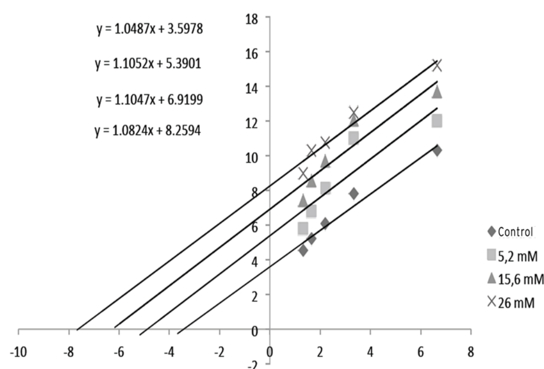

Figure 3. Lineweaver-Burk graph for Kanamycin.

the enzyme, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done. The molecular weight was determined to be 29 kDa. Similar results have been observed for the enzyme from different sources.

For example, human erythrocyte CA is 29 kDa [16], bovine erythrocyte CA is 29 kDa [17], bovine stomach CA is 30 kDa[18], sheep erythrocyte CA is 30 kDa [21], Pseudomonas fragi CA is 31 kDa [22] and rainbow trout liver CA is 31 kDa [20].

The enzyme was seen to exhibit the highest activity at 40°C in a study of temperatures between 5°C-70°C. (Ceyhun et al.2011) found that the optimum temperature of European sebass liver CA enzyme as 25°C [19]. (Beydemir et al. 2006) found that the optimum temperature of rainbow trout lens CA enzyme as 22,5°C [23] and (Demir et al. 1997) found that the optimum temperature of CA enzyme which was purified from the carrot's leaf and root as 75°C [24]. According to this; sheep liver CA II enzyme which optimum temperature was 40°C had a higher degree of optimum temperature than rainbow trout lens CA enzyme and European sebass liver CA enzyme . And it was seen that the optimum temperature of sheep liver CA II enzyme was lower than the optimum temperature of CA enzyme purified from the carrot.

In this study, K_M and V_{max} values were calculated for NPA by Lineweaver-Burk graph. KM constants were calculated as 0.29 mM, Vmax values as 3.5978 μmol x min-1 for NPA. Kanamycin's inhibitory effect on sheep liver CA II was investigated. and IC_{50} parameters of Kanamycin were determined for sheep liver CA II. Kanamycin inhibited the enzyme

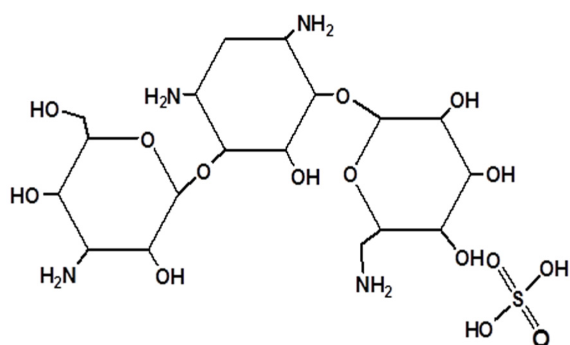

Figure 4. Chemical Structure of Kanamycin sulfate.

activity at low concentrations. and IC50 graphs show that Kanamycin inhibits CA in uncompetitive manner (Figure 2 and Figure 3).

Consequently, we purified carbonic anhydrase from sheep liver for the first time, and analyzed characteristic features. Our results are in good agreement with others reported in literature.

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