

## Purification and Characterization of Carbonic Anhydrase from Human Erythrocyte Plasma Membrane

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**Abstract:** Carbonic anhydrase is the basic enzyme in inhalation function. Until now no research had been done to determine whether CA is in the human erythrocyte plasma membrane or not.

Carbonic anhydrase (CA) was purified from human erythrocyte plasma membrane and described in this study. For this purpose, the blood samples taken from young human test subjects were hemolyzed, then the membrane fraction was separated, and this fraction was repeatedly washed. The enzyme (CA) was removed from the membrane with buffered TritonX-100 (1%), which was purified with a factor of 119.19 by affinity chromatography.

The CA obtained from the erythrocyte membrane has esterase activity as well as hydratase activity. The  $V_{max}$  and  $K_M$  of the

enzyme for the substrate (p-nitrophenyl acetate) are  $1.517 \cdot 10^{-1} \mu\text{M/L} \cdot \text{min.}$  and 1.78 mM, respectively. The purification degree of the enzyme was controlled by SDS-PAGE (3-10%), which showed one distinct band. It was determined that the enzyme was active within the pH range of 4-10, and that the optimal pH was 7.5. The temperature at which it showed activity was 5-70°C, and optimal temperature was 35°C. The molecular weight of CA was found to be ~ 36,600 by gel filtration. On the other hand, sulphanilamide, KSCN and  $\text{NaN}_3$  inhibited the enzyme.

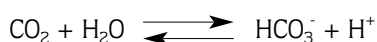
Finally, CA was shown to be present in human erythrocyte plasma membrane and this enzyme is optimized.

**Key Words:** Carbonic anhydrase, Plasma membrane, sulphanilamide, KSCN and  $\text{NaN}_3$

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### Introduction

The carbonic anhydrase isoenzymes are zinc-containing metalloenzymes that catalyze the reversible hydration of carbon dioxide.



They vary in their subcellular localization, with cytoplasmic (CA-I, CA-II, CA-III and CA-VII) (1,2) cell surface membrane (CA-IV) (3,4,5), mitochondrial (CA-V) (6), and secretory (CA-VI) (7,8,9) forms, all of which have been described before. The cytoplasmic isoenzymes have been studied in the greatest detail. Recently, poor yields of the membrane-bound enzyme (CA-IV) from cumbersome purification procedures have prevented extensive studies on the structure/function relationship of the membrane-bound isoenzyme.

The first membrane-associated CA purified to homogeneity was obtained from bovine lung (3). It was

characterized as a disulfide bond-containing glycoprotein with an apparent molecular weight of 52,000 and designated CA-IV to distinguish it from the three known cytoplasmic isozymes CA-I, CA-II and CA-III (3). Bovine CA-IV had the unique property of being stable for up to several hours in 1-5% SDS solution, which facilitated its isolation by affinity chromatography. However, the enzyme was unstable in SDS after 24 h and could not be characterized extensively. Several years later, a different type of purification of a membrane-bound carbonic anhydrase from human kidney membranes was reported (4,10). The apparent molecular weight was initially reported to be 68,000 (10), but more recent purification by this method yielded an inactive polypeptide with a Mr of 34,400 on SDS-PAGE (4).

This study aimed to shed light on whether CA-IV is present in a human being's erythrocyte membrane or not. Previous studies report that CA-IV is available only to lung and kidney-cell membranes. Therefore, membrane-bound CA (CA-IV) has been purified from human erythrocyte membrane and characterized.

## The Experiments

### Obtaining Blood Samples and Preparation of Erythrocyte Membranes

Twenty blood samples were obtained from 20 different human beings, and these samples were placed in bottles with ACD. The samples were centrifuged at 5,000 rpm for 15 min. The white blood cells and plasma were removed. The packed red cells were washed three times with saline (0.9% NaCl) and they were lysed with chilled water, yielding destroyed plasma membranes. The membrane fraction was obtained with centrifugation of 20,000 rpm for 1 hour, washing the fraction and centrifugation was continued until the colorless content was obtained. The final material was treated with 0.05 M Tris-SO<sub>4</sub> (pH: 7.4) and TritonX-100 (1%) and the membrane particles were destroyed in an ultrasound dismembrator for four hours, followed by centrifugation (20,000 rpm for 30 min). The solution was dialyzed against distilled water for 2 days and against a buffer (0.05 M Tris-SO<sub>4</sub>, pH: 7.4) for 1 day. After the dialysis process, the pH of the solution was adjusted to 8.7 with solid Tris (11).

### Enzyme Purification

The enzyme was purified with a sepharose-4B-L-tyrosine-sulphanilamide affinity column. The column was balanced with solution of 25 mM Tris-HCl/ 0.1 M Na<sub>2</sub>SO<sub>4</sub>, pH: 8.7. The hemolysate was applied to the column. The column was then washed with 400 mL of solution of 25 mM Tris-HCl/ 22 mM Na<sub>2</sub>SO<sub>4</sub>, pH: 8.7, resulting in a significant amount of adsorption of CA-IV on affinity gel. Using a buffer, we carried out the elution of CA-IV from the column (0.1 CH<sub>3</sub>COONa/ 0.5 M NaClO<sub>4</sub>, 0.01 mM EDTA, and pH: 5.6). The elution was stopped at the point where no further absorbance was obtained at 280 nm. The column was then rebalanced (12).

### Protein Determination

After scanning at 280 nm, the tubes with significant absorbance were pooled and a quantitative protein determination was done by the Coomassie brilliant blue G-250 method (13).

### Enzyme Activity Determination

Esterase and hydratase activities were determined for the isoenzyme.

### CO<sub>2</sub>-Hydratase Activity Determination

Two mL of veronal buffer (pH: 8.2), 0.2 mL of brometymol blue (0.004%), 0.8 mL of diluted enzyme, and 2 mL of a CO<sub>2</sub> solution (saturated 0°C) were mixed. The time ( $t_c$ ) interval was determined between the addition of CO<sub>2</sub> solution and the occurrence of a yellow-green color. The same interval was recorded without an enzyme solution ( $t_0$ ). The activity was calculated from the formula given below (14),

$$1 \text{ Wilbur-Anderson Unit} = (t_0 - t_c) / t_c$$

### Esterase Activity Determination

The principle of this determination is that the substrate of CA (p-nitrophenylacetate) 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<sub>4</sub>, pH: 7.4) and 1.5 mL of substrate were mixed in a cuvette and, 3 min later, the absorbance was measured (348 nm, 25°C). A blank measurement was obtained by preparing the same cuvette without the enzyme solution (15).  $V_{max}$ ,  $K_M$  and optimal pH were determined through 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 0.1 ml and buffer was added to make up a total volume of 3 ml in the determination of  $V_{max}$ ,  $K_M$  values.  $V_{max}$  and  $K_M$  values were determined from the Lineweaver-Burk graph.

### Enzyme Activity Determination in Inhibitor

For this purpose, sulfanilamide, NaN<sub>3</sub> and KSCN were used as inhibitors. The Rickly method was used for the determination of the hydrolysis activity of the enzyme (14).

### SDS-PAGE Electrophoresis

The purity of isoenzymes eluted from the affinity column were controlled by SDS polyacrylamide gel electrophoresis (16). Bovine erythrocyte carbonic anhydrase was purified by affinity chromatography and used as a standard (12).

### Molecular Weight Determination with Gel Filtration

For this purpose, sephadex G-150 was incubated with distilled water at 90°C for 5 hours and then poured into a column (3 cm x 70 cm). The column was balanced for 24 hours with the buffer (0.05 M Na<sub>3</sub>PO<sub>4</sub>, 1 mM dithiothreitol, pH: 7) until no absorbance at 280 nm was

obtained. A protein standard solution was added to the column and standard graphics were obtained. The concentration of protein solution was 0.2 mg/ml. The standard proteins and CA-IV were eluted under the same conditions in separate steps. The flow rate through the column was 20 mL/h (17).

## Discussion

The purpose of this study, was to, determine the presence of carbonic anhydrase in the human erythrocyte plasma membrane.

The enzyme was purified from bovine lung and kidney cell membranes, and the authors wanted to know whether it was present in human red cell plasma membranes. It was reasonable to assume that the cells have the enzyme in their membranes.

For this purpose, the cells were hemolyzed; the hemolysate was washed repeatedly in order to quantitatively remove the cytoplasmic CA from the medium. The enzyme was removed from the membrane with TritonX-100, and was purified by affinity chromatography (Table). At the end of this study, the presence of the enzyme was determined and in the second part of study it was characterized.

It was detected that the enzyme had a high hydratase activity. This can be seen from the activity-absorbance graphics (Figure 1). It was also seen that the enzyme has an effect on p-nitrophenylacetate, giving us  $V_{max}=1.517 \cdot 10^{-1} \mu\text{M/L.min}$  and  $K_M=1.78 \text{ mM}$  values for the enzyme.

The optimum pH value of CA from human erythrocyte plasma membrane was 7.5. CA had activity in the pH range of 6.5-7.5, which is the same for CA from bovine erythrocyte plasma membrane, which has an optimum pH of 7.5 (18) (Figure 2). We know that blood pH is 7.4.

The optimum temperature for human erythrocyte plasma membrane CA was 35°C (Figure 3). Other mammalian CA optimum temperatures are ~37°C (18).

The Mr of the CA from human erythrocyte, determined by gel filtration chromatography, was ~36,600. This is higher than the Mr of the human erythrocyte CAs (CA-I and CA-II, 30,000), but near to that of bovine erythrocyte plasma membrane CA (52,000) (18) (Figure 4). The result, for the human erythrocyte plasma membrane is different from that of bovine erythrocyte plasma membrane CA. But the result is similar to human leucocyte plasma membrane CAs (19).

Steps	Volume (ml)	Activity EU/ml	Total Activity		Protein mg/ml	Specific Activity EU/mg protein	Purification fold
			EU	%			
Hemolysate	80	1.29	103.2	100	20.215	0.0638	-
Purified Enzyme	20	4	80	77.5	0.526	7.604	119.19

Table: Carbonic anhydrase from human erythrocyte membrane.

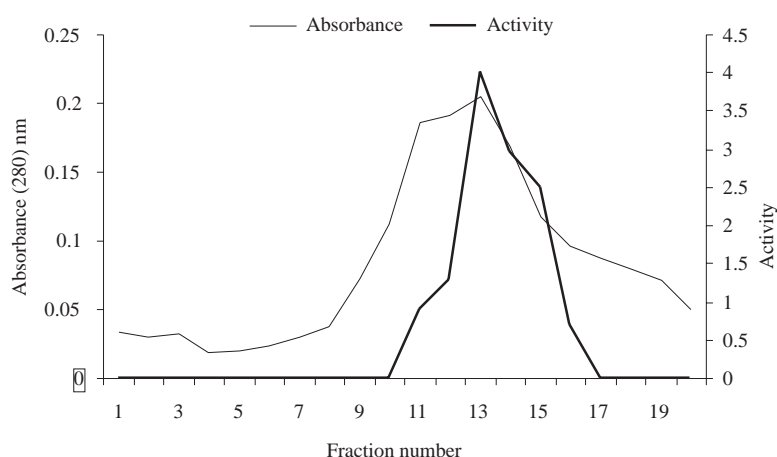


Figure 1. The activity-absorbance graphs of carbonic anhydrase enzyme from Sepharose-4B-L-Tyrosine-Sulphanilamide affinity chromatography; elution with  $\text{NaCH}_3\text{COO}/\text{NaClO}_4$  (pH=5.6), column 1.5 x 35 cm, flow rate 15 ml/h, elution volume 5 ml.

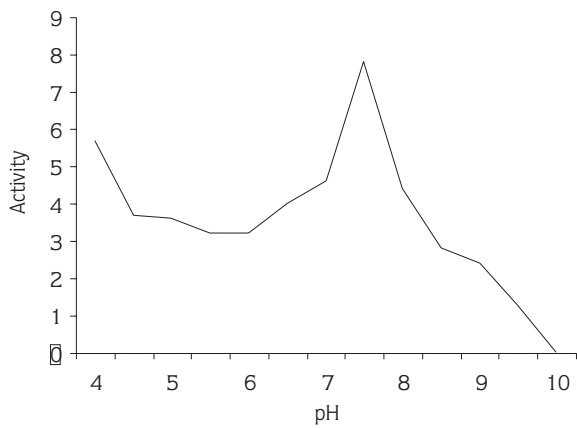


Figure 2. Activity of carbonic anhydrase from human erythrocyte membrane in Tris- acetate buffer of pH= 4-10.

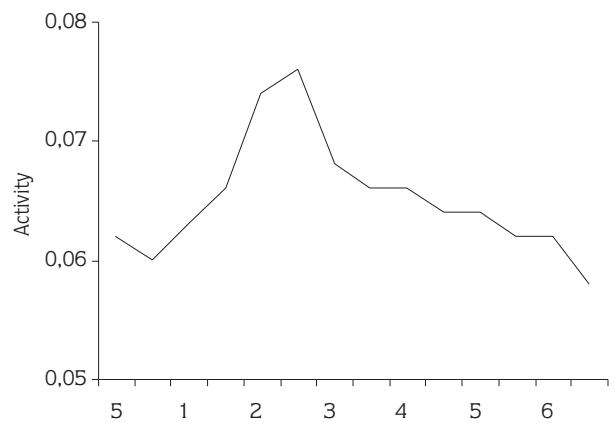
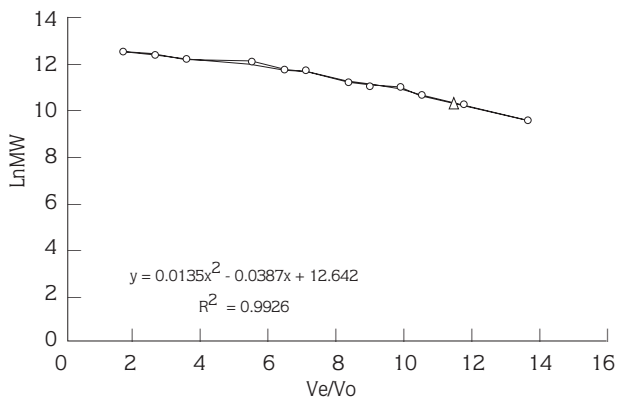


Figure 3. Effect of temperature on purified carbonic anhydrase from human erythrocyte membrane.



1	Hemoglobin	<i>monomer</i>	16.000
2	Hemoglobin	<i>dimer</i>	32.000
3	Hemoglobin	<i>trimer</i>	48.000
4	Hemoglobin	<i>tetramer</i>	64.000
5	Albumin	<i>monomer</i>	66.000
6	Hemocyanin	<i>monomer</i>	70.000
7	Albumin	<i>dimer</i>	132.000
8	Hemocyanin	<i>dimer</i>	140.000
9	Albumin	<i>trimer</i>	198.000
10	Hemocyanin	<i>trimer</i>	210.000
11	Albumin	<i>tetramer</i>	264.000
12	Hemocyanin	<i>tetramer</i>	280.000

Figure 4. Gel-filtration analysis of carbonic anhydrase from human erythrocyte membrane. The chromatography was on a Sephadex G-150 column in 0.05 M sodium phosphate, 1 mM dithiothreitol, pH 7.0. ( $V_e$  elution volume,  $V_o$  column void volume)

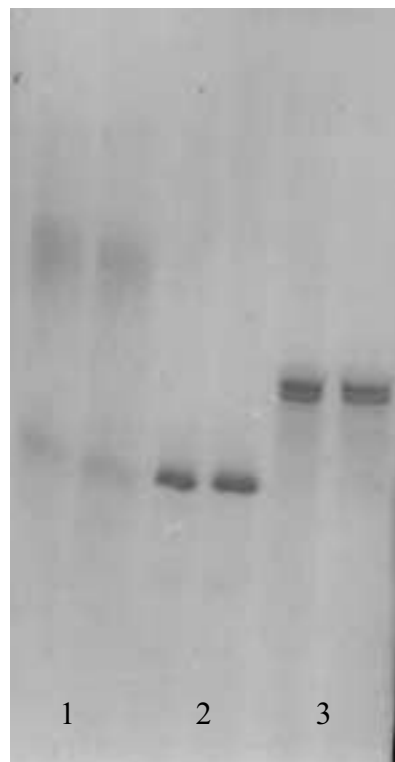


Figure 5. SDS-PAGE electrophoretic pattern of human erythrocyte plasma membrane carbonic anhydrase. Bovine membrane CA (1), bovine erythrocyte CA (2), human erythrocyte membrane CA (3).

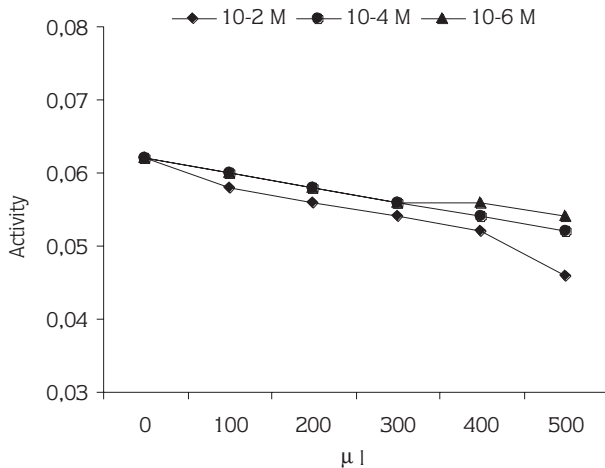


Figure 6. Effect of sulphanilamide on purified carbonic anhydrase from human erythrocyte membrane.

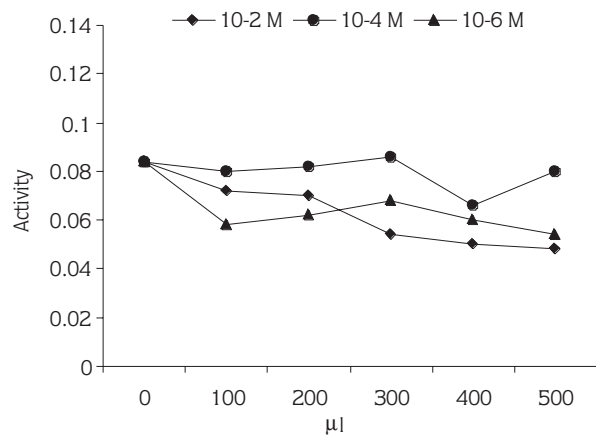


Figure 7. Effect of KSCN on purified carbonic anhydrase from human erythrocyte membrane.

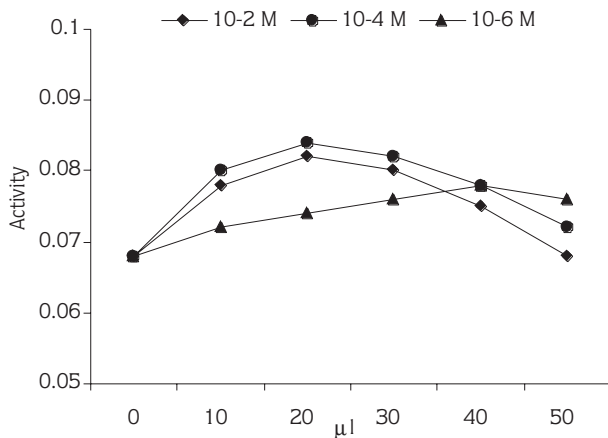


Figure 8. Effect of  $\text{NaN}_3$  on purified carbonic anhydrase from human erythrocyte membrane.

Sulfanilamide, KSCN and  $\text{NaN}_3$  inhibited the activity of carbonic anhydrase (Figures 6- 8). This result is parallel to all known mammalian CA result (20).

The function of the enzyme in the membrane may be the transport of electrolytes such as  $\text{CO}_2$ ,  $\text{H}^+$ ,  $\text{HCO}_3^-$ , and  $\text{Cl}^-$  (9). The enzyme has a very important in the transport activity for the membrane and it is necessary to study its of physiological importance. In this enzyme there may be a pump.

The isolation of CA from human erythrocyte membrane is not an easy procedure; but a complicated and, an important phenomenon, since one can think that CA is in all cell membranes. Studies on this subject are underway.

Bovine CA was used as a standard for 3-10% SDS electrophoresis, and the Mr of each subunit of carbonic anhydrase was 36,600 (Figure 5). The result is different from that of bovine erythrocyte plasma membrane CA, human erythrocyte CA I and CA II isoenzymes. But the result is similar to human leucocyte plasma membrane CAs (19).

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