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# A New Method for Purification of Carbonic Anhydrase Isozymes by Affinity Chromatography

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**Abstract**—A new affinity gel for purification of carbonic anhydrase isozymes was prepared using EUPERGIT$^R$ C-250L derivatized with *p*-aminobenzenesulfonamide, an inhibitor of carbonic anhydrase. The binding capacity of the affinity gel was determined at different temperatures, pH values, elution buffers, and ionic strengths. Human carbonic anhydrase isozymes (HCA I and HCA II) and bovine carbonic anhydrase (BCA) were purified in high yields from erythrocytes.

*Key words*: EUPERGIT$^R$ C-250L, carbonic anhydrase, affinity chromatography

Carbonic anhydrase (CA) is a zinc-containing metalloenzyme that is widespread in nature and catalyzes the reversible hydration of $CO_2$ to $HCO_3^-$ and $H^+$. In mammals, CA plays an important role in respiration by facilitating the transport of $CO_2$. In plants, carbonic anhydrases are involved in the photosynthetic fixation of $CO_2$. Mammalian erythrocytes contain two distinct forms of CA, distinguished by differences in their catalytic activities. Carbonic anhydrase isozymes differ in their subcellular localization, with cytoplasmic (CA I, CA II, CA III, and CA VII), cell surface membrane (CA IV), mitochondrial (CA V), and secretory (CA VI) forms, all of which have been described [1, 2].

Affinity chromatography is a powerful and generally applicable technique that is distinctly advantageous for the rapid purification of a substance from a complex mixture of proteins [3]. This technique takes advantage of the high affinity of many proteins for specific ligands or chemical groups. Methods for purifying CA-I, CA-II, and BCA from different tissues by affinity chromatography have been described. Aromatic and heteroaromatic sulfonamides used as ligands in these methods are specific and strong inhibitors of CA [4-6]. A large number of affinity gels, using a variety of matrices, spacer arms and ligands, have been described in the literature [7-10].

EUPERGIT$^R$ C-250L has been reported to be useful as a matrix for the purification of several proteins by virtue of its functional oxirane group, in spite of a rather strong nonspecific adsorption during the initial cycles [11-13]. The chemical structure of the matrix offers high chemical stability in aqueous medium over a wide pH range. Also, changes in pH and ionic strength have no effect on matrix swelling [14].

The present report describes the successful purification of carbonic anhydrase (EC 4.2.1.1) from erythrocytes using an affinity adsorbent consisting of EUPERGIT$^R$ C-250L coupled with an enzyme inhibitor. To achieve high purification a selective elution step had to be applied.

## MATERIALS AND METHODS

**Materials.** EUPERGIT$^R$ C-250L was obtained from Rohm Pharma Chem. Co. (Germany) and *p*-aminobenzenesulfonamide from Merck (Germany). Blood samples with acid-citrate-dextrose (ACD) were obtained from humans and bovines. All other chemicals were of analytical grade.

**Preparation of affinity gel.** One gram of EUPERGIT$^R$ C-250L was suspended with 5 ml of phosphate buffer, pH 7.5, for 72 h in a shaker at room temperature. After the incubation, 2 g *p*-aminobenzenesulfonamide in 10 ml of cold 1 M HCl was added to the suspension. The mixture was stirred with a magnetic stirrer and maintained at pH 7.5 for 3 h at room temperature (see Scheme). The coupled EUPERGIT$^R$ C-250L derivative

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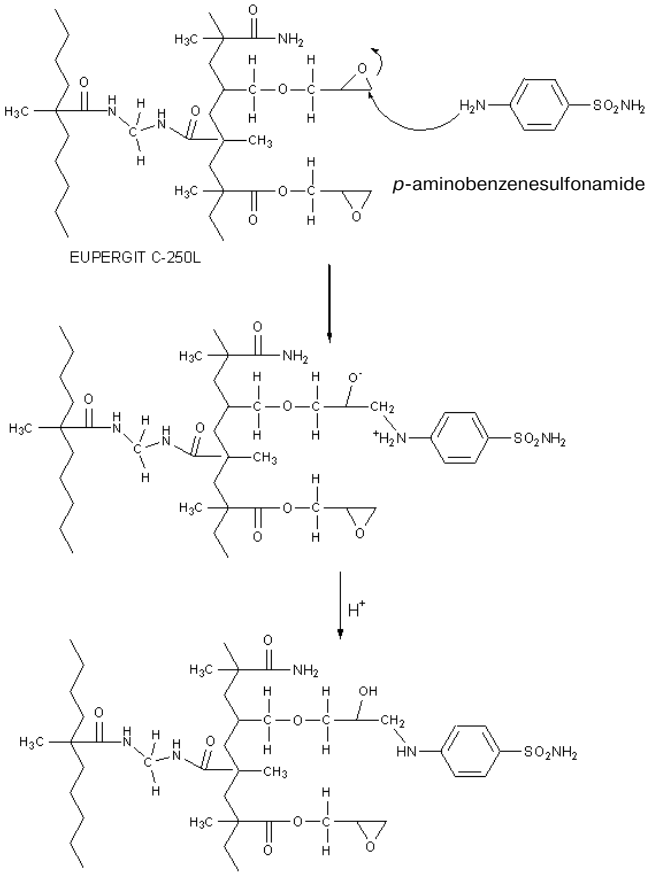


Synthesis of the affinity gel

**Scheme**

was washed with 1 liter of distilled water followed by 200 ml of 0.05 M Tris-sulfate, pH 7.5.

**Purification of carbonic anhydrase from bovine and human erythrocytes.** Bovine blood samples were obtained from the Balikesir slaughterhouse using bottles containing anticoagulant (ACD). The blood samples were centrifuged at 1850*g* for 20 min and the plasma and buffy coat were removed. After washing the packed red cells three times with NaCl (0.9%), the erythrocytes were hemolyzed with cold water. The ghosts and intact cells were removed by centrifugation at 18,900*g* for 30 min at 4°C [4]. The hemolysate was applied to the EUPERGIT$^R$ C-250L-*p*-aminobenzenesulfonamide affinity column equilibrated with 25 mM Tris-HCl/0.1 M $Na_2SO_4$ (pH 7.0). The affinity gel was washed with 25 mM Tris-HCl/22 mM $Na_2SO_4$ (pH 7.0), and CA isozymes were eluted under different elution conditions.

**Protein determination.** After scanning at 280 nm, the tubes with significant absorbance were pooled and a quantitative protein determination was then performed by the Coomassie Brilliant Blue G-250 method [15].

**Enzyme assay.** Carbonic anhydrase activity was measured by the Maren method [16], which is principally based on the determination of the time required for solution pH to decrease from 10.0 to 7.4 due to hydration of $CO_2$.

**SDS-PAGE of carbonic anhydrase.** The purity of isozymes HCA I, HCA II, and BCA from the affinity column was assessed by SDS polyacrylamide gel electrophoresis according to the method of Laemmli [17].

## RESULTS AND DISCUSSION

In this study, a new affinity gel was prepared for the purification of CA isozymes from a variety of sources. EUPERGIT$^R$ C-250L was selected as a matrix due to its long operational life, stability to mechanical stress, and possession of favorable flow rates. These features are particularly important during routine purifications for large-scale production.

Several analogs of sulfonamide have been demonstrated to possess good binding affinities for carbonic anhydrase [18]. According to this, *p*-aminobenzenesulfonamide was chosen as a ligand, since it is a specific and strong inhibitor of CA. The gel was synthesized by means of a consecutive reaction (Scheme). *p*-Aminobenzenesulfonamide was bound to the oxirane groups on EUPERGIT$^R$ C-250L by means of a covalent amide bond. The oxirane groups on EUPERGIT$^R$ C-250L also serve as spacer-arms on the affinity gel. Accordingly, the requirement for any activation method has been avoided. In the literature there are several types of methods that have been used with different matrices and different spacer arms that undergo activation methods. It is known that affinity gels prepared with these activation methods can undergo partial deterioration in the course of the reaction [2, 4, 5].

The present paper deals with the use of a weakly hydrophobic matrix with non-activated spacer arms onto which the ligand is coupled. The affinity gel has good flow properties without any adverse effects of the environmental conditions. HCA I, HCA II, and BCA were purified using the affinity gel with different elution buffers. The most suitable elution buffers were 0.1 M $CH_3COONa$/0.5 M $NaClO_4$ (pH 5.6), for BCA and HCA I; and 0.1 M NaCl/25 mM $Na_2HPO_4$ (pH 6.3), for HCA II (Fig. 1).

The eluates were characterized by protein determination at 280 nm and assaying $CO_2$ hydratase activity for HCA I and HCA II (Fig. 2) and for BCA (Fig. 3). Specific activities for HCA (as HCA I + HCA II) and BCA were calculated by using hemolysate and purified enzyme solution. There was a 258-fold purification of HCA and a 478-fold purification of BCA. These values are very similar to those for a Sepharose-4B-L-tyrosine-sulfonylamide affinity gel. In addition, the capacity of the prepared affinity gel is higher than some of the reported affinity gels, and equivalent to others [5, 6]. The purity of our enzyme preparation was assessed by SDS-PAGE. The

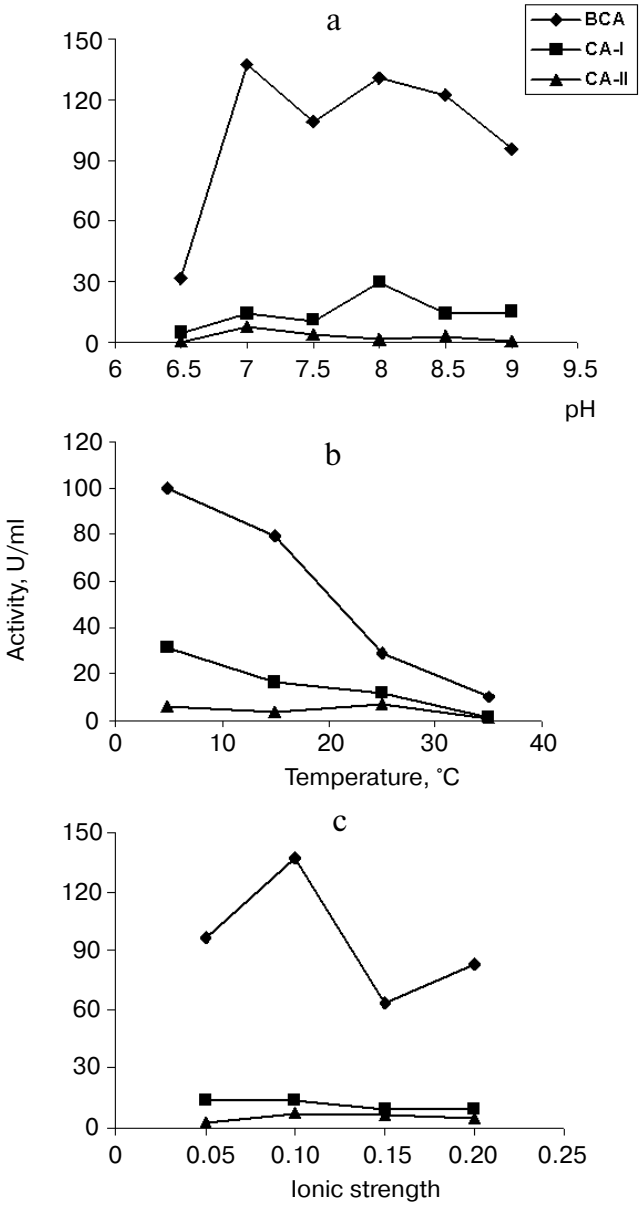


**Fig. 1.** Effect of pH (a), temperature (b), and ionic strength (c) on purification of BCA and HCA.

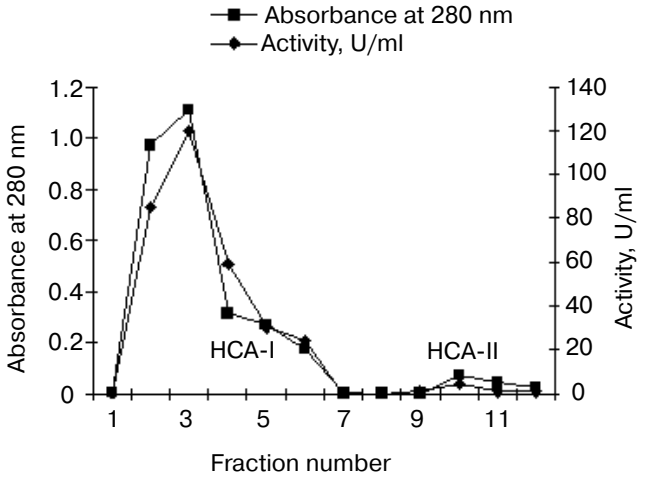


**Fig. 2.** Purification of HCA I and HCA II using the affinity gel. The column (1.36 × 10 cm) was eluted with 0.1 M NaCl/25 mM $Na_2HPO_4$ (pH 6.3) buffer for HCA I, and 0.1 M $CH_3COONa$/0.5 M $NaClO_4$ (pH 5.6) buffer for HCA II. The flow rate was 20 ml/h, and the fraction volume was 5 ml.

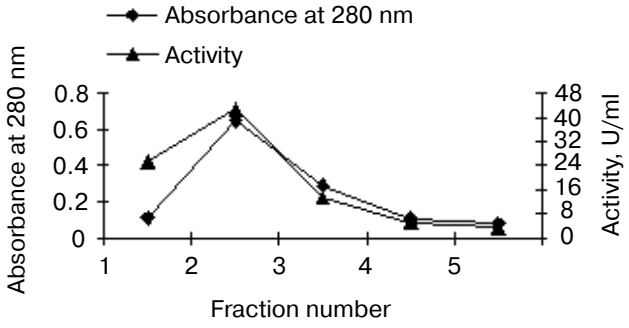


**Fig. 3.** Purification of BCA using the affinity gel. The column (1.36 × 10 cm) was eluted with 0.1 M $CH_3COONa$/0.5 M $NaClO_4$ (pH 5.6) buffer at 20 ml/h flow rate with fraction volume of 5 ml.

purified bovine and human isozymes migrated as single bands in all cases, with apparently identical molecular masses (Fig. 4).

The binding capacities of the affinity gel for the HCA I and HCA II isozymes and BCA were determined at different temperatures, pH values, and ionic strengths (Fig. 1). Maximum binding was achieved at 5°C with pH 7.0 and ionic strengths around 0.1. These results are similar to those from other studies in the literature [2, 4, 5].

Thus, the EUPERGIT$^R$ C-250L-*p*-aminobenzenesulfonamide affinity gel is shown to be favorable for the purification of HCA I, HCA II, and BCA in active form.

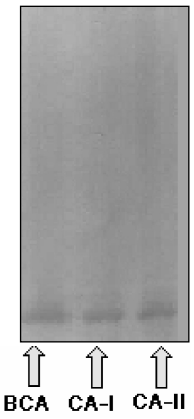


**Fig. 4.** SDS-PAGE pattern of CA isozymes.

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