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Characterization of functionally active immobilized carbonic anhydrase purified from sheep blood lysates

Maitreyee Chandra [a,1], Abdul Waheed [b], Rakesh K. Singh [c,*]

[a] Department of Biological and Health Sciences, Diablo Valley College, Pleasant Hill, CA, United States
[b] Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, MO, United States
[c] Department of Food Science and Technology, University of Georgia, Athens, GA, United States

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ABSTRACT

Carbonic anhydrase (CA) catalyzes the reversible reaction of hydration of CO_2 to bicarbonate and the dehydration of bicarbonate back to CO_2. Sequestration of CO_2 from industrial processes or breathing air may require a large amount of highly active and stable CA. Therefore, the objectives of the present study were to purify large amounts of CA from a cheap and easily accessible source of the enzyme and to characterize the enzymatic and kinetic properties of soluble and immobilized enzyme. We recovered 80% of pure enzyme with a specific activity of 4870 EU/mg protein in a single step using sheep blood lysates from slaughter house waste products and CA specific inhibitor affinity chromatography. Since affinity pure CA showed both anhydrase and esterase activities, we measured the esterase activities for enzymology. The Michaelis–Menten constant, K_M, pH optimum, activation energy, and thermal stability of soluble enzymes were 8×10^{-2} M, 7.3 pH, 7.3 kcal/mol and 70 °C, respectively.

The immobilization of the enzyme to Affigel-10 was very efficient and 83% of purified enzyme was immobilized. The immobilized enzyme showed a K_M of 5×10^{-2} M and activation energy of 8.9 kcal/mol, suggesting a better preference of substrate for immobilized enzyme in comparison to soluble enzyme. In contrast to soluble enzyme, immobilized enzyme showed relatively higher activity at pH 6–8. From these results, we concluded that a shift in pH profile toward acidic pH is due to modification of lysine residues involved in the immobilization process. The immobilized enzyme was stable at higher temperatures and showed highest activity at 80 °C. The activity of immobilized enzyme in a flow reactor at 0.5–2.2 ml/min flow rate was unaffected. Collectively, results from the present study suggested the application of blood lysate waste from animal slaughterhouses for purification of homogeneous enzyme for CO_2 capture in a flow reactor.

1. Introduction

In a close workplace or in closed loop anesthesia, control of the respiratory gas environment, especially CO_2, is very challenging because of a low concentration of CO_2 requiring a high volume of CO_2 scrubbing [1–4].

In the mammalian body, different tissues, e.g., lung and kidney, encounter similar challenges for CO_2 capture, transport, and removal at a very low concentration of CO_2 in a large volume. This barrier is overcome by an enzyme, carbonic anhydrase (CA; carbonate dehydratase, carbonate hydrolase, EC 4.2.1.1). The basic physiological function of the CA enzyme is to catalyze the hydration of CO_2 to produce HCO_3 and H which regulate the acid–base balance in tissues and biological fluids. Carbonic anhydrases are zinc metalloenzymes that catalyze the interconversion of carbon dioxide and bicarbonate ion ($CO_2 + H_2O \leftrightarrow H^+ + HCO_3{}^-$). They occur in the form of fifteen isoenzymes, which differ in their locations, distributions, kinetic properties and in their sensitivity to inhibitors. They have an apparent molecular weight of 29–55 kDa [5]. Among the cytosolic isoenzymes CA I and CA II represent the major nonhemoglobin proteins of the erythrocyte [6].

CA I (previously known as CA B) is the most abundant isoenzyme (11.6 mg/g hemoglobin) with a low enzyme activity. It is sensitive to iodide ion and sulfonamides. The second isoenzyme, designated carbonic anhydrase II (CA II), also known as CA C is present in lesser amounts. It is referred to as the "high activity" isoenzyme because of its higher specific activity. The human CA II isoenzyme has a turnover number for CO_2 hydration equal to $1.3–1.9 \times 10^6$/s, which is the highest known for any enzyme [7]. In comparison with CA I, CA II is more sensitive to sulfonamides but, unlike CA I, it is

* Corresponding author at: Department of Food Science & Technology, University of Georgia, 100 Cedar Street, Athens, GA 30602-2610, United States.
Tel.: +1 706 542 1084; fax: +1 706 542 1050.
E-mail address: rsingh@uga.edu (R.K. Singh).

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resistant to iodide ion. Other mammalian species have distinct high and low activity carbonic anhydrase isoenzymes, although only the high activity form occurs in bovine erythrocytes [8]. For practical purposes, the esterase activity of CA II is determined instead of the anhydrase activity [9].

X-ray crystallographic studies of CA II have revealed that the active site of the enzyme contains a conical cleft of 15 Å depth. While one side of the cavity is formed by hydrophobic residues, the other side contains hydrophilic residues, including Thr 199 and Glu 106. The zinc ion, which is located at the bottom of this cleft, is tetrahedral coordinated to the imidazoles of three histidine residues (His 94, His 96 and His 119) and to a water molecule (called the "zinc water" that ionizes to a pK around 7 [10]. This zinc coordination polyhedron is a conserved feature among carbonic anhydrases. It is difficult to accurately measure the very fast reaction rate of enzymic hydration of CO_2 by carbonic anhydrase (CA). The active form of CA has a "zinc water" ligand which has a pK_a around 7 [10]. The active form of the enzyme for hydration of CO_2 shows the ligand in the H—O—H form (i.e., protonated) and this will be 50% in the protonated form at pH = pK_a (i.e., around 7). Below the pK_a over 50% of the enzyme will be in the deprotonated (OH^-) form and will therefore catalyze the reverse reaction (dehydration back to CO_2 and water).

In addition to the reversible hydration of carbon dioxide, carbonic anhydrase also possesses other catalytic activities [11–16]. Thus, CA serves not only as anhydrase for CO_2, acetaldehyde and related carbonyl compounds, but also as an esterase for p-nitrophenyl acetate (p-NPA) [17].

The carbonic anhydrase catalyzed hydrolysis of p-NPA exhibits certain similarities with the enzymatically catalyzed hydration of CO_2. The pH-rate profile of carbonic anhydrase catalyzed hydrolysis of p-NPA is sigmoidal in shape below pH 9.0, which is analogous to that observed in the reversible hydration of CO_2 [18,19]. The esterase activity of CA with substrate p-nitrophenyl acetate has been correlated with the enzymatically catalyzed hydration of CO_2 by CA. Thus, there seems to be a close correspondence with pH dependence of the CO_2 hydration reaction, suggesting that these various catalytic properties are shared by the same active site.

Over the years, CA has been purified from a number of sources. One of the cheapest and most readily available sources of CA is mammalian blood. There are about 12 g of carbonic anhydrase per liter of mammalian red cells [20,21]. Various established purification protocols of CA are present in the literature [8,20,22,23]. However, the most commonly used purification method involves the use of single step inhibitor affinity chromatography, which gives high yields of the enzyme from a wide variety of sources [24–26].

Studies on the effects of thermal denaturation on the catalytic properties of carbonic anhydrase have revealed that below 60 °C, the enzyme is very stable and it recovers almost all of its biological activity [27]. Between 60 and 65 °C, a drastic decrease in the biological activity occurs while above 65 °C, the residual activity drops to zero only after a few minutes.

The stability of the purified enzyme can be greatly increased by immobilizing it on to a support. The advantages of enzyme immobilization include increased stability, recycling, and ease of recovery, all of which are helpful in applications to flow reactor design, biosensors, and affinity chromatography [28]. CA has been immobilized on a number of supports [29–33]. In general, all these immobilizations led to increased stability of the enzyme both in terms of pH and temperature effects.

Beaded agarose gels are most commonly used as immobilization matrix. Agarose gels provide high porosity beads with good mechanical strength and flow properties. Immobilization using N-hydroxysuccinimide ester derivatives of agarose (Affigel-10) provides a rapid method of immobilization under mild conditions (pH 6–8), and for proteins result in good retention of biological activity upon immobilization. Affigel-10 reacts with amine-containing ligands, forming stable amide bonds between ligand and matrix. Therefore, in this study, Affigel-10 was used as a support for immobilizing purified CA.

The objectives of this study are the following: (1) develop an application for a slaughterhouse waste product for CO_2 capture, (2) extract and purify CA in large quantities from blood lysates, (3) immobilize CA on a support and determine the kinetic constants of purified soluble and immobilized CA, (4) investigate the effect of pH and temperature on the activity of both soluble and immobilized enzyme, (5) carry out stability studies on the immobilized CA, and (6) investigate the application of immobilized enzyme in CO_2 capture in a continuous flow catalytic converter.

2. Materials and methods

2.1. Materials

Bovine serum albumin, Tris base, bicinchoninic acid, Coomassie Brilliant Blue R-250, 2-mercaptoethanol, isopropanol, phenol red and ethanolamine were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol, HCl, H_2SO_4, sodium sulfate and glycine were purchased from Mallinckrodt Chemical, Inc. (Paris, KY). Sodium bicarbonate and sodium acetate were purchased from E.M. Science, Inc. (Gibbstown, NJ). Sodium hydroxide was purchased from J.T. Baker, Inc. (Phillipsburg, NJ) and sodium perchlorate was obtained from GFC Chemicals, Inc. (Columbus, OH). Affigel-10, protein molecular weight standards, 40% Acrylamide/Bis solution and TEMED were purchased from Biorad (Hercules, CA). Copper sulfate, urea and glycerin were purchased from Fischer Scientific Company (Fair Lawn, NJ). All chemicals were of analytical grade. Dialysis tubing and Centricon-10 micro concentrators were purchased from Spectraphor (Laguna Hills, CA) and Amicon (Danvers, MA), respectively.

2.2. Preparation of homogenous blood sample

The mammalian erythrocyte cells (RBC) are a rich source of carbonic anhydrase. Large samples of bovine, sheep and swine blood were obtained from the Purdue University slaughter facility for isolation of enzyme. CA from sheep blood had been previously purified, characterized, and found to contain highly active enzyme [34]. Therefore, it was decided to use sheep blood for this study. Fresh blood collected in trays was frozen for a period of four days at −20 °C. The complete stock of frozen blood was freeze dried to maintain homogeneity of samples for the entire project. The blood was weighed before and after freeze drying to determine the amount of moisture content. Freeze-dried blood was initially coarsely ground in a mortar pestle and then finely powdered in an analytical mill (Model AI0S2, Tekmar Company, Cincinnati, OH). The powdered blood was stored in air tight containers at room temperature and was later used for future experiments. The freeze dried blood was further vacuum dried in a vacuum oven (Model 5831, Narco E series, Portland, OR) to ensure that it had no moisture.

2.3. Preparation of affinity-chromatography gel

The affinity matrix was prepared from Affigel-10 by the method of Zhu and Sly [24] with slight modifications by coupling the ligand, para-aminobenzene sulfonamide, which is a carbonic anhydrase inhibitor, to Affigel-10. The entire process of washing the gel and coupling it with the ligand solution was completed within 20 min as suggested by the Biorad Affigel manual (Biorad, Hercules, CA). The slurry was gently rocked in a rotor (Model PR70, Hoefer Scientific Instruments, San Francisco, CA) for 1 h at room temperature and for 4 h at 4 °C. The reaction was blocked by adding 0.1 ml of 1 M ethanolamine HCl (pH 8.0) per ml gel and the mixture was incubated for 1 h at room temperature. The affinity gel was packed in a column and washed extensively and progressively with 50 mM Tris sulfate (pH 8.0), water and 0.1 M sodium acetate (pH 5.6). Finally, the affinity resin was washed with 50 mM Tris sulfate (pH 8.0). Before use, the column was equilibrated with several volumes of 10 mM Tris sulfate (pH 9.0). When not in use, the column was stored at 4 °C.

2.4. Purification of carbonic anhydrase from sheep erythrocyte

Keeping in mind the application of slaughterhouse waste product for bioremediation, we chose sheep blood for this study. The entire purification process can be divided into 2 stages: (a) extraction of the crude enzyme; and (b) purification using affinity chromatography.

2.5. Extraction of crude enzyme

Prior to extraction, the dried blood was solubilized by adding three times the required volume of 50 mM Tris sulfate, pH 8.0, buffer. The solubilized blood was stirred for 30 min at room temperature to lyse the RBC, and the blood pH was