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Short communication

An automated method for measuring the operational stability of biocatalysts with carbonic anhydrase activity

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ABSTRACT

The operational stability of an enzyme can be quantified by its half-life, or the length of time after which 50% of its original activity has degraded. Ideally, continuous methods for measuring half-lives are preferred but they can be expensive and relatively low throughput. Batch methods, while simple, cannot be used for all enzymes. For example, batch reactions can be difficult when there is a gas phase reactant or when there is significant product or substrate inhibition. Here we describe a repeated-batch method for measuring the half-life of carbonic anhydrase (CA)-based biocatalysts by automated periodic switching between a forward and reverse reaction. This method is inexpensive and can be multiplexed for high-throughput analysis of enzyme variants. Several purified CA enzymes as well as whole-cell biocatalysts with engineered CA activity were evaluated with this method. The results indicate a significant increase in operational stability is achieved upon immobilization of CA in the cellular periplasm of *Escherichia coli*.

1. Introduction

The stability of an enzyme or biocatalyst is a critical parameter. It can dictate the proper storage, optimal reaction conditions, and functional lifetime of a biocatalyst. However, the term "stability" covers a broad range of properties including thermal stability, pH stability, and operational (or kinetic) stability [1–5]. The stability of an enzyme is dependent on several phenomena including the structure of the protein, hydrophobic interactions, ionic interactions, hydrogen bonding, and other weak interactions [6–9]. Previously, researchers have explored methods to stabilize enzymes via directed evolution [10], rational mutagenesis of surface residues [11,12], the introduction of non-native disulfide bonds [13], and various immobilization techniques [14]. In most of these studies, the thermal stability of the enzymes is being measured and is assumed to be related to the overall stability of the catalyst, including the operational stability. However, this may not always be a valid presumption since the thermal stability does not account for the reaction being catalyzed [4]. Instead, the operational stability should be measured independently.

Ideally, the operational stability would be measured in a continuous reactor system such as a plug-flow reactor (PFR) or continuous stirred tank reactor (CSTR) under the process conditions desired. Such experiments would yield relevant stability data for biocatalysts to be used in processes [4]. But, these experiments are expensive and low throughput. Conversely, simpler batch-wise measurements can be performed, and these experiments generally involve mixing a large amount of substrate with the enzyme and allowing the reaction to progress for many half-lives of the enzyme [15]. Batch measurements are typically less expensive, but cannot be used for all types of biocatalysts. For example, the batch method is limited when there is significant substrate or product inhibition, or when substrates cannot be added in large quantities at the onset of the reaction. Thus, a repeated-batch method provides a compromise between these approaches. In such a system, the activity of a biocatalyst is measured in a series of batch reactions within a fixed range of substrate concentration [4]. By comparing these activities, the point at which 50% of the activity has degraded can be identified as the half-life of the biocatalyst. Here we develop an automated repeated-batch method for measuring the half-lives of carbonic anhydrase (CA; EC 4.2.1.1) biocatalysts.

CAs are well-studied, Zn^{+2}-binding metalloenzymes that catalyze the reversible hydration of carbon dioxide (CO_2) to bicarbonate (HCO_3^-) and carbonate (CO_3^{-2}) ions [16] according to the mechanism shown below [17]:

$$EZnH_2O \rightleftharpoons EZnOH^- + H^+ \quad (1)$$

$$EZnOH^- + CO_2 \rightleftharpoons EZnHCO_3^- \quad (2)$$

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$$EZnHCO_3^- + H_2O \rightleftharpoons EZnH_2O + HCO_3^- \quad (3)$$

$$HCO_3^- \rightleftharpoons CO_3^{-2} + H^+ \quad (4)$$

In this scheme, the reversible deprotonation of bicarbonate (Eq. (4)) occurs spontaneously as a function of pH, and is not catalyzed by the enzyme. Nearly ubiquitous in nature, CAs are among the fastest known enzymes with turnover numbers (k_{cat}) ranging from 10^4 to 10^6 s^{-1} [18,19]. While most of the prior research on CA has focused on isolation and characterization of these enzymes, recent work has explored immobilization of CAs using yeast [20] and *Escherichia coli* cells [21,22] for use as whole-cell biocatalysts. These catalysts have the benefits of enzyme stabilization and enhanced recyclability, while maintaining CO_2 hydration activity. In addition to these studies, CA enzymes have been investigated as mediators for carbon capture modalities, specifically carbon mineralization [23]. This technique involves the conversion of CO_2 into carbonate ions and subsequent precipitation with divalent cations such as Ca^{+2}. For example, in situ carbon capture from the flue gas of coal-fired power plants has been proposed and studied as a potential industrial application of CA enzymes [24]. Other applications for CA enzymes include biosensors [25], CO_2 fixation for artificial photosynthesis [26], and for biofuel production [27]. Despite the breadth of research performed on CAs, operational stabilities have not yet been reported. Some variants of CA possess alternative activities such as esterase activity [28] or peroxidase activity upon substitution of the bound Zn^{+2} ion with an Mn^{+2} ion [29]. For these special cases, measurement of the stability by a batch method would be possible. However, most forms of CA do not possess these alternative activities, and these activities are not related to the hydration activity of CA. Thus, determining the operational stability of CA using esterase or peroxidase activity may not be possible, or may lead to irrelevant results.

In this report, a method for measuring the operational stability of CA-based biocatalysts is described. The CA isoforms measured were bovine carbonic anhydrase from erythrocytes (BCA), carbonic anhydrase beta (Cab from *Methanobacterium thermoautotriphicum*; PDB: 1G5C) [30], and carbonic anhydrase methanosarcina (Cam from *Methanosarcina thermophila*; PDB: 1THJ) [31]. In addition to the purified forms of these enzymes, Cab and Cam were also tested after immobilization via periplasmic expression using two different periplasmic leader peptides (gIII and pelB), as previously described, to generate whole-cell biocatalysts [21]. These biocatalysts were selected for the various benefits they present. BCA was chosen because it is a commercially available CA that has been studied by several groups in the past. Cab and Cam were selected because they differ in their intrinsic thermal stabilities. Further, since these two isoforms were used in whole-cell biocatalysts, they provide us with the opportunity to examine the versatility of our technology, as well as examine the effects of periplasmic immobilization on operational stability.

2. Materials and methods

2.1. Materials

All chemicals, including BCA, were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Nylon tubing and push-to-connect fittings and joints for all gas lines were purchased from McMaster-Carr (Robbinsville, NJ).

2.2. Cloning, expression, and purification

Cloning, expression, and purification of all constructs were described previously [21]. Cab and Cam used in these experiments were purified from the pET-24a vector with no periplasmic leader sequence. The whole-cell biocatalysts used were BLR-gCab, BLR-pCab, BLR-gCam, and BLR-pCam where "g" indicates the gIII leader peptide is being used, while "p" indicates the pelB leader peptide is used [21].

2.3. Apparatus

A schematic of the apparatus can be seen in Fig. 1A. The apparatus consists of 4 main components: an ethylene glycol-filled water bath with a 250 mL plastic bottle (reaction vessel) and two 1 L plastic bottles (saturation vessels), an OMEGA USB-4718 Data Acqusition Module (DAQ; OMEGA Engineering, Inc., Stamford, CT), gas flow from compressed prepurified N_2 and bone dry CO_2 cylinders (TechAir, White Plains, NY), and a LabView Virtual Instrument (VI; National Instruments, Austin, TX) to control gas switching and record data. The pressure at each regulator outlet is continuously monitored using a pressure transducer (Omegadyne Inc., Sunbury, OH) connected to the DAQ. For each gas, flow is routed through a 24 V, 7 W, 175 psi solenoid valve (Peter Paul Electronics Co., Inc., New Britain, CT) before being passed through a saturation vessel containing 950 mL deionized water (DI H_2O) using a stainless steel gas sparger (Valco Instruments Co., Inc., Houston, TX). This hydrates the gas to reduce liquid loss in the reaction vessel. The solenoid valves are connected to the DAQ and switching between the gases is done using a 12 V, 15 A power relay (Tyco Electronics Corp., Wilmington, DE), also connected to the DAQ. Other components wired to the DAQ are a PHB-213 pH meter (OMEGA Engineering, Inc., Stamford, CT) and an immersion thermocouple (McMaster-Carr, Robbinsville, NJ). The hydration containers are linked via a T-joint and attached to the output is a fritted glass sparger (ACE Glass, Inc., Vineland, NJ).

2.4. Stability measurements

The filled saturation vessels and reaction vessel containing 100 mL of 100 mM Tris-HCl pH 9.0 were placed in the glycol bath set to the desired temperature (30 °C for this study). The pH probe, thermocouple, and glass sparger were placed in the reaction vessel and the top was wrapped in Parafilm. This reduced the loss of volume by evaporation in the reaction vessel. The VI was activated to initiate control over the apparatus. The relay was plugged in to start gas flow and the outlet pressure of each tank was adjusted to 6 psi. After the desired pH range was entered, data acquisition was started and the baseline was collected for 30–60 min, or until it stabilized. A pH range of 6.9–7.75 was chosen for this study for its consistency and reproducibility under the conditions used. This is within the effective range of Tris buffer which has an approximate pKa of 7.92 at 30 °C. However, the pH bounds can be set to any values, as long as the pH probe and buffer being used are effective within that range. Then, the desired catalyst was added to the reaction vessel (15–38 μg/mL for purified enzymes or 4.6×10^8–1.2×10^9 cells/mL for whole-cell biocatalysts). The reaction vessel was wrapped in Parafilm and the reaction was allowed to proceed for 24–48 h. For each run, 2 data files were generated: a trace of pH against process time, and the temperature, pressure, and the upswing and downswing periods against process time. The "downswing period" was defined as the amount of time required to traverse the defined pH range during the forward reaction (CO_2 sparging). The "upswing period" is defined the same way, but for the reverse reaction (N_2 sparging). Sample data obtained using this system can be seen in Fig. 1B and C.

2.5. Data analysis

To represent the operational stabilities of the biocatalysts tested, the half-life was selected as the relevant metric. For each