

Codon-optimized Carbonic Anhydrase from *Dunaliella species*: Expression and Characterization

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Abstract—Carbonic anhydrases (CAs) has been focused as biological catalysis for CO₂ sequestration process because it can catalyze the conversion of CO₂ to bicarbonate. Here, codon-optimized sequence of α type-CA cloned from *Dunaliella species*. (DsCAopt) was constructed, expressed, and characterized. The expression level in *E. coli* BL21(DE3) was better for codon-optimized DsCAopt than intact sequence of DsCAopt. DsCAopt enzyme shows high-stability at pH 7.6/10.0. In final, we demonstrated that in the Ca²⁺ solution, DsCAopt enzyme can catalyze well the conversion of CO₂ to CaCO₃, as the calcite form.

Keywords—Carbonic anhydrase, Codon-optimization, *Dunaliella species*, CO₂ sequestration

I. INTRODUCTION

ANTHROPOGENIC activities is cause of accumulation of greenhouse gases in the atmosphere. However, natural capacities - forest storage, ocean storage, and natural carbonate mineralization are not enough to maintain CO₂ level revoked by abrupt industrialization, population growth, energy consumption, and so on. Recently, many researchers have focused on the development of CO₂ sequestration processes, for example post-combustion carbon capture and storage (PCCCS). In this commercial PCCCS processes, various amine-based compounds have been used [1]. Although such chemical materials have good CO₂ absorption capacities, they have several drawbacks such as corrosion, solvent loss, generation of heat-stable salts, and high-energy consumption during regeneration [2]. At present, researchers are looking for more efficient ways to control the atmospheric CO₂ concentration [3]. In such view-point, much attention has been paid to biological CO₂-sequestration technology, in particular an enzyme-based CO₂ capture system [4, 5]. This enzyme-based system is prominent for development of efficient PCCCS process because enzyme-catalyzed reactions have high chemo-, regio-, and stereo- selectivity for CO₂ capture [6].

Carbonic anhydrase (CA; EC 4.2.1.1) is such biological catalysis for reversible hydration of CO₂ (CO₂ + H₂O ↔ HCO₃⁻). CA is important in biological systems because the natural hydration of CO₂ (un-catalyzed) is slow at ambient condition.

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CA is fundamental to many biological processes like photosynthesis, respiration, pH homeostasis and ion transport [7]. Presently CAs are divided into three main classes, α , β and γ , which are supposed to be evolutionarily independent [8]. The existence of additional δ [9] and ζ - classes of CAs have also been reported [10]. Recently, several reports showed that CA genes from two or even all three known classes are identified even in some prokaryotes or multiple genes from the same class are contained [8].

Nowadays, aquatic organisms have been focused as new biological resource to find novel enzymes, proteins, and organic materials because aquatic organisms have been evolved under unique or extreme conditions like high-salinity, high/low temperature, and dark or low illumination. Most of CA enzymes exhibit catalytic activity only under mild conditions, and they may get structural/functional damage when exposed to high temperatures, extreme pH or high salinity. In contrast, CA enzymes from aquatic halophiles or thermophiles maintain properly-folded structures and/or show functions optimally under extreme salt concentrations [11,12] or elevated temperature [13].

Most of the reports describe that α -type carbonic anhydrase (α CA) from halo-tolerant green alga *Dunaliella salina* maintains its functionality over nearly the entire range of salinities [14, 15]. However, the acquired amount of purified enzyme fractions is not sufficient or the bacterial recombinant expression / purification systems are not set up well, so that its accurate characterization has not been studies well. In this study, we focused on DsCA from *Dunaliella species* growing in hypersaline environment. Codon-optimized sequence of the CA (DsCAopt) was constructed and its efficient expression/purification system was set up. We characterized some of its enzymatic properties like pH stability, CO₂ sequestration ability. Results obtained here would be useful for further development of biological CO₂ converting/capturing processes.

II. MATERIAL METHODS

A. Strains, Plasmids, and Reagents

The *E. coli* hosts XL1-BlueMRF [$\Delta(mcrA)182$, $\Delta(mcrCB-hsdSMR-mrr)172$, *endA1*, *supE44*, *thi-1*, *recA*, *gyrA96*, *relA1*, *lac, λ* , {F,*proAB*, *lacI^qZ* Δ M15,*Tn10(Tc)^r*}] was purchased from Staratagen, and BL21(DE3) [(F, *ompT*, *hsdS*

($r_B^- m_B^-$), *gal*, *dcm* (DE3)) and the expression vectors pETDuet-1 were obtained from Novagen (Madison, WI). DNA polymerase (ExTaq) was purchased from TaKaRa. Restriction enzymes were obtained from New England Biolabs. Ampicillin, bromophenol blue and *p*-nitrophenylacetate were obtained from Sigma-Aldrich Co., Vernal buffer-5X from Lonza (Walkersville, MD USA), IPTG from Duchefa (Haarlem, The Netherlands), and TritonX-100 from Yakuri Pure Chemicals (Osaka, Japan). EDTA-free protease inhibitor (Halt Protease Inhibitor Cocktail) and protein assay reagents were purchased from Thermo Scientific. All the molecular biology reagents and enzymes used in this study were of the highest grade.

B. Construction of Expression Plasmid Vector

Alpha-type CA gene, DsCA, cloned in T-easy vector from *Dunaliella* species has been kindly obtained from Professor ES Jin's group at Hanyang University, Korea. The codon-optimized sequence, DsCAopt, was synthesized by Bionics (Seoul, Korea). The leader sequence was removed and the initiation codon was added. The sequence of DsCAopt was amplified by PCR with the forward [5'-GAA TTC GAT GGT GAG CGA ACC GCA TGAT-3' (underlined: *EcoRI* site)] and reverse [5'-AAG CTT TCA CGC CGC GCC GTT ATAG-3' (underlined: *HindIII* site)] primers. The reaction conditions are as follow: 94°C for 7 min, followed by 30 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 1 min, and finally 74°C for 10 min. Amplification reaction volume is 50 μ L composed of 0.5 μ L of ExTaq Polymerase (2.5 Unit), 5 μ L of 10x ExTaq buffer, 4 μ L of dNTP (2.5 mM), 1 μ L of forward primer (100 pmol), 1 μ L of reverse primer (100 pmol), 1 μ L of template (2 μ g/ml) and 37.5 μ L of ddH₂O. The PCR product was separated by agarose gel-electrophoresis and was extracted using PCR extraction kit, Expin™ GEL SV (GeneAll Biotechnology, Korea). The purified DNA fragment was ligated into pGEM-T Easy vector (Promega, Madison, WI, USA) and introduced into competent *E. coli* XL 1 Blue MRF. Recombinant plasmids were isolated from positive clones using mini-prep DNA extraction kit - Exprep™ Plasmid mini (GeneAll Biotechnology, Korea) and the insert was confirmed by DNA sequencing. After treatment of *EcoRI* and *HindIII* on the mini-prepared plasmids, the fragment of 1611 bp was recovered and subcloned into *EcoRI-HindIII* site of pETDuet-1 vector. The resulting pETD- DsCAopt expression vector was introduced into competent *E. coli* BL21 (DE3). Other DNA recombinant methods were performed as described by Sambrook *et al.* [16].

C. Expression and Purification of Recombinant DsCAopt enzyme

For expression analysis, *E. coli* BL21 (DE3) harboring the pETD-DsCAopt plasmid was grown in LB-medium supplemented with ampicillin (100 μ g/ml) on an orbital shaker (180 rpm) at 37°C. When the OD₆₀₀ of the culture reached a value of 0.6, isopropyl β -D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM and the culture was grown for 6 h at 37°C or for 24 h at 20°C. Induced culture was

harvested by low speed centrifugation, washed and re-suspended in Tris-SO₄ buffer (50 mM, pH 7.6), followed by sonication with Digital Sonifier-250 (Branson Ultrasonic Co., Mexico) for cell breakage. The supernatant and pellet fractions were separated by centrifugation at 12,000 rpm for 30 min at 4°C and then the protein expression was analyzed by SDS-PAGE (12% gels) as described by Laemmli [17].

For purification of the recombinant protein, approximately 10 g of pelleted *E. coli* cells from 20°C culture was suspended in 40 mL of lysis buffer containing Tris-SO₄ (25 mM, pH 7.6), Triton X-100 (0.1%), and 1 mL of EDTA-free protease cocktail. Cells were thawed in ice-cold water bath and DNase I, MgSO₄, and ZnSO₄ were added to the bacterial lysate (final concentrations: 20 μ g/mL, 15 mM, and 0.5 mM, respectively). The lysate was spun at 15,000 rpm for 30 min in an ultracentrifuge (Gyrozen 1730MR, Incheon, Korea) at 4°C and the resulting supernatant was subjected to affinity-purification. The cell extract was loaded onto Talon resin column (HisPur™ Cobalt Spin Column) pre-equilibrated with equilibration buffer [Na-phosphate buffer (50 mM, pH 7.6) containing NaCl (300 mM)], and eluted with the elution buffer [Na-phosphate buffer (50 mM, pH 7.6) containing NaCl (300 mM) and imidazole (150 mM)] according to company manual. The different fractions having single band corresponding to correct size were collected and dialyzed extensively against Tris-SO₄ buffer (25 mM) containing ZnSO₄ (10 μ M) and concentrated using Amicon Ultra centrifugal filter (Millipore). The entire purification procedure was carried out at 4°C. The purified protein was analyzed by SDS-PAGE (12% gels) as described by Laemmli [17]. Coomassie Brilliant Blue R-250 (Bio-Rad) was used for gel staining and then treated several times by de-staining solution until the proper protein bands appear. Protein quantity was determined by spectrophotometer at 595 nm according to the Bradford method, using bovine serum albumin as the standard [18].

D. Carbonic Anhydrase Assay

CO₂ hydration assay was performed by recording pH variation in presence of indicator. Two mL of veronal buffer (pH 8.2), 0.2 mL of bromothymol blue (0.004%), 0.8 mL of diluted enzyme, and 2 mL of a CO₂ solution (saturated at 0°C) were mixed. The time (t_c) interval was determined between the addition of CO₂ solution and the occurrence of a yellow-green color. In the same way, the time interval was recorded without an enzyme solution (t_0). The activity was calculated from the formula given below [19]. One Wilbur-Anderson Unit = ($t_0 - t_c$) / t_c

Esterase activity of carbonic anhydrase was measured by following the change in absorbance at 348 nm of *p*-nitrophenylacetate (pNPA) to *p*-nitrophenylate ion ($\Delta\epsilon = 5000 \text{ m}^{-1}\text{cm}^{-1}$) over a period of 3 min at 4°C using a UV-VIS spectrophotometer (UVmini - 1240, Shimadzu Co.) according to the method described by Verpoorte [20]. The enzymatic reaction volume is 3.0 mL composed of 1.4 mL of Tris-SO₄ buffer (50 mM, pH 7.6), 1 mL of *p*-nitrophenylacetate (3 mM), 0.5 mL of ddH₂O and 0.1 mL of enzyme solution.

A reference measurement was obtained by preparing the same cuvette without enzyme solution.

E. CO₂ Sequestration

The hydration of CO₂ reportedly involves the transfer of hydrogen ions between the active sites of the enzyme and the surrounding buffer [21]. CO₂-saturated solution used as substrate was prepared by introducing CO₂ (standard grade) at ~1 bar (100 kPa) into 500 ml of MilliQ-grade pure water in the tube with a rubber cork for 1 h at 4°C. CO₂-saturated solution (10 ml) was mixed with 1 ml of Tris-SO₄ buffer (1 M, pH 8.3) containing free DsCAopt (100 µg) for 15min. The bicarbonate solution was released into another vessel containing 10 ml of CaCl₂ solution (final concentration: 10.0 mM). To the mixture, 2 ml of Tris-SO₄ buffer (1.0 M, pH 9.5) was immediately added. The reaction mixture was incubated at 35°C and at 45°C, each for 5min, to allow precipitation of CaCO₃. The precipitated CaCO₃ was filtered and dried at 60°C. The resulting CaCO₃ was weighed, and the amount of sequestered CO₂ (gram equivalent of CO₂ present in calcium carbonate) was calculated [22]. The results were corrected in terms of mg CaCO₃ formed by subtracting the amount from the control experiment carried out in the absence of enzyme.

III. RESULTS

DsCA cloned from *Duneliata species* consists of 1770 bp. DsCAopt gene (codon-optimized and leader sequence removed, 1611bp) was cloned in pETD-DsCAopt was expressed well in *E. coli* BL21(DE3), and the proteins was obtained as soluble form. The soluble portion of the expressed DsCAopt was further optimized at 20°C. The expressed amount of soluble DsCAopt enzyme is 27.79 mg/L (at 20°C under 1.0 mM of IPTG induction). The crude extract of BL21(DE3) cell expressing DsCAopt was subjected to the affinity column and the differentially-eluted fractions were analyzed by SDS-PAGE for purification procedures. A protein fraction that eluted with 50 mM of sodium phosphate buffer (pH 7.6) contained a protein band of ~59 kDa, as expected for the DsCAopt enzyme fused to the moiety of N-terminal His-tag sequences.

DsCAopt enzyme shows esterase activity measured by using p-nitrophenylacetate (pNPA) as substrate. The K_m (M-M constant) and V_{max} (the maximum reaction rate) were obtained by Lineweaver-Burk plotting estimations [23]. Apparent values of K_m and V_{max} were 0.09095 mM and 3.303 × 10⁻⁸ mM min⁻¹, respectively (K_{cat} value: 3.63166 × 10⁻⁸ min⁻¹).

CO₂ hydration activity was measured by pH change as a function of time. DsCAopt enzyme exhibited a specific activity of 712 WAU/mg proteins. The pH was determined from the absorbance of the reaction mixture using bromophenol blue [19], varying pH 8.3 to pH 6.0. CA activity is expressed in Wilbur-Anderson (WA) units per milligram of protein.

The effect of pH on stability of purified DsCAopt enzyme was investigated in three different buffer systems, 50 mM Citrate phosphate buffer (pH 5.0–6.5), Tris-SO₄ buffer (pH 7.0–8.5) and glycine-NaOH buffer (pH 9.0–10.0). The purified enzyme was found to retain 100% activity at pH 7.6.

Interestingly, more than 80% enzyme activity was also retained at the range of pH 10.0-11.0, even after 24 h incubation at 4°C (Fig. 1).

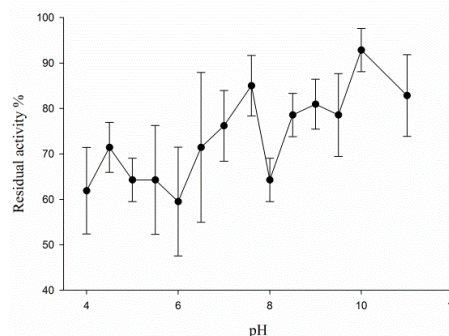


Fig.1 Effect of pH on stability of purified Dt-aCAopt enzyme. The pH stability of the enzyme was determined by incubating the enzyme in different pH buffers for 24h at 4°C. The residual enzyme activity was measured according to the standard enzyme assay. 100% CA activity is equivalent to that of 172 U/mg protein

However, 50-70% activity retained at the range of pH 4.0-7.0. The purified DsCAopt enzyme was found to be stable at pH range 7.6 and 10.

Carbonic anhydrases has been focused as biological catalysis for CO₂ sequestration process. Here, we also investigated the ability of DsCAopt to mediate CO₂ to CaCO₃ formation under Ca²⁺ solution. CaCO₃ precipitation using free DsCAopt enzyme was carried out in Tris-Cl buffer (1.0 M, pH 10.0). CaCO₃ was precipitated from the hydrated CO₂ solution by addition of 4% CaCl₂ solution. The precipitated CaCO₃ was recovered by filtration, weighed and the gram equivalent of CO₂ present in the calcium carbonate was calculated.

IV. DISCUSSIONS

We carried out multiple alignments with CAs from similar *Duneliata species*. DsCA from *Duneliata species* was found to share 43% identity with DCAI (AAF22644) and 99% with dCAI (AAC49378) from *Duneliata salina* (Fig 2).

In preliminary attempts, we testified several vector systems such as pQE series and pET series, and also employed several *E. coli* hosts such as *E. coli* BL21(DE3), *E. coli* M15 [pREP4] and *E. coli* SG13009 [pREP4] to express DsCA gene. Mostly, in the transformed cells with DsCA gene expression system (non codon-optimized), the yield of the expressed CA protein as soluble form was quite low (negligible), even as insoluble form. To increase the obtained yield of soluble and active DsCA, we screened the effects of different parameters; growth media [LB, 2X YT or Super Broth (SB)], IPTG concentrations (0.1-2mM), growth temperature (20-37°C), and cell disruption methods (sonication, freezing, and thawing in the presence or absence of detergents such as Triton X-100 and lysozyme). But, most of the attempts were in vain (data not shown). The acquired amount of soluble DsCA enzyme is still negligible. We constructed the codon-optimized sequence of DsCA (DsCAopt) by chemical

synthesis to expression in *E. coli*. The leader sequence (first 162 bp) was removed and transcription initiation codon (ATG) was added before codon sequence of 55th amino acid. The codon-optimized DsCAopt gene (1611bp) was cloned into pETDuet-1vector. The recombinant DsCAopt was expressed well as soluble form in *E. coli* BL21(DE3) (pETD-DsCAopt).

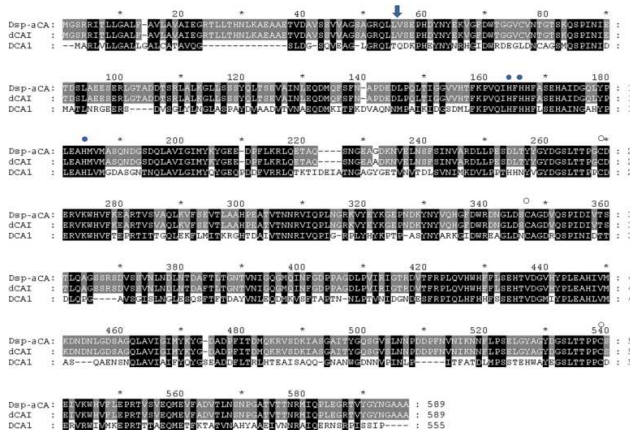


Fig. 2 Multiple amino acid sequence alignment: DsCA is CA under study from *Dunaliella sp.* dCAI duplicated carbonic anhydrase (accession number AAC49378), DCA1 duplicated carbonic anhydrase (accession number AAF22644) from *Dunaliella salina*. Downward arrow represents proteolytic cleavage site for maturation of peptide. Filled circles- zinc-ligand binding His residues; open circles- cysteines involved in disulfide bond formation.

We investigated the kinetic properties utilizing esterase activity of Dt-aCAopt enzyme considering Micheilis-Menten equation [$V = V_{max}/(K_m + [S])$]. The changes of the reaction rate (V) of the enzyme at 4°C and pH 7.5 were measured several times according to the concentration of pNPA ($[S]$). Dt-aCAopt has apparent values of K_m and V_{max} were 0.09095 mM and 3.303×10^{-8} mM min⁻¹, respectively (K_{cat} value: 3.63166×10^{-8} min⁻¹). α -CA from *Oncorhynchus mykiss* (rainbow trout) has apparent K_m and V_{max} for pNPA are 0.40 mM and 0.097 μ mol min⁻¹, respectively [24]. Similarly, α -CA from *Dicentrarchus labrax* (teleost fish) has 0.44 mM of K_m and 0.0249 μ mol min⁻¹ of V_{max} [25]. For the same α -CA, K_m was reported as 1.595 mM and V_{max} as 0.509 μ mol min⁻¹ for pNPA hydrolysis (26). DsCAopt CA has more affinity to pNPA substrate (lower value of K_m), but lower maximum rate (lower value of V_{max}) than the above mentioned CAs. The purified DsCAopt enzyme was found to be stable at pH range 7.6 and 10. Similar results have been found documented. The extracellular CA from *M. cationoplastes* showed two peaks of activity at pH 7.5 and 10.0 [27]. In contrast CA from *H. pylori* showed high acid tolerance, functioning optimally in the acidic environment of human stomach [28]. All these facts indicate to the remarkable functional diversity of CA and the ability of this enzyme to perform different roles for the organisms surviving in extreme microenvironments and diverse ecological niche.

CaCO₃ has three crystalline mineral polymorphs including rhombic calcite, needle-like aragonite, and spherical vaterite [29, 30].

Calcite is the dominant polymorph at high pH and low temperature and is the most stable at room temperature under atmospheric conditions. While vaterite and aragonite are mostly produced at low pH and high temperature, they can transform to stable calcite. Calcite is especially abundant in nature, environmentally harmless, and widely utilized in industry due to its regular crystal size and smooth surface [31]. FE-SEM image revealed that the morphologies of CaCO₃ crystal obtained by precipitation of hydrated CO₂ (catalyzed by DsCAopt enzyme) were similar to the morphologies described previously [32]. SEM image revealed rhombohedral calcite crystals. XRD analysis of precipitated CaCO₃ also indicates the formation of a calcite phase. The diffraction peaks occurred at 23.07, 29.5, 36.1, 39.5, and 43.3°, corresponding to the calcite crystal faces (012), (104), (110), (113), and (202), respectively and the representative crystal surface of calcite was $2\theta = 29.42$ data not shown).

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