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Improvement of the phospho*enol*pyruvate carboxylase activity of *Phaeodactylum tricornutum* PEPCase 1 through protein engineering

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ABSTRACT

In order to mitigate CO_2 accumulation and decrease the rate of global warming and climate change, we previously presented a strategy for the development of an efficient CO_2 capture and utilization system. The system employs two recombinant enzymes, carbonic anhydrase and phospho*enol*pyruvate carboxylase, which were originated from microalgae. Although utilization of this integrated system would require a large quantity of high quality PEPCase protein, such quantities could be produced by increasing the solubility of the *Phaeodactylum tricornutum* PEPCase 1 (PtPEPCase 1) protein in the *Escherichia coli* heterologous expression system. We first expressed the putative mitochondria targeting peptide- and chloroplast transit peptide-truncated proteins of PtPEPCase 1, mPtPEPCase 1 and cPtPEPCase 1, respectively, in *E. coli*. After affinity chromatography, the amount of purified PEPCase protein from 500 mL of *E. coli* culture was greatest for cPtPEPCase 1 (1.99 mg), followed by mPtPEPCase 1 (0.82 mg) and PtPEPCase 1 (0.61 mg). Furthermore, the enzymatic activity of mPtPEPCase 1 and cPtPEPCase 1 showed approximately 1.6-fold (32.19 units/mg) and 3-fold (59.48 units/mg) increases, respectively. Therefore, cPtPEPCase 1 purified using the *E. coli* heterogeneous expression system could be a strong candidate for a platform technology to capture CO_2 and produce value-added four-carbon platform chemicals.

1. Introduction

Because of the continuously increasing use of fossil fuels and levels of exhaust emissions since the Industrial Revolution, greenhouse gas levels in the atmosphere, especially CO_2, have been increasing and contributing to global warming and climate change. Therefore, worldwide scientific efforts have attempted to mitigate CO_2 accumulation. One useful solution among the various schemes for CO_2 abatement is the capture and utilization of CO_2 (CCU), in which waste CO_2 is recycled and converted into valuable chemicals through biocatalysis [1–3].

Photosynthetic plants and microalgae have developed a physiological CO_2 fixation process for a wide range of CO_2 concentrations [4,5]. Importantly, marine microalgae in particular have the capacity to fix CO_2 that produces organic compounds under conditions of lower CO_2 concentration. In order to fix a lower concentration of CO_2 around the unicellular microalgae efficiently, these organisms have developed a carbon dioxide concentration mechanism (CCM). Carbonic anhydrase (CA; EC 4.2.1.1) is an important biocatalyst in the CCM process via its stimulation of the CO_2 hydration reaction that produces bicarbonate, as well as the reverse dehydration reaction from bicarbonate to CO_2 in a concentration-dependent manner [6]. Another significant biocatalysis, ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco; EC 4.1.1.39) is a fundamental enzyme in the carbon fixation reaction of the Calvin cycle. However, this enzyme has a relatively slower reaction rate and reacts with CO_2 and O_2 as substrates in a concentration-dependent manner. The oxygenase reaction rate of Rubisco increases as temperature increases, as the solubility of O_2 is relatively greater than that of CO_2 at higher temperatures [7]. This photorespiration reaction of Rubisco reduces the carbon fixation rate and also attenuates the efficiency and effectiveness of photosynthetic energy [8]. Therefore, Rubisco would appear to be a poor candidate for a biocatalyst contributing to CO_2 abatement.

Another biocatalyst specific to CO_2 fixation is phospho*enol*pyruvate carboxylase (PEPCase; EC 4.1.1.31). PEPCase catalyzes the conversion of phospho*enol*pyruvate (PEP) to oxaloacetate (OAA) using bicarbonate. Unlike Rubisco, PEPCase is not affected by, nor does it react with O_2 [9]. Additionally, it has a higher affinity for CO_2 than Rubisco [9]. The lower K_m value of PEPCase as compared to Rubisco reflects a higher CO_2 fixation rate [10]. PEPCase is also an anaplerotic enzyme that provides OAA and/or malate to replenish tricarboxylic acid (TCA) cycle intermediates in all organisms with the exception of animals and

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fungi [11]. The PEPCase product OAA can also be converted into succinate, a very valuable C4 chemical. Thus, PEPCase could be an important enzyme in the production of C4 chemicals and in the mitigation of CO_2.

There have been many attempts to reduce CO_2 levels via conversion to useful chemical feedstocks. Among these strategies, we studied the CO_2-utilizing bioconversion system of CA and PEPCase purified from marine microalgae [12]. The heterogeneously expressed and purified recombinant CA and PEPCase proteins have demonstrated the possibility of CO_2 capture and utilization for conversion to the C4 chemical compound OAA using integrated sequential CA-PEPCase enzyme systems. This previous study utilized the expression and purification of diatom *Phaeodactylum tricornutum* PEPCase 1 (PtPEPCase 1) for the CA-PEPCase bioconversion system [12]. In order to further study the CA-PEPCase system, it was imperative to prepare a large quantity of high quality PEPCase proteins via modification of the expression or purification methods.

Plants and microalgae acquired plastid via the evolutionary process of endosymbiosis. Most organellar proteins are encoded by the host genome and the precursor proteins of plastid proteins are translocated to their target organelle. These precursor proteins have an N-terminal extension sequence referred to a transit peptide. These N-terminal presequences are classified based on their targeting either to mitochondria or to plastid such as chloroplast. Despite the number and variety of transit peptides, they all share a characteristic α-helix structure as well as amphipathic sequences [13,14]. The current study was performed to analyze the amino acid sequence of PtPEPCase 1 and to develop a technical approach that could increase protein expression levels from the heterogeneous expression system of *Escherichia coli* by eliminating the N-terminal presequence in the protein and purify PEPCase proteins with enhanced activity via the expression of mutated PtPEPCase 1.

2. Materials and methods

2.1. Cloning, construction, and transformation of the mutated PtPEPCase 1

The full-length cDNA of the open reading frame encoding *P. tricornutum PEPCase 1* (*PtPEPCase 1*, XM_002180991) was cloned as *Nde* I and *Pst* I inserts into the pCold I DNA cold-shock expression system (Takara Bio Inc., Japan) as previously described [12]. Specific primers were designed for expression of the N-terminal truncated mutated isoforms of the PtPEPCase 1 protein. Forward primers used for the m-type mutant protein (mPtPEPCase 1) and the c-type mutant protein (cPtPEPCase 1) of PtPEPCase 1 were 5′-CATATGACGGGTTGTCCGTGGAG-3′ and 5′-CATATGCAAGCGTCGACGGTATCC-3′, respectively. The *Pst* I-reverse primer 5′-CTGCAGTTAACCAGTGTTGCCCATTC-3′ was used as a reverse primer. The underlined nucleotide sequences indicate restriction enzyme sites for *Nde* I or *Pst* I for directional cloning into pCold I expression vectors. PCR fragments of *mPtPEPCase 1* and *cPtPEPCase 1* were amplified with the cDNA of *PtPEPCase 1* by a Pfu Plus DNA polymerase (Elpis Bio, Korea). The *Nde* I/*Pst* I fragment was cloned into a likewise-digested pCold I expression vector to create pCold I-mPtPEPCase 1 and pCold I-cPtPEPCase 1, respectively (Fig. 1a). To construct expression vectors having EYFP and putative chloroplast transit peptide (cTP) fused with EYFP, EYFP were amplified using the forward primer [5′-CATATGAGCAAGGGCGAGGAGCTG-3′, *Nde* I site is underlined, for pCold I-EYFP construct or 5′-GGTACCATGAGCAAGGGCGAGGAGGAGCTG-3′, *Kpn* I site is underlined, for pCold I-cTP:EYFP construct] and the reverse primer [5′-TCTAGATTACTTGTACAGCTCGTCCATG-3′, *Xba* I site is underlined]. The sequence of cTP was amplified from *PtPEPCase 1* with *Nde* I-forward primer [12] and cTP reverse primer [5′-GGTACCTTGCAATGGAGAAGCACCAA-3′, *Kpn* I site is underlined]. The *Nde* I/*Xba* I fragment of *eyfp* or *Kpn* I/*Xba* I fragment of *eyfp* and *Nde* I/*Kpn* I fragment of cTP region from PtPEPCase 1 were cloned into an appropriately digested pCold I expression vector to create pCold I-EYFP and pCold I-cTP:EYFP vector, respectively (Fig. 3a). The sequence of the inserted DNA was verified (Macrogen, Korea) and the confirmed constructs were transformed in *E. coli* BL21-CodonPlus (DE3)-RIPL (Agilent Technologies, USA) prior to selection on a Luria broth (LB) agar plate containing 100 μg/ml of ampicillin.

2.2. Expression and purification of different types of recombinant PtPEPCase 1

The *E. coli* transformant cells harboring pCold I-PtPEPCase 1, -mPtPEPCase 1, and -cPtPEPCase 1 were grown aerobically overnight at 37 °C and inoculated at OD_{600} = 0.05 in 500 mL of LB medium containing ampicillin (100 μg/ml) at 37 °C with shaking at 250 rpm. Cells were allowed to grow until the OD_{600} reached 0.4 prior to incubation for 30 min at 15 °C without shaking. These pre-incubated *E. coli* cells were treated with 0.8 mM isopropyl-β-D-thiogalactoside (IPTG, Sigma, USA) and induced at 15 °C for 24 h by shaking at 250 rpm.

Induced cells were harvested by centrifugation at 4000 × *g* for 10 min. Pellets were washed with lysis buffer (20 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 5% (v/v) glycerol, 20 mM KCl, 10 mM $MgCl_2$, 10 mM imidazole) containing 0.1% (v/v) Triton X-100 and resuspended with 15 mL of lysis buffer per 500 mL of culture. Resuspended *E. coli* cells were treated with 15 mg of lysozyme (Sigma, USA) and incubated at 4 °C for 30 min. Cells were then sonicated on ice with 2-s pulses at 3-s intervals 60 times using a Misonix Ultrasonic Liquid Processor Q500 (Q Sonica LLC, CT, USA) at 20% amplitude. Sonicated cells were centrifuged for 30 min at 10,000 × *g* at 4 °C. The soluble fraction of the supernatant was used for purification of His-tagged recombinant proteins under native conditions with Ni-NTA agarose resin (Qiagen, USA) according to the manufacturer's protocol. 50% slurry of Ni-NTA agarose resin (1.5 mL) was carefully loaded onto the bottom of a Poly-Prep Chromatography Column (Bio-Rad Laboratories, USA) and equilibrated with lysis buffer containing 10 mM imidazole. Soluble fractions were loaded onto the Ni-NTA agarose column. The column was washed twice with 15 mL of lysis buffer containing 20 mM imidazole. Recombinant proteins were eluted 4 times with 0.5 mL elution buffer (50 mM Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 5% (v/v) glycerol) containing 250 mM imidazole. Eluted fractions were evaluated using 8% SDS-PAGE stained with Bio-Safe Coomassie G-250 Stain (Bio-Rad Laboratories, USA). Protein content was determined using a protein assay reagent (Bio-Rad Laboratories, USA).

2.3. Immunoblotting analysis, enzyme activity, and enzyme stability of purified PEPCases

Immunoblotting analysis was performed as previously described in a study that confirmed the purity of PEPCase proteins [12]. Rabbit anti-PEPCase polyclonal serum was obtained from Agrisera AB (AS09 458, Sweden). The PEPCase activity assay was performed essentially as described by O'Leary *et al.* [15] and was analyzed by the coupled enzyme method with malate dehydrogenase (MDH) [12,15]. PEPCase activity at 25 °C was calculated from the rate of NADH oxidation by measurement of the absorbance at 340 nm using a UV/visible spectrophotometer (Ultrospec 2100 pro; Amersham Pharmacia Biotech, USA) equipped with a Peltier heated cell holder for fixed temperature control between 20 and 50 °C. The reaction medium consisted of 100 mM Tris–HCl (pH 8.0), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.1 mM NADH, 50 μM Acetyl-CoA, 3 U porcine heart malate dehydrogenase, and 4 mM phospho*enol*pyruvate (PEP). All reagents were purchased from Sigma–Aldrich (USA). One unit of PEPCase activity was defined as the amount of enzyme required to produce 1 μmol of OAA per minute [15]. Heat stability of the purified PEPCase was determined by assaying the enzyme activity at various temperatures (20–50 °C). Reaction mixture without the protein sample was placed in the cuvette of a spectrophotometer heated cell holder. After incubation at a fixed temperature for 5 min, the sample was added to the cuvette, and the decreased rate of absorbance at 340 nm was measured for 1 min. The pH stability was determined using assay conditions with Tris–HCl reaction buffer from pH 7 to 10 incubated at 25 °C. In the MDH activity assay, the same reaction buffer used in the PEPCase assay was employed with the exception of the use of 4 mM OAA as the substrate. In order to minimize the level of dissolved bicarbonate in the PEPCase activity assay media, doubly deionized water was boiled and then was bubbled with nitrogen gas. The assay buffer of Tris was acidified to pH 2 and bubbled with nitrogen gas above 1 h [16]. After that, the pH was adjusted to 8.0 with saturated NaOH. The K_m values for PEP substrate were calculated using nonlinear regression analysis software (SigmaPlot Version 12.0).

3. Results and discussion

3.1. Construction of the protein expression vectors for engineering P. tricornutum PEPCase 1

In the *E. coli* heterologous expression system, over-expressed proteins often form insoluble aggregates referred to as inclusion bodies [17]. Results from a previous study showed that sufficient enzyme activity of PEPCase itself could be purified from soluble lysate under native conditions when the recombinant full length PtPEPCase 1 was expressed using the cold shock expression system [12]. However, the amount of partially purified PtPEPCase 1 protein was relatively low because highly induced proteins were detected as insoluble forms in the total lysate. Thus, in order to utilize the purified PEPCase protein, it was necessary to prepare large quantities of high quality PEPCase proteins by increasing the solubility of the recombinant PEPCase 1 protein.