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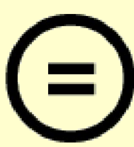

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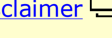

Increased Reaction Efficiency of Enzyme Complexes Based on Irreversible Covalent Bonding Systems

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Bibliographical note: This thesis was previously published in the *Process Biochemistry* (Sun et al., 2022, 120: 195 - 201).

Increased Reaction Efficiency of Enzyme Complexes Based on Irreversible Covalent Bonding Systems

A Master's Thesis
Submitted to the
Graduate School of Sungshin University

in partial fulfillment of the requirements
for the degree of Master of Food Science

[HaBin Sun]

[11, 2022]

This is to certify that we have examined the Master's Thesis of HaBin Sun Submitted to Department of Next Generation Applied Sciences

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The Graduate School of Sungshin University

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The Graduate School of Sungshin University

ABSTRACT

Increased Reaction Efficiency of Enzyme Complexes Based on Irreversible Covalent Bonding Systems

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Carbonic anhydrase (CA) and cyanase can be a solution to the reduction of CO_2 and cyanide that adversely affect the environment. However, the high temperature inactivation of enzymes and their dependence on bicarbonate limits their industrial applications. Therefore, this problem was overcome by changing the structure of the enzyme using the Catcher/Tag system with irreversible covalent bonding and combining them with each other. Cyclization and polymer formation of *hm*CA recovered its activity even at a high temperature of 70 °C or higher compared with the wild type, and polymeric *hm*CA showed a result that was more than 4 times different from that of the control in $CaCO_3$ production through enzymatic reaction. This suggests Thatcher the binding

of enzymes by the Catcher/Tag system improved thermal and structural stability and could be a new strategy to overcome the existing limitations. The subsequent binding of cyanase and *hm*CA increased cyanide degradation compared to when cyanase alone was used, but the degradation reaction efficiency was lowered compared to the condition in which the two enzymes were not linked. The cause of these results is that the formation of the complex in a linear form in the binding through the peptide module brought a limitation in distance, resulting in decreased efficiency. To improve this, the complex was engineered to form a circular structure. As a result of measuring the reaction efficiency of the enzyme, it showed more than two times higher activity than the condition in which cyanase alone was used, and it was confirmed that the reaction efficiency was improved compared to the linear complex formed by mixing Catcher, cyanase, and *hm*CA. In conclusion, an enzyme complex with reduced HCO $LSUB3LSUP-$dependence and improved reaction efficiency was prepared in the presence of the same concentrations of oxidized cyanide and HCO $LSUB3LSUP-$ than that in the presence of cyanase alone.

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Ⅰ. INTRODUCTION

The impact of the COVID-19 pandemic has brought about a global economic crisis, and CO_2 emissions decreased by 5.2 % in 2020, but despite this decline, global energy-related CO_2 emissions remained at 31.5 Gt, which is the largest annual amount released into the atmosphere (Le Quéré et al., 2020). In 2021, the prospects for economic growth increased, as many major economies accelerated COVID-19 vaccinations and initiated broad fiscal responses to the economic crisis. This has led to a rebound in energy demand, and global energy-related CO_2 emissions are supposed to increase by 4.8 % as coal, oil, and gas demand recover along with the economy (International Energy Agency, 2021). The increase in CO_2 is the cause of climate change and global warming, and countries around the world are trying to reduce CO_2 by introducing and expanding renewable energy, researching various strategies such as development of fossil fuel alternatives, carbon capture and conversion (CCU), and clean energy development (The Government of the Republic of Korea, 2020).

Carbonic anhydrase (CA) is used as a CO_2 reduction method in this study. This enzyme catalyzes the conversion of CO_2 and H_2O to HCO *LSUB*3*LSUP*− and H^+, and most CA active parts contain Zn^{2+}, which is classified as metal enzymes, with a total of six types: α, β, ɣ, δ, ζ, and ɳ (Angeli et al., 2020; Badger & Price, 1994; Lionetto et al., 2016). *Hydrogenovibrio marinus* is a marine bacterium capable of producing CA. It is a gram-negative marine bacterium that tolerates medium temperatures of 10-50 °C, is chemically independent and uses H_2 and CO_2 as nutrients (Jo et al., 2020a; Nishihara et al., 2001). Halogen-resistant *hm*CA , found in

this microorganism, exhibits excellent activity and stability, generally operates at high salinity (or electrolyte) conditions, and is an attractive candidate for biomimetic carbon capture and storage (CCUS), which frequently encounters flue gas impurities (Jo et al., 2018; Power et al., 2016; Qi et al., 2018; Savile & Lalonde, 2011; Srikanth et al., 2017). In addition, *hm*CA produced a high yield with excellent solubility conferred by N′-terminal expansion in *E. coli*, which is advantageous for building a bacterial whole-cell catalytic system (Jo et al., 2020b). Therefore, the CA from *Hydrogenovibrio marinus* can be used as a biomimetic for sequestering CO_2 as part of a CO_2 capture system in coal and natural gas thermal power plants (Wu et al., 2010). However, there are aspects to consider when using a CA. Because this enzyme is a protein, it can be inactivated if it exceeds a certain temperature, so temperature safety should be considered. Since the flue gas is 40～60 ℃, CA must be able to withstand the temperature above that. To overcome this limitation, a peptide module that performs irreversible covalent bonding was used to modify the structure of the enzyme (Sun et al., 2022).

Second, cyanide is a compound containing a C≡N functional group, a molecule composed of a single nitrogen atom with a triple bond (Jaszczak et al., 2017; Kuyucak & Akcil, 2013). These cyanides are found in nature as cyanated glycosides in the seeds of fruits such as apricots and peaches and in plants such as cassava roots (Barceloux, 2009; Jones, 1998) and can also be produced by microorganisms and insects. Cyanide in various states is highly toxic, and when intoxicated with this substance, symptoms such as headache, vomiting, and dizziness may occur in the

beginning, followed by paroxysm, slow heart rate, hypotension, unconsciousness, and cardiac standstill (Anseeuw et al., 2013). The reason for this is that cyanide is a potent cytochrome c oxidase (COX, also known as Complex IV) inhibitor, so its addiction interferes with oxidative oxidation and interferes with cellular respiration, resulting in a form of histotoxic hypoxia (Leavesley et al., 2008). Currently, various activities such as metal extraction, electroplating, and aluminum work emit wastewater containing heavy metals such as cadmium, copper, chromium, zinc, and cyanide, which negatively affects the water quality environment, increasing the risk (Smith & Heath, 1979). A common method used for wastewater treatment is alkaline chlorination (Mapstone, 1978). However, chlorination of cyanide can create additional environmental concerns as it produces highly toxic intermediates such as cyanogen chloride and other toxic organochlorines. Therefore, eco-friendly methods such as the use of activated sludge or microorganisms are adopted instead of chemical treatment (Ebbs, 2004; Papadimitriou et al., 2009), but this also has a disadvantage that the survival and activity of microorganisms may be weakened by high concentrations of cyanide in industrial wastewater (Sharma & Philip, 2014).

Research uses cyanase as a method for cyanide removal. It is an enzyme that converts cyanate, an oxide of cyanide, to ammonia and carbon dioxide in a HCO*LSUB*3*LSUP*−dependent manner and is found in bacteria and plants (Johnson & Anderson, 1987; Sung & Fuchs, 1988). In addition, in many environments where this compound is present, cyanase becomes an important component for plants and bacteria as some microorganisms

can overcome toxicity by breaking down oxidized cyanide through this enzyme (Schlachter et al., 2017). However, since the activity of the enzyme is highly dependent on the presence or absence of HCO$LSUB3LSUP-$, a previous study applied a method of combining cyanase and carbonic anhydrase to overcome this bottleneck (Ranjan et al., 2018)(Fig. 1).

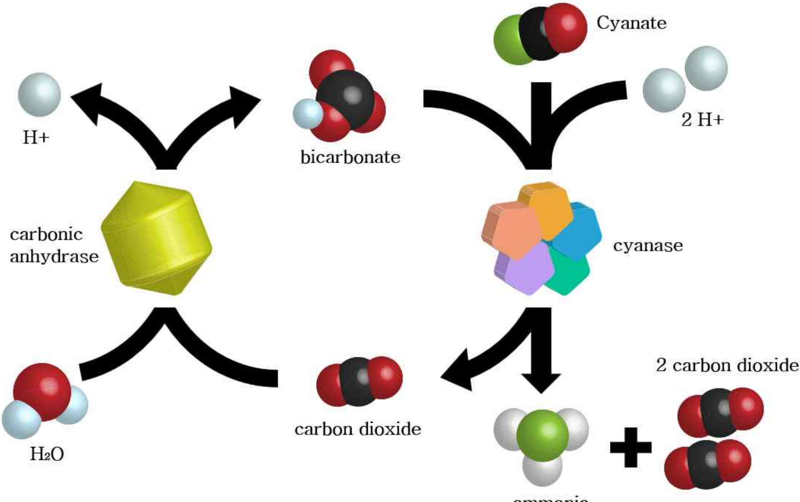

Fig. 1. Schematic diagram of cyanate detoxification of cyanase and carbonic anhydrase. Cyanase relies on HCO*LSUB*3*LSUP*− to convert the toxic cyanate to ammonia and carbon dioxide, while carbonic anhydrase transforms carbon dioxide and water to HCO*LSUB*3*LSUP*−. Therefore, the action of these two enzymes is necessary to detoxify cyanate.

In this study, these two enzymes were combined as a solution to the cyanase problem, and by polymerization and immobilization, various advantages such as improved structural stability and reuse were attempted (Mohsen Dehnavi et al., 2015; Ranjbakhsh et al., 2012; Zakeri et al., 2012). The Catcher/Tag system was used for the structural modification of *hm*CA and the immobilization of two enzymes. In this system, peptide tags and protein partner Catchers react spontaneously to form intermolecular isopeptide bonds (Zakeri et al., 2012). When recombinantly inserted into DNA encoding a target protein to form a fusion protein, the proteins bound to the system can be covalently linked when mixed. This enables bioconjugation between two proteins, which is limited or impossible with direct fusion due to problems related to protein folding, sub-optimal expression host, and specialized posttranslational modifications (Brune & Howarth, 2018). The detected Catcher/Tag pairs have been studied extensively and the types developed are diverse (Curves, 2013; Halide & Eq, 2017; Keeble et al., 2022; Tan et al., 2016; Veggiani et al., 2016). SpyCatcher/SpyTag and several others, including SnoopCatcher/SnoopTag, SdyCatcher/SdyTag, DogCatcher/DogTag, have also been developed from the cell surface adhesion proteins of *Clostridium perfringens*. These multiple Catcher/Tag systems were used to bind together enzymes together, thereby reducing HCOLSUB3LSUP−dependence and increasing reaction efficiency, resulting in effective cyanide removal. By using these multiple Catcher/Tag systems to form an enzyme complex, it is possible to reduce the HCOLSUB3LSUP−dependence of cyanase and improve the enzyme reaction efficiency, along with thermal and structural stability.

Ⅱ. MATERIALS AND METHODS

2.1. Strains and Media

Escherichia coli DH5α (Invitrogen, CA, USA) was used as a host strain for all plasmid compositions containing recombinant genes, and *E. coli* Rosetta (DE3) strain (Merk Millipore, MA, USA) was used as a host strain in protein expression. The vectors pCold II plasmid (Takara, Shiga, Japan) were used for cloning and expression of Catcher-Catcher and Catcher-*hm*CA, Tag-*hm*CA, and Tag-Cyn genes, and the media used for experiment were LB broth and LB agar, and the composition was yeast extract 5 g/L, tryptone 10 g/L, sodium chloride 10 g/L, agar 15 g/L. All strains were incubated for one day in a 37 °C incubator. All primers used in this study were provided by Cosmogenetech and the template was also manufactured by the same company (Table 1).

Table 1. List of microbial strains and plasmids used in this study

Strain or Plasmid	Description	References
Bacterial Strains		
Escherichia coli DH5α	*F- deoR endA1 gyrA96 hsdR17(rk-mk+) recA1, relA1 supE44 thi-1(lacZYA-argF) U169 (Phi80lacZdelM15)*	Invitrogen
Escherichia coli Rosetta (DE3)	*F- ompT gal dcm lon hsdSB (rB- mB-)λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pRARE*	Novagen
Plasmids		
pColdⅡ	$CspA_p$-TEE-$CspA_t$, Amp^R	Takara
pColdⅡ Cyclic-*hm*CA	$CspA_p$-TEE-SpyCatcher-*hm*CA-SpyTag-$CspA_t$, Amp^R	This study
pColdⅡ Polymeric-*hm*CA SpyTag	$CspA_p$-TEE-SpyTag-*hm*CA-SpyTag-$CspA_t$, Amp^R	This study
pColdⅡ Polymeric-*hm*CA SpyCatcher	$CspA_p$-TEE-SpyCatcher-*hm*CA-SpyCatcher-$CspA_t$, Amp^R	This study
pColdⅡ SpyCatcher-SnoopCatcher	$CspA_p$-TEE-SpyCatcher-SnoopCatcher-$CspA_t$, Amp^R	This study
pColdⅡ SpyTag-*hm*CA	$CspA_p$-TEE-SpyTag-*hm*CA-$CspA_t$, Amp^R	This study
pColdⅡ SnoopTag-Cyn	$CspA_p$-TEE-SnoopTag-Cyanase-$CspA_t$, Amp^R	This study
pColdⅡ SpyCatcher-ClopCatcher	$CspA_p$-TEE-SpyCatcher-ClopCatcher-$CspA_t$, Amp^R	This study
pColdⅡ	$CspA_p$-TEE-SpyTag-*hm*CA-$CspA_t$,	This study

SpyTag-*hm*CA	Amp^R	
pColdⅡ ClopTag-Cyn	CspA$_p$-TEE-ClopTag-Cyanase -CspA$_t$, Amp^R	This study
pColdⅡ SnoopCatcher -ClopCatcher	CspA$_p$-TEE-SnoopCatcher -ClopCatcher-CspA$_t$, Amp^R	This study
pColdⅡ SnoopTag-*hm*CA	CspA$_p$-TEE-SnoopTag-*hm*CA-CspA$_t$, Amp^R	This study
pColdⅡ ClopTag-doc-Cyn	CspA$_p$-TEE-ClopTag-dockerin-Cyanase-CspA$_t$, Amp^R	This study
pColdⅡ Cyn-doc-ClopTag	CspA$_p$-TEE-Cyanase-dockerin -ClopTag-CspA$_t$, Amp^R	This study
pColdⅡ SpyCatcher-*hm*CA -ClopCatcher	CspA$_p$-TEE-SpyCatcher-*hm*CA -ClopCatcher-CspA$_t$, Amp^R	This study
pColdⅡ SpyTag-doc-Cyn-ClopTag	CspA$_p$-TEE-SpyTag-dockerin -Cyanase-ClopTag-CspA$_t$, Amp^R	This study

2.2. Construction of recombinant *hm*CA and Cyn plasmids

The cyanase genes from *Pseudomonas stutzeri* and carbonic anhydrase genes from *Hydrogenovibrio marinus* were amplified using primers prepared with *Sac I* and *Kpn I* restriction sites and partial vector DNA regions, respectively (Table 2).

Table 2. List of primers used in this study

Name	Sequence
SCA P1	ATATGAGCTCGATTACGACATCCCAACGACCGAAAACCTGTATTTTAGGGCGCGATGGTCGATACTCTGA
SCA P2	*CATTGCTATGAATGTGGGCG*TCTCCTTTGGTTGCCTTTCCG
SCA P3	*CGCCCACATTCATAGCAATG*CCCCATTGATT
SCA P4	ATATGGTACCCTACTTCGTGGGTTTATAGGCATCGACCATAACGATATGAGCGCCTCCGCTGCCACCACTCCCGTAATATTGATAGTAAC
CCA P1	ATATGAGCTCGATTACGACATCCCAACGAC
CCA P2	*CGACCATCGCGCCTCCGCTG*CCA
CCA P3	*CAGCGGAGGCGCGATGGTCG*ATACTCTGA
CCA P4	GAATTCGGATCCCTCGAGATATGGTACCCTAAATGTGGGCGTCTCCTTT
TCA P1	ATATGAGCTCGATTACGACATCCCAACGACCGAAAACCTGTATTTCAGGGCGCTCATATCGTTATGGTCGATGCCTATAAACCCACGAAGGGTTCAGGGGGTTCCGGTCATAGCAATGCCCCATTGA
TCA P2	ATATGGTACCCTACTTCGTGGGTTTATAGGCATCGACCATAACGATATGAGCGCCTCCGCTGCCACCACTCCCGTAATATTGATAGTAAC
SSC P1	CATCATCATATGGAGCTCGCGATGGTCGATACTCTGAG
SSC P2	*GCTTCATATGAATGTGGGCG*TCTCCTT
SSC P3	*CGCCCACATTCATATGAAGC*CGCTGCG
SSC P4	GGATCCCTCGAGGGTACCCTATTTCGGCGGTATCGGTT
SpCA P1	CATCATCATATGGAGCTCCATAGCAATGCCCCATTGAT
SpCA P2	GGATCCCTCGAGGGTACCTCACTTCGTGGGTTTATAGGCATCGACCATAACGATATGAGC
SnCyn P1	CATCATCATATGGAGCTCATGATCAGCAGTCGTGAACA
SnCyn P2	GGATCCCTCGAGGGTACCTCACATATGGGAAAACTGGGC

	GATATTGAATTTATTAAAGTGAACAAAGGTAGTGGT GCCTCCGCTGCCACCACTCCC
SpCC P1	CATCATCATATGGAGCTCGCGATGGTCGATACTCTGAG
SpCC P2	*CCGGAAGATTAATGTGGGCG*TCTCCTT
SpCC P3	*CGCCCACATTAATCTTCCGG*AAGTTAAAGATGG
SpCC P4	GGATCCCTCGAGGGTACCCTAGTAATCCTTATCTGTATC A
ClCyn P1	CATCATCATATGGAGCTCATGATCAGCAGTCGTGAACA
ClCyn P2	GGATCCCTCGAGGGTACCTCAAGGTTTTTCAACTACAAG AGTTTGAGCTTTATCATTTTTATCTTCATGTTTAACAA CTTGCTTTGTATCGCCTCCGCTGCCA
SnCC P1	CATCATCATATGGAGCTCCATATGAAGCCGCTGCGTGG
SnCC P2	*TGTGGGCG*TCCATATGAAGCCGCTGCG
SnCC P3	*CGCCCACA*TTAATCTTCCGGAAGTTAAAGATGG
SnCC P4	GGATCCCTCGAGGGTACCCTAGTAATCCTTATCTGTATC A
SnCA P1	CATCATCATATGGAGCTCCATAGCAATGCCCCATTGAT
SnCA P2	GGATCCCTCGAGGGTACCTCACATATGGGAAAACTGGGC GATATTGAATTTATTAAAGTGAACAAAGGTAGTGGTGA AAGTGGTGCCTCCGCTGCCA
CyndC P1	CATCATCATATGGAGCTCATGATCAGCAGTCGTGAACA
CyndC P2	*CAGCGGATCCGTAGCTCTTG*TAGGGCAG
CyndC P3	*CAAGAGCTACGGATCCGCTG*GCTCC
CyndC P4	GGATCCCTCGAGGGTACCTCAAGGTTTTTCAACTACAAG AGTTTGAGCTTTATCATTTTTATCTTCATGTTTAACAA CTTGCTTTGTATCGCCTCCGCTGCCACCACTCCCTAAAA GCATTTTTTTAAGAACAGCTAAAT
CdCyn P1	CATCATCATATGGAGCTCGATACAAAGCAAGTTGTTAA ACATGAAGATAAAAATGATAAAGCTCAAACTCTTGTAG TTGAAAAACCTGGGAGTGGTGGCAGCGGAGGC
CdCyn P2	*TGCTGATCATGCCTCCGCTG*CCA

Primer	Sequence
CdCyn P3	*CAGCGGAGGCATGATCAGCA*GTCGTGAAC
CdCyn P4	GGATCCCTCGAGGGTACCTCAGTAGCTCTTGTAGGGCA
SCCA P1	CATCATCATATGGAGCTCGCGATGGTCGATACTCTGAG
SCCA P2	*CATTGCTATGGAATTCCCCA*GAACCAGCAGCGGAGCCAGCGGATCC
SCCA P3	*TGGGGAATTCCATAGCAATG*CCCCATTGA
SCCA P4	*CCGGAAGATTGCCTCCGCTG*CCA
SCCA P5	*CAGCGGAGGCAATCTTCCGG*AAGTTAAAGATGG
SCCA P6	GGATCCCTCGAGGGTACCCTAGTAATCCTTATCTGTATCA
SCCyn P1	CATCATCATATGGAGCTCGCTCATATCGTTATGGTCGATGCCTATAAACCCACGAAGGGATCCGCTGGCTCCGCTGCTGGTTCTGGGGAATTC
SCCyn P2	GGATCCCTCGAGGGTACCTCAAGGTTTTTCAACTACAAGAGTTTGAGCTTTATCATTTTTATCTTCATGTTTAACAACTTGCTTTGTATCGCCTCCGCTGCCACCACTCCCGTAGCTCTTGTAGGGCAG

*Italics indicate the overlapping part of the primer.

*Underline means restriction enzyme site

A three-step PCR process was performed to bind each enzyme and Catcher/Tag gene, and the conditions were as follows; First PCR was performed with denaturation at 98 °C for 3 minutes and 10 seconds, annealing at 52 °C for 5 seconds, and elongation at 68 °C for 15 seconds, and a total of 30 cycles were repeated. In the overlap PCR process, purified PCR solutions without primer and template were added, and under the same temperature conditions as before, denaturation was performed for 5 minutes and 30 seconds, annealing was performed for 25 minutes, and elongation was repeated for a total of 10 cycles. After that, the bound gene was amplified through Final PCR, and the DNA obtained through the experiment was purified and then the concentration was measured together with the restriction enzyme-treated vector DNA purification solution. To compose the mixture required for cloning, we calculated using the bp length of vector and insert and vector mass to obtain the insert mass in a ratio of 1:3, and then divided the measured concentration value to obtain the addition amount. NEBtools application was used for calculation and the vector mass was set to 100. During the cloning process, the EZ-Fusion *LSUPTM* HT Cloning kit (Enzynomics, Daejeon, Korea) was used, the mixture composition ratio was set as described in the manual, and the prepared solution was subjected to thermal shock in a 50 °C water bath for 15 minutes. Then, it was transformed into *E. coli* DH5α competent cells.

2.3. Expression and purification of enzymes

Plasmids separated from the positive clones pColdⅡ-Catcher, pColdⅡ-*hm*CA, and pColdⅡ-Cyn were transformed into *E. coli* Rosetta (DE3) and incubated at 37 °C in 5 mL of an LB medium with ampicillin (50 μg/mL) and chloramphenicol (1 μL/mL) added for one day. The preculture medium was inoculated into 300 mL LB-ampicillin-chloramphenicol medium and cultured at 37 °C until the absorption rate (A*LSUB*600) reached 0.2. The expression of the genes of pColdⅡ-Catcher, pColdⅡ-*hm*CA, and pColdⅡ-Cyn was induced by adding 1M isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 16 °C for one day. After that, the microorganism was extracted by centrifuging it at 4 °C and 4,000 rpm for 15 minutes, and then removing the supernatant, it was suspended in lysis buffer (50 mM NaH*LSUB*2PO*LSUB*4, 300 mM NaCl, 20 mM imidazole, pH 8.0) and crushed the microorganism in ice for 7 minutes with ultrasonicator. After releasing the protein, centrifugation was performed under the same conditions as before to remove cell debris and purify the obtained supernatant. Purification of recombinant *hm*CA and cyanase enzymes was performed using a Ni-NTA column (Qiagen, USA), and lysis buffer, wash buffer (50 mM NaH*LSUB*2PO*LSUB*4, 300 mM NaCl, 20 mM imidazole, pH 8.0), elution buffer (50 mM NaH*LSUB*2PO*LSUB*4, 300 mM NaCl, 20 mM imidazole, pH 8.0) were used together.

2.4. SDS-PAGE and western blot analyses

SDS-PAGE and western blot analysis were carried out to confirm whether the extracted purified solution contains the target enzyme. 10 μL of protein sample were centrifuged for 5 minutes at 13,000 rpm after being heated for 5 minutes at 100 °C, and electrophoresis was performed on 15 % SDS-PAGE to analyze the size of the enzyme. The isolated protein band was confirmed through western blot using an anti-His-HRP conjugate antibody, and InVision*LSUPTM* His-Tag reagent(Thermo Fisher Scientific Korea, Seoul, Korea) was used together in the experiment, and the manufacturer's manual was followed (novex).

2.5. Thermostability Test

To compare the thermal stability of the expressed wild *hm*CA protein and cyclic *hm*CA protein, the reaction time was measured by dividing it into short and long periods of time through quantification of soluble fractions, and the results were shown through CO_2 hydration analysis using colorimetric methods (Hatlem et al., 2019; Wu et al., 2010). Samples measured for a short period of time were cultured at 25, 37, 55, 75, 90, and 100 °C, reacted at each temperature for 10 minutes, cooled to 10 °C, and centrifuged at 4 °C and 17,000 rpm for 30 minutes. Subsequently, 6X SDS loading buffer was added to the supernatant, heated at 95 °C for 7 minutes, and loaded onto a 10 % SDS-PAGE. Each gel contained three protein control samples intended to be used to standardize the concentration of different gels. The recovery rate was calculated from the band intensity relative to the control sample of that gel and was defined as 100 % recovery. In this case, band intensity analysis was carried out using ImageJ (version 1.52a; National Institutes of Health, USA). Samples of long-term measurements were investigated at high temperatures of 40~80 °C, incubated at each temperature for 6 hours, immediately cooled on ice, and added to a disposable cuvette containing 20 mM Tris buffer (pH 8.3) supplemented with 100 μM phenol red. This reaction was conducted inside the spectrometer at 4 °C by mixing 400 mL of prepared CO_2 saturated deionized water with cold ice water, and the change in absorbance was tracked at 570 nm. It took time (t) to lower the

absorbance from 1.20 (pH 7.5) to 0.18 (pH 6.5), and this time was measured., and the time (t_0) for the unanalyzed reaction was also tested by adding the equivalent empty buffer instead of the enzyme sample (Jo et al., 2020b). Standard tests were used to evaluate the activity, and the pretreatment activity was explained as 100 %.

2.6. Enzymatic Conversion of Carbon Dioxide

To measure CA's ability to convert CO_2 into calcium carbonate ($CaCO_3$), 2 mL of Tris buffer (1 M, pH 8.0), 23 mL of CO_2 saturated water, 23 mL of 2 % (v/v) calcium chloride solution, and 2 mL (0.5 mg/mL) of an enzyme phosphate (50 mg/mL) were all included in a 50 mL total reaction mixture container. The conversion reaction was performed at room temperature for 10 minutes, and bovine serum albumin (BSA) was used as a control. Centrifugation was performed to recover the formed precipitate after 10 minutes, and dry powder was obtained by freeze drying. Subsequently, the weight of the $CaCO_3$ precipitate was measured to figure out the amount of carbonate accumulated during the enzyme reaction (T. Sharma & Kumar, 2021).

2.7. Enzyme assays

The cyanase assay was modified and performed according to Anderson's method (Anderson, 1980; Kebeish & Al-Zoubi, 2017). A mixture of an enzyme tablet buffered with Na*LSUB*2CO*LSUB*3-NaHCO *LSUB*3 buffer (pH 9.16) and phosphate-buffered sealine (PBS) buffer, 50mM potassium-phosphate buffer (pH 7.7), and 20 mM sodium carbonate was produced in Table 3 and 4.

Table 3. Proportions of reaction samples tested in this study.

Amount (μL) \ Sample	Control	*hm*CA	Cyanase	Catcher
20 mM Sodium cyanate	5	5	5	5
PBS buffer	20	20	20	20
50 mM Potassium phosphate buffer(pH 7.7)	175	165	165	165
Cyanase	0	0	10	0
*hm*CA	0	10	0	0
Catcher	0	0	0	10

Table 4. Proportions of reaction samples tested in this study.

Amount (µL) \ Sample	*hm*CA +Cyanase	*hm*CA +Catcher	Cyanase +Catcher	*hm*CA +Cyanase +Catcher
20 mM Sodium cyanate	5	5	5	5
PBS buffer	20	20	20	20
50 mM Potassium phosphate buffer(pH 7.7)	155	155	155	145
Cyanase	10	0	10	10
*hm*CA	10	10	0	10
Catcher	0	10	10	10

When a Catcher is connected to both sides of *hm*CA, the reaction efficiency was analyzed only with 4 samples excluding the Catcher under the conditions. Afterwards, incubate at 27 °C for 5 min and terminate the reaction by adding 150 µL of Nessler reagent to 150 µL of the mixture. The amount of ammonia released was measured as absorbance at 420 nm.

Ⅲ. RESULTS AND DISCUSSION

3.1. Expression of subunits and the assembly of *hm*CA complexes

Cyclization and polymer strategies have been introduced to impart thermal and structural stability to *hm*CA to be used as a CO_2 reduction strategy (Sun et al., 2022). The pColdⅡ Cyclic-*hm*CA Plasmid, pColdⅡ Polymeric-*hm*CA SpyTag Plasmid, and pColdⅡ Polymeric-*hm*CA SpyCatcher Plasmid completed through genetic recombination were expressed as proteins, and SDS-PAGE and western blot were carried out to compare the level with the existing enzyme (Fig. 2).

(A) (B)

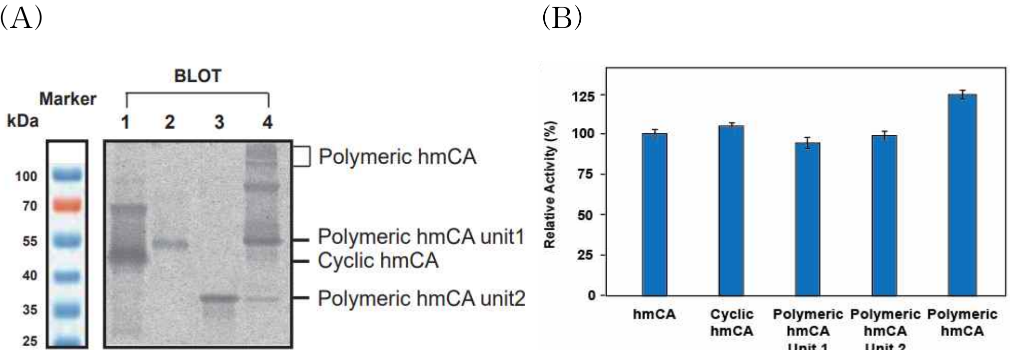

Fig. 2. Comparison of SDS-PAGE profiles and activities of recombinant enzymes. (A) Results of western blot after protein SDS-PAGE treatment. Line 1 is a cyclic *hm*CA protein of SpyCatcher + *hm*CA + SpyTag DNA, and line 2 is a polymeric *hm*CA unit 1 protein expressing the SpyCatcher + *hm*CA + SpyCatcher gene. Line 3 represents polymeric *hm*CA unit 2 expressing SpyTag + *hm*CA + SpyTag DNA, and line 4 represents polymeric *hm*CA unit 1 and unit 2 combined. It can be seen that all purified proteins form bands at the expected positions, and M is a protein marker. (B) As a result of measuring the activity of the expressed enzyme, it can be seen that the expression level of polymeric *hm*CA is higher than that of other enzymes while all of them show similar activities.

The expected size of Cyclic *hm*CA is 50.8 kDa (the size calculated by genetic map analysis is SpyCatcher 11.9 kDa, *hm*CA + SpyTag 35.5 kDa, and the total size is 47.4 kDa), so it is estimated that the band located between 40 kDa and 55 kDa of the marker is an enzyme protein. When the same experiment was performed on the proteins of Polymeric *hm*CA unit 1 and Polymeric *hm*CA unit 2 that will form the polymer, it was confirmed that bands appeared at around 55 kDa and around 35 kDa of the markers (Polymeric *hm*CA unit 1 57.8 kDa, Polymeric *hm*CA unit 2 36.8 kDa), respectively. In addition, when a standard curve of protein concentration was drawn using Bradford's reagent and the activity of the expressed enzyme was measured, recombined *hm*CA such as Cyclic *hm*CA showed similar levels to wild *hm*CA, especially polymeric *hm*CA's enzyme activity was higher than other enzymes. These results indicate that the modification of the structure to be cyclized through covalent bonds does not decrease the activity of the enzyme, and that the formation of the polymer can enhance the action of the protein more than the existing activity.

3.2. Thermostability of cyclic and polymeric *hm*CA

To check whether the stability of *hm*CA over temperature and time was enhanced compared to that of the wild-type enzyme, quantification of soluble fractions and a colorimetric method through CO_2 hydration assay was performed. Figure 3A shows the short-term response results calculated using ImageJ. When wild *hm*CA, cyclic *hm*CA, and polymeric *hm*CA were incubated in phosphate buffered saline for a short period of time over a wide range of temperatures, all three enzymes showed quite maintaining their original activity at a temperature in the range of 20～30 °C. However, wild *hm*CA decreased to 68 % and 55 % from the point above 40 °C and was found to be fully agglomerated from 70 °C, while cyclic *hm*CA and polymeric *hm*CA showed stability in maintaining catalytic activity recovery between 90～100% even above 70 °C (Fig. 3A).

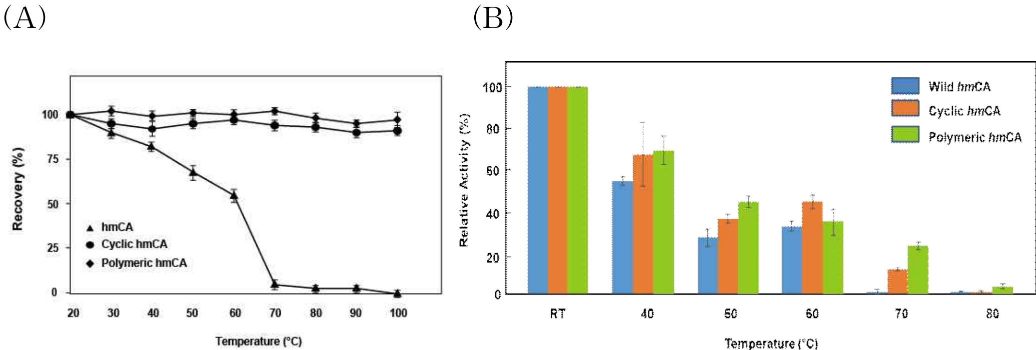

Fig. 3. Comparison of thermal stability of three types of *hm*CA. (A) In short-term reaction, wild *hm*CA shows a sharp decrease in recovery from 40～50 °C, whereas cyclic *hm*CA and polymeric *hm*CA maintain their structures during all reaction temperatures. (B) All three types of *hm*CA show decreased activity when exposed to reaction temperature for a long period of time, but recombinant *hm*CA forming cyclization shows activity even at the temperature at which wild-type is inactivated.

In SDS-PAGE gel, wild *hm*CA did not show a band from 70 °C, but cyclized and polymerized *hm*CA formed a band around 55 kDa at all temperatures. In the long-term reaction, SpyCatcher protein and *hm*CA + SpyTag protein were mixed in a 1:1:1 ratio using 20 mM Tris buffer (pH 8.3) and the experiment was carried out (Fig. 3B). At 40 °C, wild *hm*CA, cyclic *hm*CA, and polymeric *hm*CA had activities of 55.1 %, 68.3 %, and 69.8 %, respectively, and at 50 °C they were 27.3 %, 36.1 %, 44.3 %, and at 60 °C, 33.1 %, 44.6 %, and 35.5 %. As the reaction temperature increased, each enzyme showed a difference in activity according to its structure. At 70 °C, wild *hm*CA had almost no activity at 1.1%, whereas cyclic *hm*CA and polymeric *hm*CA were 10.4 % and 22.6 %, showing that they act even at higher temperatures than wild *hm*CA. At 80 °C, all of the activity values were as low as 4 % or less. Analysis of the overall results showed that all three *hm*CA decreased in activity as the temperature applied to the enzyme increased, but the recombinant *hm*CA reacted at temperatures above 50 °C where wild *hm*CA slowly lost activity, and additionally, polymeric *hm*CA was found to have higher thermal stability than cyclic *hm*CA.

As seen from the above results, it is possible to maintain stability against heat when cyclization and polymerization of *hm*CA are formed. In previous research, the N'- and C'-terminal are the most flexible parts of the protein and connecting these regions by protein-protein interaction has been investigated as a promising method for thermal stabilization (Gao & Ming, 2022; Xu et al., 2020). Due to this principle, the cyclization of carbonic anhydrase by the Catcher/Tag system can maintain the enzyme

activity even at high temperatures. In addition, the fact that polymeric *hm*CA exhibited a higher thermal stability than cyclic *hm*CA is likely due to the difference in the number of irreversible covalent bonds. Compared to a cyclic protein having only one bond, a polymeric protein is composed of several bonds and is able to maintain a stronger structure at different temperatures.

3.3. Enzymatic Conversion of Carbon Dioxide

Finally, the reaction efficiency of CA acting in the conversion of CO_2 to $CaCO_3$ in the presence of calcium ions was investigated. BSA was used as a control that did not show CO_2 precipitation in the experiment, and the CO_2 conversion efficiency of CA was investigated by estimating the quantity of produced $CaCO_3$. The experiment's findings showed that the test reaction using crude CA converted CO_2 significantly more than the control. Wild-type *hm*CA formed 11.23 mg $CaCO_3$/mg protein. The CO_2 conversion efficiency of polymeric *hm*CA (47.21 mg $CaCO_3$/mg protein) was much higher than that of cyclic *hm*CA (27.67 mg $CaCO_3$/mg protein). $CaCO_3$ is frequently used as a filler in many different industries, including those that deal with food, medicine, paper, paint, and plastics. The isolated bacterium's ability to create powerful CA, which can be employed for CO_2 conversion both in the lab and on a large scale, was thus validated.

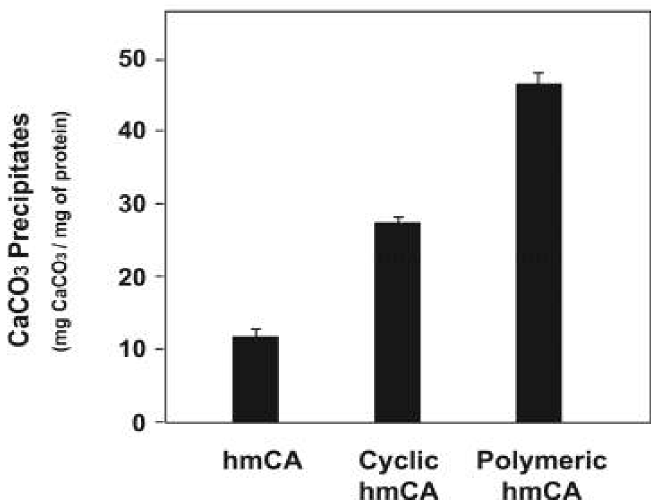

Fig. 4. Comparison of the efficacy of converting CO_2 into $CaCO_3$ of three *hm*CAs. When the amount of synthesized $CaCO_3$ is calculated, polymeric *hm*CA and cyclic *hm*CA generate 2 times and 4 times more $CaCO_3$ than wild *hm*CA, respectively, so it can be confirmed that the complex-forming *hm*CA has the greatest CO_2 conversion efficiency.

3.4. Design of recombinant cyanase and *hm*CA

As a way to reduce cyanase's HCO*LSUB*3*LSUP*− dependence, it was intended to bind to *hm*CA, and for this purpose, a peptide module that performs irreversible covalent bonding was introduced. A multi-step PCR process was performed to construct the recombinant enzyme complex, and as a result, two types of Catcher/Tag systems were able to obtain genes linked to cyanase and *hm*CA enzymes. The gene containing the Catcher and the tag gene with dockerin inserted, which require overlap PCR, amplified each DNA to be combined by first PCR, and then performed an experiment according to the principle of overlap PCR to fuse two and three DNA fragments. Then, the entire linked gene was amplified once more through 3rd PCR, and general PCR was used for enzyme + Tag DNA that does not require a multi-step PCR process. All of the recombinase genes to be used in the study were amplified successfully and bands were formed at each position consistent with the expected size (Fig. 5, 6, 7).

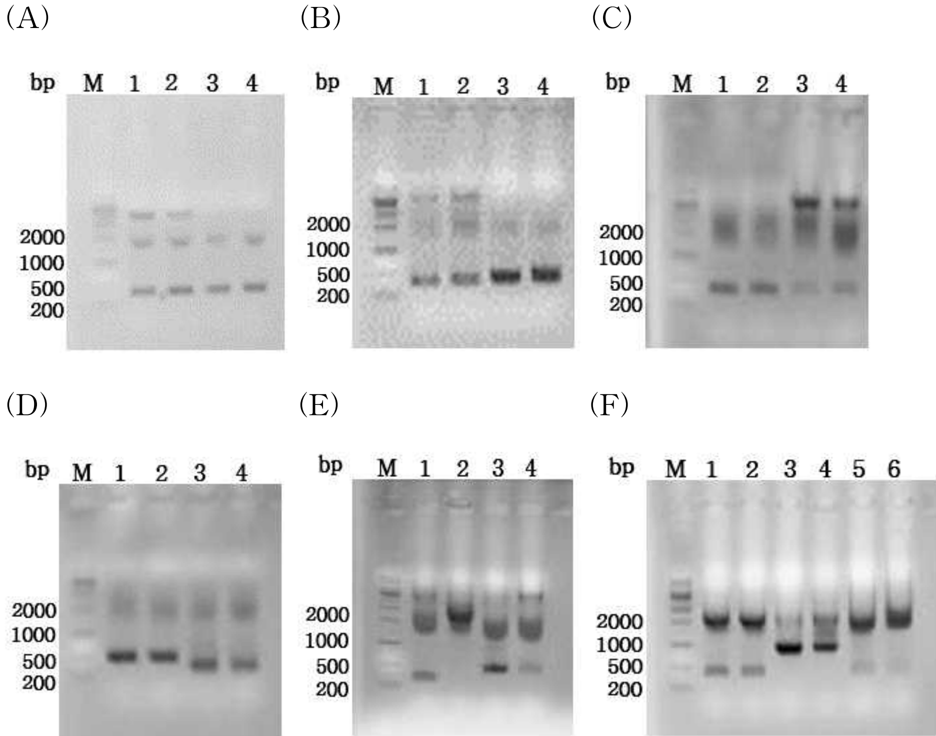

Fig. 5. Analysis of primary PCR results of enzyme genes by agarose gel electrophoresis. 5 μL of PCR reaction is blended with 1 μL of 6X Loading buffer and separated on a 0.8 % agarose gel. It can be observed that most of the DNA forms a band near 500 bp, and it can be confirmed that this is a position similar to the length of each gene. (A) Lines 1-2 are SpyCatcher and lines 3-4 are SnoopCatcher. (B) Lines 1-2 are SpyCatcher, Lines 3-4 are ClopCatcher, (C) Lines 1-2 are SnoopCatcher and Lines 3-4 are ClopCatcher. (D) line 1-2 is cyanase, line 3-4 is dockerin + ClopTag and (E) line 1-2 is ClopTag + dockerin, line 3-4 is cyanase. (F) Lines 1-2 are SpyCatcher, lines 3-4 are *hm*CA, lines 5-6 are ClopCatcher.

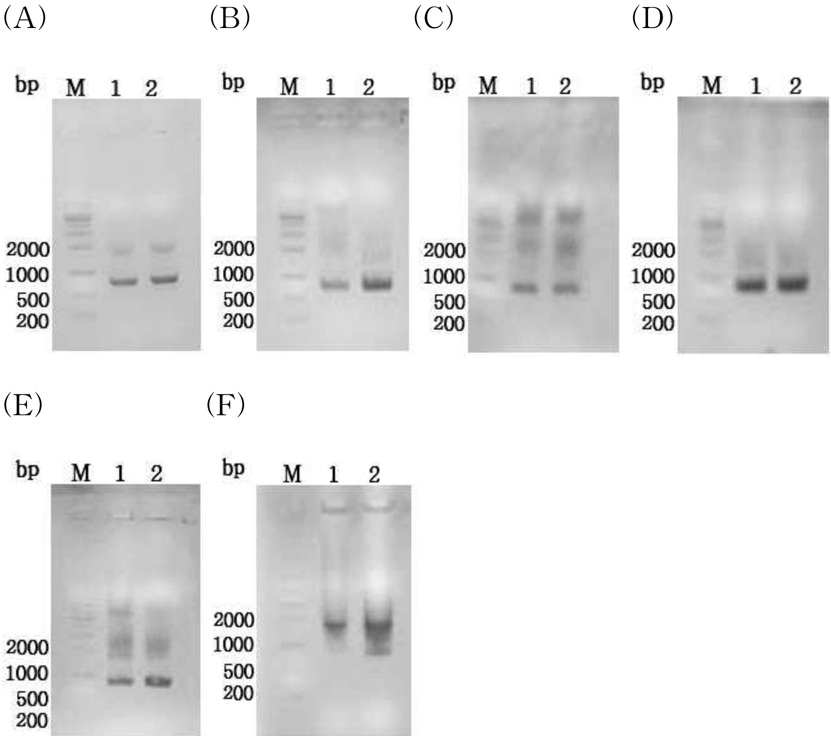

Fig. 6. Analysis of final PCR results of recombinant genes by agarose gel electrophoresis. 5 μL of PCR reaction is blended with 1 μL of 6X Loading buffer and separated on a 0.8 % agarose gel. It can be observed that the bound enzyme DNA forms bands near 500～1,000 bp and 1,000 bp, which are similar to the expected length, indicating that the recombinant gene required for the study was amplified correctly. (A) Lines 1-2 represent SpyCatcher + SnoopCatcher and (B) Lines 1-2 represent SpyCatcher + ClopCatcher. (C) Lines 1-2 are SnoopCatcher + ClopCatcher and (D) Lines 1-2 are Cyanase + dockerin + ClopTag. (E) Lines 1-2 represent ClopTag + dockerin + Cyanase and (F) Lines 1-2 are SpyCatcher + *hm*CA + ClopCatcher.

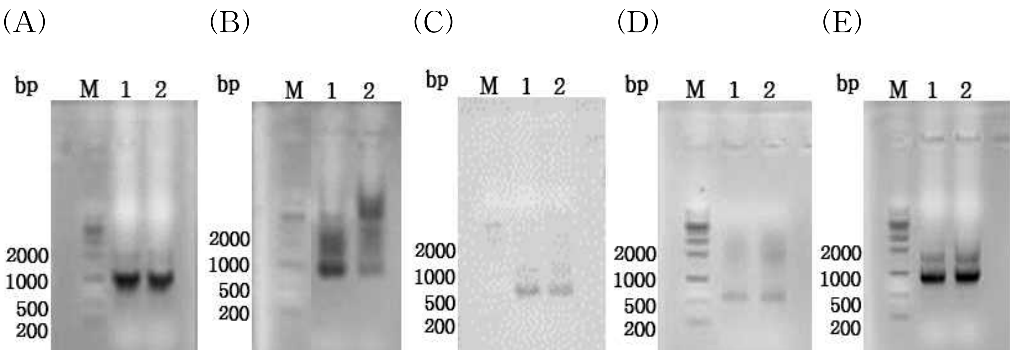

Fig. 7. Analysis of PCR results of recombinant genes by agarose gel electrophoresis. 5 μL of PCR reaction is blended with 1 μL of 6X Loading buffer and separated on a 0.8 % agarose gel. It can be observed that the bound enzyme DNA forms a band near 500~1,000 bp and 1,000 bp, which are similar to the expected length, indicating that the recombinant gene required for the study was amplified correctly. (A) Lines 1-2 represent *hm*CA + SpyTag and (B) Lines 1-2 are *hm*CA + SnoopTag. (C) Lines 1-2 represent Cyanase + SnoopTag, (D) Lines 1-2 are Cyanase + ClopTag, (E) Lines 1-2 represent SpyTag + dockerin + Cyanase + ClopTag.

Each enzyme DNA size is as follows; SpyCatcher 333 bp, SnoopCatcher 342 bp, ClopCatcher 372 bp, SpyTag 39 bp, SnoopTag 63 bp, ClopTag 69 bp, cyanase 450 bp, *hm*CA 882 bp, SpyCatcher + SnoopCatcher 687 bp, SpyCatcher + ClopCatcher 717 bp, SnoopCatcher + ClopCatcher 726 bp, *hm*CA + SpyTag 942 bp, Cyanase + SnoopTag 531 bp, *hm*CA + SnoopTag 966 bp, Cyanase + ClopTag 537 bp, Cyanase + dockerin + ClopTag 732 bp, ClopTag + dockerin + Cyanase 732 bp, SpyCatcher + *hm*CA + ClopCatcher 1644 bp, SpyTag + dockerin + Cyanase + ClopTag 792 bp. When checking the first PCR results, it can be observed that most genes form bands near 500 bp of the marker, and after overlap PCR and 3rd PCR, they were located near 500～1,000 bp or 1,000 bp. In addition, it was confirmed that bands appeared at positions similar to the length of each enzyme gene after undergoing general PCR.

It seems that overlap PCR played a major role in the multi-step PCR process that made it possible to construct a recombinant plasmid. In this experiment, which aims to bind protein and peptide genes to target enzyme DNA, primers are constructed with complementary 5′ overhangs of other molecules. Therefore, the DNA is expanded after annealing, and the two DNA molecules made in this way are mixed and the gene is amplified using only the primer of the fabric. The gene obtained through PCR was purified and then inserted into the pColdⅡ vector to finally complete the recombinant plasmid.

3.5. Expression of subunits and the assembly of complexes

Expression of recombinant cyanase, *hm*CA, and Catcher-Catcher forming a complex was confirmed along with their size and purity by performing SDS-PAGE and western blot after purification (Fig. 8).

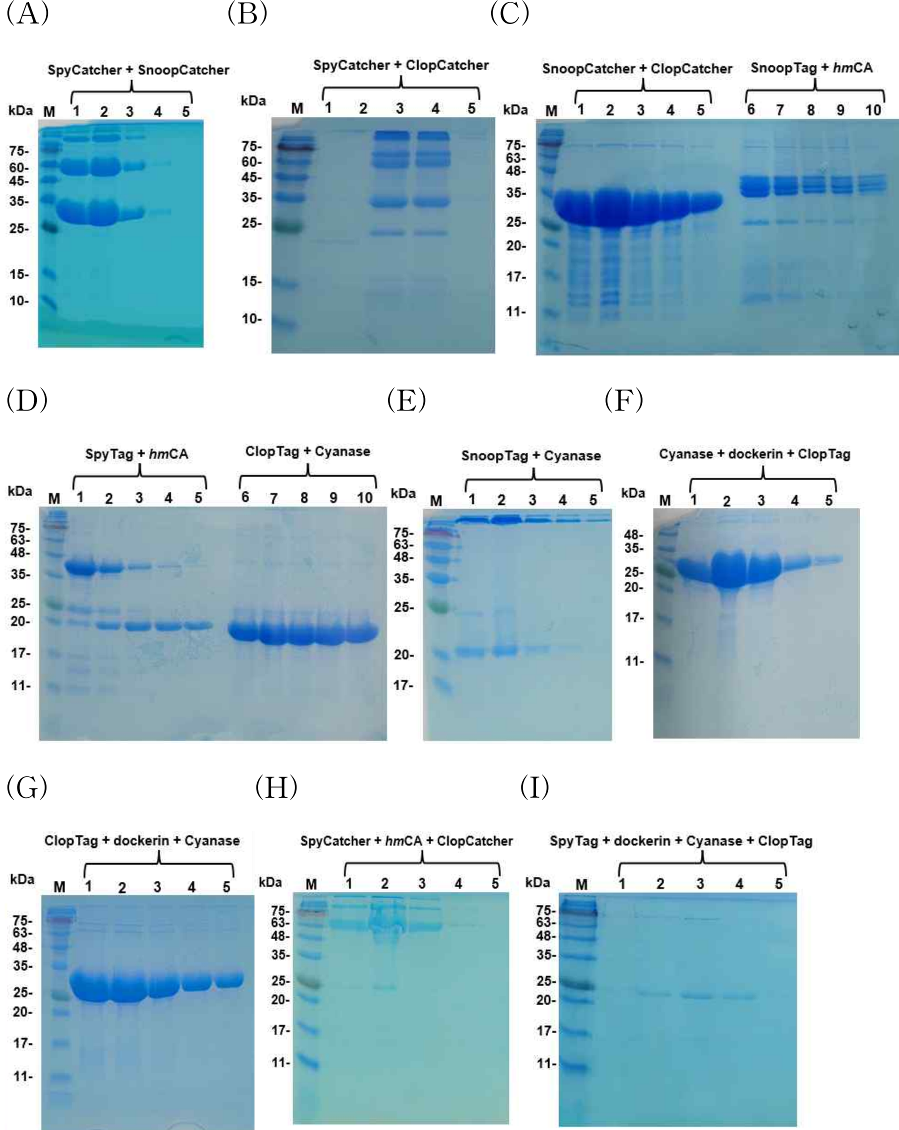

Fig. 8. SDS-PAGE profile of recombinant enzymes. It can be confirmed that all of the purified proteins form a band at the expected position, and M is a protein marker.

The molecular weight of the enzyme protein expected through genetic map analysis is as follows; SpyCatcher + SnoopCatcher 25.42 kDa, SpyCatcher + ClopCatcher 25.70 kDa, SnoopCatcher + ClopCatcher 26.87 kDa, *hm*CA + SpyTag 35.10 kDa, Cyanase + SnoopTag 16.66 kDa, *hm*CA + SnoopTag 36.15 kDa, Cyanase + ClopTag 19.76 kDa, Cyanase + dockerin + ClopTag 26.40 kDa, ClopTag + dockerin + Cyanase 26.40 kDa, SpyCatcher + *hm*CA + ClopCatcher 59.88 kDa, SpyTag + dockerin + Cyanase + ClopTag 28.32 kDa, SpyCatcher + *hm*CA + ClopCatcher 59.87 kDa, SpyTag + dockerin + Cyanase + ClopTag 28.32 kDa. The bands of the proteins observed through the experiment were formed around ~20 kDa, 25~35 kDa, ~63 kDa of the marker, and it can be confirmed that this is consistent with the expected molecular weight mentioned above. In addition to the molecular weight of the bound enzymes, it can be seen that several bands appeared at other positions of the marker. It seems that the enzymes combine to form a complex, or the fragments appear as bands as the linked proteins are degraded. In the results of SpyCatcher + SnoopCatcher, complexes of different sizes were formed upstream of the protein at 25~35 kDa. ClopCatcher and ClopTag were introduced to check whether other types of peptide modules also showed this result, and similar bands were observed. Therefore, a reaction efficiency experiment was conducted with the SpyCatcher + ClopCatcher enzyme complex, judging that similar results would be obtained in subsequent experiments.

3.6. Binding effect of cyanase and *hm*CA on cyanate degradation

The decomposition of cyanate by cyanase showed the highest number when it worked with *hm*CA. The conditions in which cyanase was included in the reacted sample were generally high, and the conditions in which Catcher + Catcher, Tag + cyanase, and Tag + *hm*CA were complexed showed higher efficiency than when cyanase was used alone, but the ammonia production was lower than those in the presence of only two enzymes(Fig. 9).

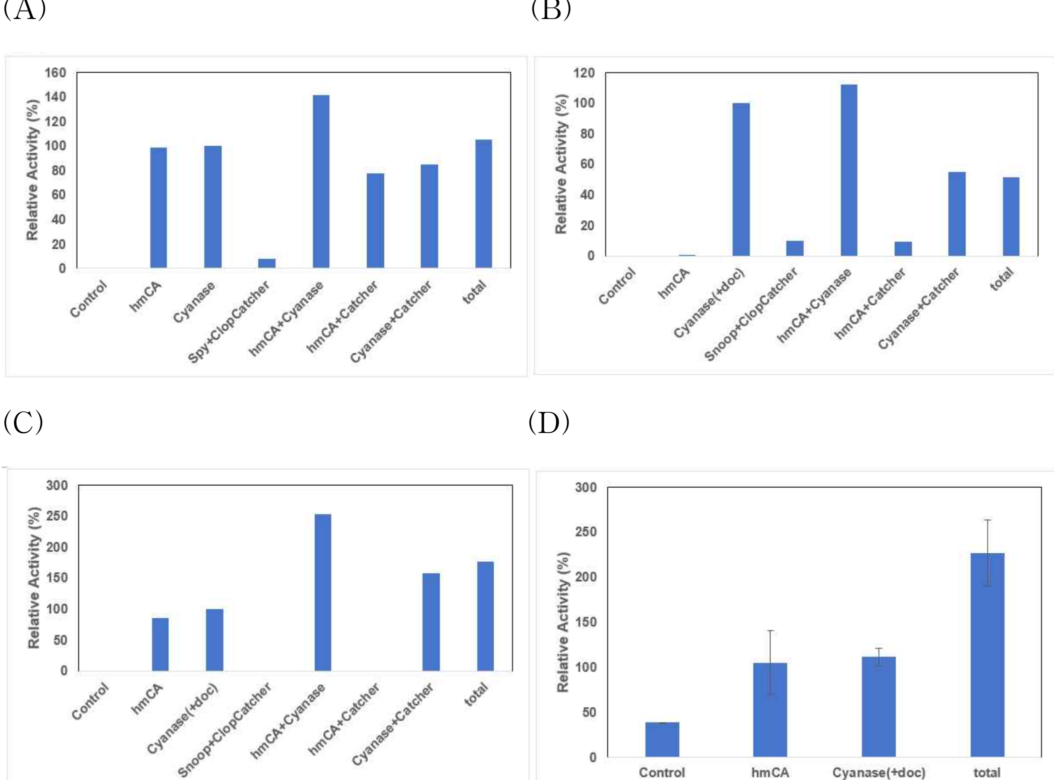

Fig. 9. (A) Spy + Clop (B) Snoop + Clop (Cyanase-Tag) (C) Snoop + Clop (Tag-Cyanase) (D) Spy + Clop (Catcher-*hm*CA, Tag-Cyanase) digestion of cyanate in samples under different conditions. In each experiment in which different types of Catcher/Tag systems were introduced, the *hm*CA + Cyanase condition showed the greatest reaction efficiency in common, and other samples containing cyanase also showed relatively high reaction. Under the condition in which the two enzymes form a complex with a circular structure, the reaction efficiency is twice as high as when cyanase alone is used.

These results suggest that when enzymes form a complex through the Catcher/Tag system, they bind in a straight line that connects side by side, leading to a restriction in distance compared to cyanase and *hm*CA alone, resulting in reduced efficiency.

Therefore, to improve this, the gene was manipulated to form a circular structure like polymeric *hm*CA rather than a linear structure, and the reaction efficiency was measured again by expressing the protein(Fig. 9D). As a result of the experiment, it was confirmed that the condition in which the two enzymes formed a complex showed more than two times higher activity than the condition in which cyanase alone was used, and the reaction efficiency was improved compared to the previous complex formed by mixing Catcher, cyanase, and *hm*CA. In addition, compared to when the two enzymes were not combined, the difference in activity with the condition using only cyanase increased more than before, suggesting that the formation of a complex with a circular structure promoted the conversion action of the enzyme. These results indicate that in the presence of the same concentrations of oxidized cyanide and HCO*LSUB*3 *LSUP*−, an enzyme complex with reduced HCO*LSUB*3 *LSUP*−dependence and improved reaction efficiency was constructed compared to cyanase alone.

Ⅳ. CONCLUSION

This study used Catcher/Tag systems with irreversible covalent bonds to cyclize and form a complex of *hm*CA, and then combined with cyanase to improve the thermal and structural stability of enzymes and increase reaction efficiency, as well as reduce the HCO*LSUB*3*LSUP*− dependent of cyanase. The structure was modified by recombination of *hm*CA and cyanase genes through a multi-step PCR process, and subunits capable of forming complexes by expressing proteins were prepared.

The *hm*CA study showed that cyclization and complex formation exhibit distinct differences at temperatures above 60 °C when compared to wild-type in terms of thermal stability. Unlike wild *hm*CA, which showed a sharp decrease in activity recovery, cyclic *hm*CA and polymeric *hm*CA recovered and maintained activity even at 90～100 °C, indicating improved enzyme stability. This means that the action of linking the N- and C-terminals helped improve the protein structure. In conclusion, we succeeded in preparing a heat-resistant enzyme that can work even at high temperatures where wild-type enzymes are inactivated. In addition, in the synthesis of $CaCO_3$ through enzymes, polymeric *hm*CA produced 4 times more than wild *hm*CA, confirming that structural modification improved stability as well as production efficiency of reactants.

A study in which cyanase and *hm*CA were combined and reacted together confirmed that the combination of the two enzymes increased

the efficiency compared to the use of cyanase alone, but the amount of ammonia produced decreased under the condition in which the complex formation was induced by adding a Catcher. Unlike the previous experiment in which the conversion efficiency of CO_2 was improved, the cyanide decomposition of cyanase and *hm*CA complex using two types of Catcher/Tag system was more inefficient than when the enzymes were not combined with each other. The reason for this is that the formation of a complex in a straight line connected side by side through a peptide module seems to have reduced efficiency due to distance limitations, and to improve this, the reaction efficiency was measured again with a complex forming a circular structure rather than a linear structure. As a result of the experiment, it exhibited more than 2 times higher activity than the condition in which cyanase alone was used, and it was confirmed that the reaction efficiency was improved compared to the previous complex formed by mixing Catcher, cyanase, and *hm*CA. In conclusion, this study demonstrates that in the presence of the same concentrations of oxidized cyanide and HCO*LSUB*3*LSUP*−, an enzyme complex with reduced HCO*LSUB*3*LSUP*−dependency and improved reaction efficiency was constructed compared to cyanase alone.

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ABSTRACT IN KOREAN (국문 요약)

Carbonic anhydrase(CA)와 cyanase는 환경에 악영향을 미치는 CO_2와 cyanide 감소의 해결방안이 될 수 있습니다. 하지만 효소가 가진 고온에서의 불활성화 및 중탄산염에 대한 의존성은 산업적 응용에서 제한을 가져옵니다. 따라서 비가역적 공유결합을 하는 Catcher/Tag 시스템을 이용하여 효소의 구조를 변화시키고 서로 결합시킴으로써 이러한 문제점을 극복하고자 하였습니다. *hm*CA의 고리화 및 중합체 형성은 야생형과 비교하여 70 ℃ 이상의 고온에서도 활성을 회복하였으며 효소 반응을 통한 $CaCO_3$ 생성에서 대조군보다 polymeric *hm*CA가 4배 이상 차이나는 결과를 보였습니다. 이를 통해 Catcher/Tag 시스템에 의한 효소의 결합이 열적, 구조적 안정성을 향상시켰으며 기존에 있던 한계를 극복하기 위한 새로운 전략이 될 수 있음을 시사합니다. 이후 진행한 cyanase와 *hm*CA의 결합은 cyanase만 단독으로 사용할 때보다 cyanide 분해가 증가하였으나 두 효소가 연결되지 않은 조건과 비교하면 분해 반응 효율이 낮아짐을 보였습니다. 이러한 결과의 원인은 펩타이드 모듈을 통한 결합에서 직선 형태로 복합체를 형성한 것이 거리상의 제한을 가져와 효율이 감소한 것으로 보이며 이를 개선하기 위해 복합체가 원형 구조를 형성하도록 조작하였습니다. 효소의 반응 효율을 측정한 결과, cyanase만 단독으로 사용한 조건보다 2배 이상의 높은 활성을 나타냈으며 Catcher와 cyanase, *hm*CA를 혼합하여 형성한 직선 형태의 복합체보다 반응 효율이 향상된 것을 확인할 수 있었습니다. 결론적으로, 같은 농도의 산화된 cyanide와 HCO_3^-이 있는 조건에서 cyanase만 있는 것보다 HCO_3^- 의존성이 감소되고 반응 효율이 향상된 효소 복합체 구축을 성공하였습니다.

감사의 글

1년 반의 석사 과정과 학위 논문을 마무리하며 이렇게 감사의 글을 쓰게 되니 감회가 새롭습니다. 설렘과 긴장감을 가득 안고 성신여자대학교 대학원에 입학했던 게 엊그제 같은데, 벌써 모든 과정을 마치고 졸업만을 앞두고 있다는 사실이 놀랍기만 합니다. 지난 1년 반이라는 시간은 저에게 항상 감사함을 느끼게 해준 시간이었고 많은 것을 배웠으며 즐거운 추억이 가득했던 시간이었습니다. 지금까지 올 수 있었던 것은 언제나 성심성의껏 가르쳐주시고 도와주시려 했던 교수님들과 지칠 때마다 큰 힘이 되어주고 의지할 수 있도록 해준 미생물연구실 친구들, 부모님 덕분입니다. 그 감사함을 이 글을 통해 조금이나마 표현하고자 합니다.

우선, 1년 반의 석사 과정 동안 부족한 저를 이끌어주시고 누구보다도 많은 도움을 주신 현정은 교수님께 진심으로 감사의 말씀을 전합니다. 연구를 진행하고 논문을 작성하는 과정에서 항상 꼼꼼한 손길로 지도해주시고 방향을 제시해주셔서 제가 보지 못한 것을 알게 해주시고 앞으로의 길에 밑거름이 되는 다양한 경험을 할 수 있도록 기회 주신 것 감사드립니다. 교수님께서 마지막까지 배려해주시고 좋은 말씀으로 격려해주신 덕분에 무사히 석사 과정을 마무리할 수 있었습니다. 교수님의 가르침을 통해 연구에 관한 많은 것을 배웠고 저에게 부족한 점이 무엇인지와 어떠한 방향으로 나아가야 할지를 깨닫고 정할 수 있었던 것 같습니다. 4년이라는 긴 시간 동안 연구실에서 얻은 배움을 잊지 않고 늘 되새기면서 앞으로의 자리에서도 최선을 다하는 연구자가 되도록 노력하겠습니다. 또한, 바쁘신 가운데에도 학위 논문 심사위원을 맡아주시고 논문을 체계적으로 다듬어 갈 수 있도록 세심하게 짚어주시면서 따뜻한 격려와 조언의 말씀 해주신 장혜원 교수님과 강태욱 교수님께도 깊은 감사를 드립니다.

함께 있는 것만으로도 큰 힘이 되고 의지가 되었던, 정말 많은 응원을 쏟아부어준 우리 미생물연구실 친구들 정말 감사합니다. 진심 어린 조언으로 더 성장할 수 있게 해주고 기댈 수 있게 해주었던 주희 언니, 힘든 시간을 함께 버텨내며 존재만으로도 든든했던 예원 언니, 어려운 일 있을 때마다 주저함이 없이 도와주고 위로해준 유경이, 연구실의 비타민이자 분위기메이커로 항상

밝게 웃어준 운정이, 똑똑하고 어른스러운 모습과 함께 주변 사람들을 잘 챙겨준 은서, 짧은 시간이었지만 좋은 추억들을 남길 수 있게 먼저 다가와 준, 언니로서 무언가를 해주고 도와준 것이 많이 없어 미안하기도 한 수민이와 지현이 모두에게 고마움을 표합니다. 함께 MT를 가고 학회에 참석한 일, 밤늦게까지 술 마시며 웃고 떠들던 일, 서로의 생일을 챙겨주고 졸업, 논문 등을 축하해주며 파티 열어주던 일, 세미나실에서 했던 많은 이야기 등 친구들과 보냈던 시간은 저에게 너무나도 소중한 추억이 되었고 지친 일상에서 힘든 시기를 견뎌낼 수 있게 한 큰 힘이 되었습니다. 자그마한 것 하나까지 배려해주고 화목한 분위기로 만들어준 모두의 노력 덕분에 제가 연구실에 잘 녹아들 수 있었고 생각을 정리하며 할 일에 집중할 수 있었습니다. 이렇게 좋은 사람들을 만나 참 감사했던 시간이었고 행복했던 시간이었습니다.

그리고 언제나 저를 믿어주시고 뒤에서 응원하며 늘 기도해주시는 사랑하는 부모님께 진심으로 감사를 드립니다. 많이 힘들고 어려울 텐데 항상 괜찮다고 말씀해주시면서 가고자 하는 길을 지지해주시고 넌 잘할 거라고 격려해주셨기에 제가 힘을 내서 여기까지 올 수 있었고 다음을 향해 나아갈 용기를 얻을 수 있었습니다. 무뚝뚝한 딸이라 표현도 많이 하지 않고 연락도 자주 드리지 못해서 죄송한 마음이 큰데 이런 저를 이해해주시고 자랑스럽다고 말씀해주셔서 정말 감사합니다. 사랑합니다.

이 글에는 다 적지 못했지만 석사 과정을 하는 1년 반 동안 힘이 되어준 많은 분들께 감사를 전하고 싶습니다. 그 고마움 잊지 않고 차근차근 전하면서 저 또한 힘이 될 수 있는 존재가 되도록 노력하겠습니다.

마지막으로, 항상 감사한 마음으로 살아갈 수 있게 해주시고 지금까지 그리고 앞으로도 저의 앞길을 인도해주시는 하나님께 감사를 드립니다.

2022년 12월 20일
선하빈 올림