Enzymatic Depolymerization of Ulvan by Using Ulvan Lyase Enzyme Complex for Effective Production of Low Molecular Weight Algal Oligosaccharides

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This is to certify that we have examined the
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ABSTRACT

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The diverse structural and functional properties of algal biomass provide the potential for biological research. To effectively utilize algal biomass, depolymerization into low-molecular-weight algal oligosaccharides is needed. To confirm the depolymerization of algal biomass, green algal ulvan was used in the experiment. Cellulosomes, one of the complex enzymatic systems produced by C. cellulovorans, have various cellulolytic subunits bound to the non-enzymatic scaffoldin CbpA via dockerin-cohesin interactions. The dockerin-cohesin interactions are high-affinity protein protein interactions. Previous studies have reported that CBM increases the proximity of the enzyme to the substrate by targeting the enzyme to the substrate upon binding and enhances the catalytic function of

carbohydrate-active enzymes (CAZymes) that degrade polysaccharides. In this study, the dockerin-cohesin interaction was used to create a highly active enzyme capable of depolymerizing into low molecular weight oligosaccharides. The chimeric enzyme was created by attaching dockerin to the C-terminus of the enzyme, and a scaffolding protein containing CBM and two cohesins was used. Then, the enzyme complex reacted with polysaccharides extracted from biomass to confirm the effect of substrate depolymerization. As a result, the excellent ulvan depolymerization efficiency of the enzymatic complex constructed by the dockerin-cohesin interaction was confirmed. The use of cellulosic dockerin-cohesin interactions in seaweed polysaccharide depolymerase complexes is efficient and will advance biotechnology research for the production of functional oligosaccharides.

CONTENTS

List of Tables

List of Figures

Ⅰ. INTRODUCTION

Seaweeds are relatively free on arable land, they grow rapidly, and are more readily available due to several desirable properties, such as the absence of structural biopolymers such as lignin and their ability to grow in brine. The main component of green algae biomass is cell wall polysaccharides, accounting for 54% of the total dry weight. Of these, ulvan is the most abundant, accounting for 29% of the total dry weight (Konasani et al., 2018b). Nevertheless, green algae have not been well studied compared to brown and red algae (He et al., 2017). In addition, green algae multiply rapidly in eutrophic waters, causing serious environmental and economic problems. Therefore, various studies using green algae are needed (Konasani et al., 2018a). Ulvan is a water-soluble polysaccharide that is primarily made up of 3-sulfated rhamnoses (Rha3S), D-glucuronic acid (GlcA), L-iduronic acid (IduA), and D-xylose (Xyl). The repeating disaccharide units in ulvan are Rha3S-GlcA, Rha3S-IdoA, and Rha3S-Xyl (Reisky et al., 2019). (Fig. 1.)

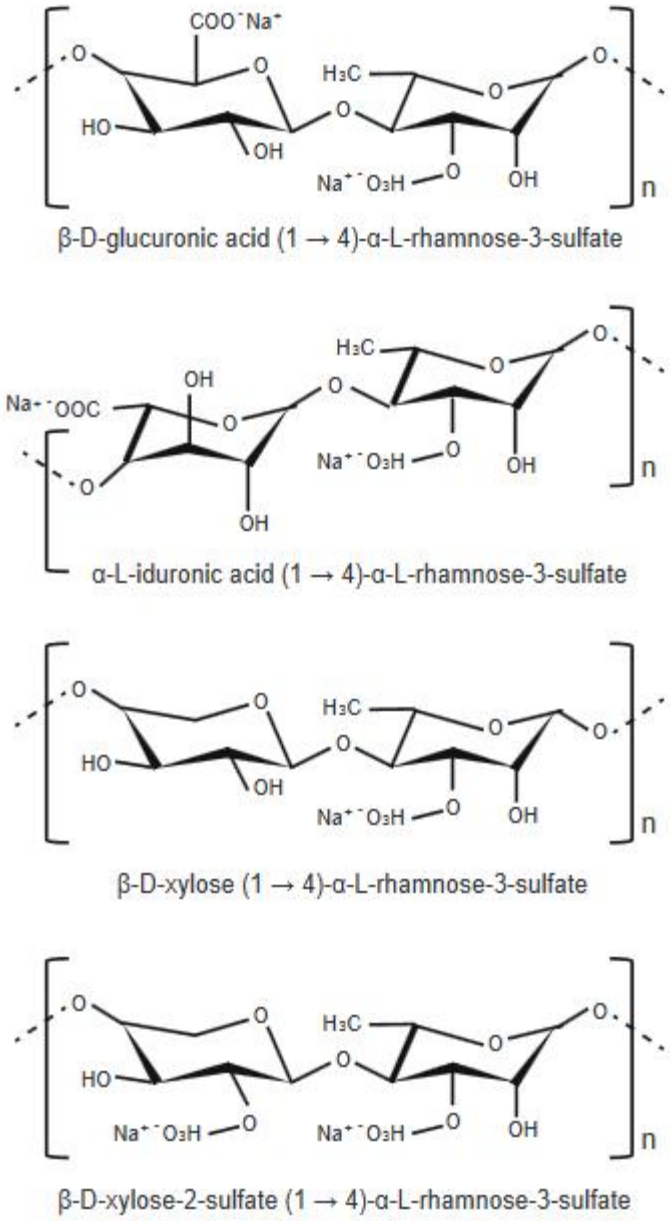

Fig. 1. Chemical structure of the ulvan repeat disaccharide

Of these, uronic acids (glucuronic and iduronic acids) are the structural components of mammalian glycosaminoglycans and chondroitin sulfate, heparan sulfate, and heparin, which have unique properties that distinguish ulvan from other marine polysaccharides. Therefore, ulvan can be utilized in many biomedical and biotechnology applications (Kidgell et al., 2019). The biological activity of ulvan is affected by its molecular weight. Compared to low molecular weight ulvan, high molecular weight ulvan has a high viscosity and low solubility, making it difficult to use. Therefore, an effective ulvan depolymerization process is needed (Gao et al., 2019). The depolymerization process by chemical hydrolysis requires high cost, causes environmental pollution, and has limitations in use due to problems such as low yield. Therefore, an environmentally friendly, energy-efficient, and economical enzymatic depolymerization process using ulvan-degrading enzymes is needed (Li et al., 2013). Ulvan lyase (FaUL) catalyzes the endolytic cleavage of the glycosidic bond between 3-sulfated rhamnose (Rha3S) and uronic acids (GlcA or IduA), resulting in the production of unsaturated 4-deoxy-L-threo-hex-4-enopyranosiduronic acid (deltaUA). This causes ulvan polysaccharides to degrade into deltaUA-Rha3S disaccharides and deltaUA-Rha3S-Xyl-Rha3S tetrasaccharides (Reisky et al., 2019). To completely degrade ulvan polymers, unsaturated beta-glucuronyl hydrolase (UGL) collaborates with ulvan lyase. It is responsible for the cleavage of unsaturated 4-deoxy-L-threo-hex-4-enopyranosiduronic acid (deltaUA). It also hydrolyzes the tetrasaccharides deltaUA-Rha3S-IduA-Rha3S and deltaUA-Rha3S-GlcA-Rha3S (Reisky et al., 2019). However, the enzymatic

depolymerization process is considered an impractical method because it takes a great deal of time for the enzymatic depolymerization of polysaccharides. Therefore, there is a need for a method to compensate for the disadvantages of enzymatic depolymerization. The construction of chimeric enzymes using carbohydrate-binding modules (CBMs) is one of the methods for improving enzyme performance (Sidar et al., 2020). Cellulosomes, one of the complex enzymatic systems produced by the anaerobic microorganism Clostridium cellulovorans, have various cellulolytic subunits bound to the nonenzyme scaffoldin CbpA through dockerin-cohesin interactions (Hyeon et al., 2014). The dockerin-cohesin interaction occurs between the dockerin domain (an enzyme that is present in all cellulosomal enzymes and not found in noncellulosomal enzymes and contains overlapping sequences) and the cohesin domain (a noncatalytic scaffolding containing repetitive sequences). It is a high-affinity protein protein interaction (Hyeon et al., 2016). CBM increases the proximity of the enzyme to the substrate by targeting the enzyme to the substrate upon binding and enhances the catalytic function of carbohydrate-active enzymes (CAZymes) that breaks down polysaccharides. That is, when the enzyme binds to the substrate, the enzyme activity increases, and the enzyme stability increases, thereby increasing the availability of the enzyme (Sidar et al., 2020). Therefore, to construct a complex enzymatic system, a small recombinant scaffoldin mCbpA containing only two cohesion modules was used instead of CbpA from C. cellulovorans containing nine cohesion modules, and an enzymatic complex was constructed using a chimeric enzyme with a dockerin domain (Hyeon et al., 2014). (Fig. 2.)

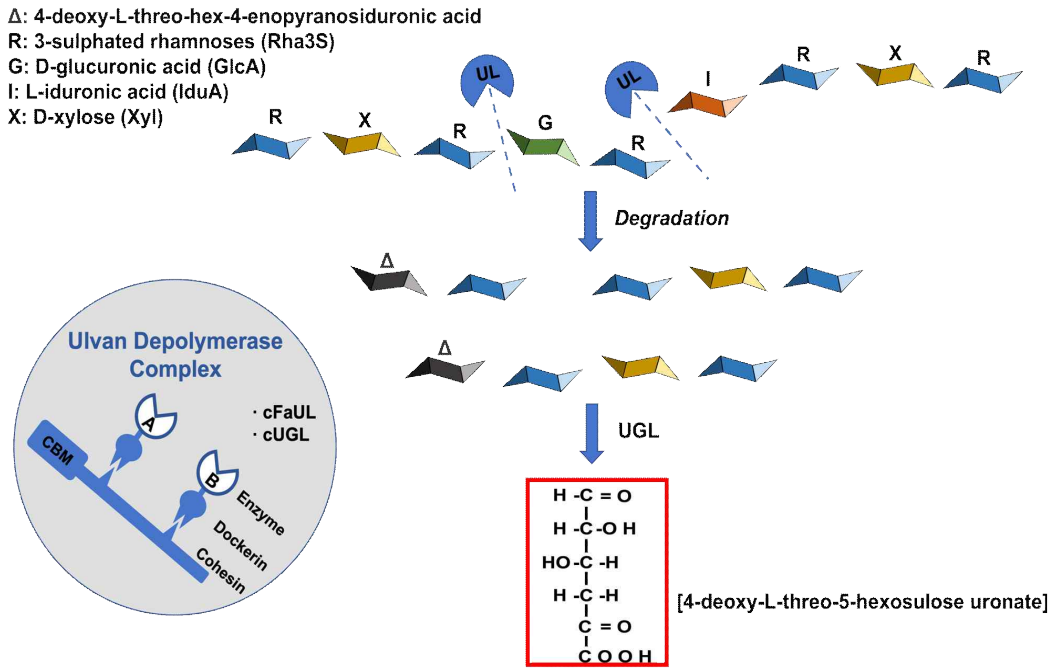

Fig. 2. Schematic illustrations of ulvan depolymerization

Currently, interest in developing new foods containing probiotic microorganisms such as lactic acid bacteria is growing. Probiotic foods have been reported to provide several health benefits, including balancing the composition of gut flora, increasing resistance to pathogens, and stabilizing the function of the gastrointestinal (GI) barrier. Some algae, such as Chlorella, have great potential in the human diet and exhibit the prebiotic potential to support probiotic growth in the host's gut when ingested (Sidira et al., 2013). Therefore, studies on the prebiotic role of lesser-known microalgae are needed. This study aimed to determine the bioavailability of ulvan as a prebiotic by effectively breaking down ulvan polysaccharides. For effective ulvan degradation, we developed a recombinant *Escherichia coli* strain with an ulvan lyase, unsaturated 3S-rhamnoglycuronyl hydrolase enzyme complex by introducing genes encoding dockerin-fused chimeric FaUL, dockerin-fused chimeric UGL, and scaffoldin mCbpA. The resulting functional ulvan depolymerase complex degraded ulvan more easily than the control, and the potential of ulvan as a prebiotic product was confirmed through the reaction between the degraded low-molecular-weight polysaccharide ulvan and lactic acid bacteria.

Ⅱ. MATERIALS AND METHODS

2.1. Strains, plasmids and media

For recombinant DNA manipulation and gene expression, *E. coli* DH5α and BL21(DE3) were used, respectively. The strains used in this study are listed in Table 1.

Table 1. Microbial strains and plasmids used in this study

Strain or plasmid	Genotype or construct	Reference or source[a]
Bacterial strains		
Escherichia coli DH5α	F^-, *deoR*, *endA1*, *gyrA96*, *hsdR17*(rk^-mk^+),*recA1*, *relA1*, *supE44*, *thi-1*, Δ(*lacZYA- argF*)*U169*, (Phi80*lacZ*delM15)	Invitrogen (Carlsbad, CA, USA)
Escherichia coli Rosseta BL21(DE3)	F^- *ompT gal dcm lon hsdS*$_B$($r_B^-m_B^-$) λ(DE3[*lacI lacUV5-T7* gene 1 *ind1 sam7 nin5*] pRARE	Invitrogen (Carlsbad, CA, USA)
Escherichia coli BL21(DE3)	F^- *ompT gal dcm lon hsdS*$_B$($r_B^-m_B^-$) λ(DE3[*lacI lacUV5-T7* gene 1 *ind1 sam7 nin5*]	Invitrogen (Carlsbad, CA, USA)
Clostridium cellulovorans	WT strain ATCC 35296	ATCC[a]
Formosa agariphila	WT strain KCTC 12365	KCTC[b]
Lactobacillus casei	WT strain ATCC 393	ATCC[a]
Transformants		
E. coli BL21 (pET22b (+) Control)	[$T7_P$-*pelB*-$T7_T$]	This study
E. coli BL21 (pColdII mCbpA)	[$CspA_P$-TEE-*mCbpA*-$CspA_T$]	This study
E. coli Rosetta (DE3) (pColdII FaUL-Doc)	[$CspA_P$-TEE-*FaUL-docB*-$CspA_T$]	This study
E. coli Rosetta (DE3) (pColdII UGL-Doc)	[$CspA_P$-TEE-*UGL-docB*-$CspA_T$]	This study
Plasmids		
pET22b (+)	$T7_P$-*pelB*-$T7_T$	Novagen (San Diego, CA, USA)
pColdII	$CspA_P$-TEE-$CspA_T$	Takara
pColdII mCbpA	$CspA_P$-TEE-*mCbpA*-$CspA_T$	This study
pColdII FaUL-Doc	$CspA_P$-TEE-*FaUL-docB*-$CspA_T$	This study
pColdII UGL-Doc	$CspA_P$-TEE-*UGL-docB*-$CspA_T$	This study

[a] ATCC, American Type Culture Collection.

[b] KCTC, Korean Collection Type Cultures

E. coli was grown at 37 °C in Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride) containing 50 μg/mL ampicillin. *Formosa agariphila* was cultured in Marine Broth 2216 medium containing 50 μg/mL ampicillin at 21 °C for 3 days. C. cellulovorans was grown anaerobically in round-bottom flasks containing the previously described medium at 37 °C.

2.2. DNA manipulation and plasmid construction

Table 2. shows the oligonucleotide primers used in plasmid construction.

Table 2. List of oligonucleotide primers used in this study.

FaUL-P1	CATATGGAGCTC*GGTACC*ATGTTTTTAGCTGTAGTAAC
FaUL-P2	CAGCGGATCCCTTTTACTATCAATTTCTTATGATACTTGTTG
FaUL-P3	GATAGTAAAAGGATCCGCTGGCTCC
FaUL-P4	CTGCAGGTCGAC*AAGCTT*TCATAAAAGCATTTTTTTAAGA
UGL-P1	CATATGGAGCTC*GGTACC*ATGAAAAACCAAGCATTAAA
UGL-P2	CAGCGGATCCCTCTTCCAGTTTTAAAACTTCACTAC
UGL-P3	ACTGGAAGAGGGATCCGCTGGCTCC
UGL-P4	CTGCAGGTCGAC*AAGCTT*TCATAAAAGCATTTTTTTAAGA
mCbpA-f	CATCATCATATG*GAGCTC*GCAGCGACATCATCAATGTC
mCbpA-r	TCTAGACTGCAG*GTCGAC*CTATATAGGATCTCCAATATTTATT

[a] Restriction enzyme sites are italicized, 10-nucleotide long 5′extension are underlined

The chimeric FaUL gene, which contains the dockerin region of C. cellulovorans EngB at the 3′ end of *E. coli* ulvan lyase FaUL, was constructed using a multistep PCR strategy in which FaUL-P1, FaUL-P2, FaUL-P3, and FaUL-P4 primers overlap. The FaUL-P2 and FaUL-P3 primers each had a 5′ extension of 10 nucleotides in length complementary to the end of the adjacent fragment of the chimeric FaUL-doc gene to fuse the different fragments together. FaUL-P1 and FaUL-P4 primers contain restriction enzyme sites for *KpnI* and *HindIII* at their 5′ and 3′ ends, respectively. Two fragments, 1503 bp and 195 bp were purified and fused together in a second PCR step. The fusion product of 1698 bp was then amplified. Prior to cloning, PCR products were isolated and purified. The insert was ligated to the *KpnI-HindIII* site of the pColdII vector to generate the pColdII FaUL plasmid. A chimeric UGL gene containing a dockerin region at the end was constructed by the above experimental method. The UGL-P1 and UGL-P4 primers contain restriction enzyme sites for *KpnI* and *HindIII* at their 5′ and 3′ ends, respectively. Two fragments, 1134 bp and 195 bp were purified and fused together in a second PCR step. Next, the 1329 bp fusion product was amplified. Prior to cloning, PCR products were isolated and purified. The insert was ligated to the *KpnI-HindIII* site of the pColdII vector to generate the pColdII UGL plasmid. Using C. cellulovorans genomic DNA as a template for PCR, a 1650 bp *SacI-SalI* DNA fragment encoding the mCbpA gene was generated using primers mCbpA-f and mCbpA-r. The 5′ and 3′ ends of these primers have *SacI* and *SalI* restriction enzyme sites, respectively. A pColdII mCbpA plasmid was obtained by introducing the mCbpA DNA fragment

into the pColdII vector using the *SacI-SalI* site.

2.3. Expression and purification of the recombinant proteins

For inoculation preparation and protein expression, *E. coli* strains were shaken at 200 rpm in Luria-Bertani (LB) medium supplemented with 50 μg/mL ampicillin in 15 mL conical tubes and 500 mL Erlenmeyer flasks with shaking and then incubated at 37 °C. The culture was then cooled to 16 °C with an optical density of 600 nm of 0.6, and isopropyl thio-β-D-galactoside (IPTG) was added to the medium to react overnight to induce the T7 promoter. Cells were collected by centrifuging them twice at 4,000 x g for 10 minutes and then suspending them in 10 mL of ice-cold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0). Following cell destruction using sonication, samples were centrifuged at 10,000 x g for 30 minutes. After applying crude cell extract supernatants to a Ni-NTA column that binds to recombinant protein with a His-tag, the column was washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0). Elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 8.0) was then used to elute the bound recombinant protein. The purified protein was dialyzed against 20 mM Tris-HCl buffer (pH 8.5), and the culture supernatant was concentrated by ultrafiltration using centrifugal filter units for the concentration and purification of biological solutions with a 10 kDa cutoff membrane. The concentrated protein concentration was measured using the Bradford method and a Quick Start protein assay kit using bovine serum albumin as a standard.

2.4. Ulvan lyase and UGL complex assembly and CBM-based enzyme recycling

Recombinant ulvan lyase protein (2.0 nmol), recombinant UGL protein (2.0 nmol), and purified mCbpA protein (2.0 nmol) were mixed in 100 μL of 5x binding buffer (125 mM sodium acetate, pH 6.0, and 75 mM $CaCl_2$) and incubated at 4 °C for 1 hour. A CBM-based purification method confirmed the assembly of ulvan lyase and the UGL complex. Purification using CBM was conducted using microcrystalline cellulose (Daejung, Korea) at a protein concentration of 10 μg per 1 mg of cellulose (Sigmacell Type 50). The binding was carried out while shaking for 1 hour at room temperature. Cellulose-bound CBM-fusion protein was centrifuged at 1,600 x g for 10 min. Nonspecific proteins attached to cellulose were removed by washing the cellulose sample 3 times (1 time with 1 M NaCl (pH 8.0) and 2 times with 20 mM Tris (pH 7.5)). The bound protein was then eluted using 50 mM Tris (pH 12.5). SDS PAGE was used to analyze the proteins in the cellulose-bound fraction.

2.5. Ulvan extraction from green algae

The green algae ulva pertusa was purchased from Parajeju (para Jeju, Jeju, Korea). The dried powder was macerated in water (6:1 distilled water/green algae powder, w/w) containing 2% cellulase. The mixture was incubated at 55 °C for 3 hours. Then, 1% hydrogen peroxide was added to the mixture and incubated at pH 4.0 for 6 hours. The mixture was lyophilized for 24 hours. Finally, 17 mL/g of 20 mM Tris-HCl (pH 8.5) was added to the mixture to extract ulvan.

2.6. Measurement of enzymatic activities

The activity of the Ulvan lyase and UGL enzyme complex was confirmed. For directly extracted ulvan, 50 μg of ulvan and 2 μg of the enzyme were mixed in 148 μL of 20 mM Tris-HCl (pH 8.5). For purchased ulvan, 20 μg of ulvan and 2 μg of the enzyme were mixed in 178 μL of 20 mM Tris-HCl (pH 8.5). The mixture was reacted at 37 °C, and the absorbance was monitored every 10 min with a nanodrop set to 232 nm. The activity of the enzyme was confirmed by measuring the formation of unsaturated oligosaccharide products in the assay mixture via an increase in absorption at 232 nm.

2.7. Effects of FaUL and UGL on the growth of *L. casei* in MRS medium

Lactobacillus casei ATCC 393 used in this study was cultured in Lactobacilli MRS medium at 37 °C. MRS medium consisted of 10 g peptone, 10 g/L meat extract, 5 g/L yeast extract, 20 g/L glucose, 2 g/L potassium phosphate (K_2HPO_4), 5 g/L sodium acetate ($C_2H_3NaO_2$), 0.2. g/L magnesium sulfate ($MgSO_4$), and 0.05 g/L manganese sulfate ($MnSO_4$). To remove residual medium components, cells were washed three times with MRS broth without glucose and inoculated with MRS broth without glucose. The MRS broth without glucosemedium comprised 10 g of Proteose Peptone, 10 g/L Beef Extract, 5 g/L Yeast Extract, 5 g/L Sodium Acetate ($C_2H_3NaO_2$), 2 g/L Dipotassium Phosphate (K_2HPO_4), 2 g/L Ammonium Citrate ($C_6H_{17}N_3O_7$), 1 g/L Polysorbate 80 ($C_{64}H_{124}O_{26}$), 0.1 g/L Magnesium Sulfate ($MgSO_4$), and 0.05 g/L Manganese Sulfate (MnSO). To determine the effect of each compound on the growth of *L. casei*, the medium was supplemented with depolymerized ulban degradation products in stages using an enzyme. Absorbance was measured every hour for 6 hours using a microplate spectrophotometer.

Ⅲ. RESULTS AND DISCUSSION

3.1. Chimeric ulvan-degrading enzyme design for enzyme complex assembly

Ulvan is a complex structure and sulfated polysaccharide. These structural properties of ulvans require several types of enzymes to act on the depolymerization of ulvans. In this study, we used two types of ulvan depolymerases that degrade in stages. The first enzyme is the endo-acting ulvan lyase of *F. agariphila*, which breaks down ulvan polysaccharides into delta UA-Rha3S disaccharides and deltaUA-Rha3S-Xyl-Rha3S tetrasaccharides. The second enzyme is unsaturated beta-glucuronyl hydrolase from *F. agariphila*, which works with ulvan lyase to completely degrade the ulvan polymer (Konasani et al., 2018c). For more effective ulvan depolymerization of enzymes, ulvan lyase and UGL complexes were assembled through the interaction of dockerin and cohesin in recombinant scaffolding proteins. Previous studies have confirmed the successful degradation of algae using enzyme complexes. In the case of red algae, when the mCbpA and cAgaB complexes were formed, it was shown that the agar-degrading activity was improved by 1.40 times higher than that of the noncomplex (Hyeon et al., 2012). In the case of brown algae, when the activity of the alginate lyase complex on alginate degradation was investigated, it was confirmed that the enzyme complex system showed 1.85 times higher activity in the degradation of alginate compared to the

noncomplex system (Jeong et al., 2021). These results suggest that the high-affinity interaction enzyme complex system using mCbpA is useful for efficiently converting marine biomass into valuable products. To enable the assembly of a heterologous ulvan lyase and UGL as a complex, the ulvan lyase and UGL catalytic domains were fused at the C-terminus with a dockerin domain from C. cellulovorans cellulosomal cellulase EngB, generating cFaUL and cUGL. In addition, the mCbpA plasmid DNA fragment was introduced into the restriction enzyme portion of the pColdII vector to obtain a pColdII mCbpA plasmid. (Fig. 3).

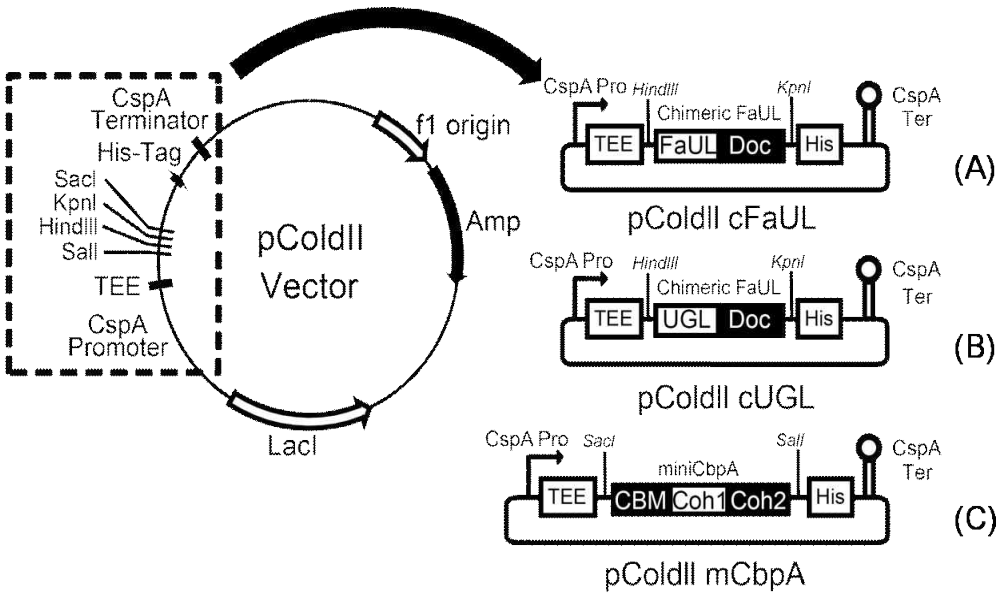

Fig. 3. Strategies for generating cFaUL, cUGL, and mCbpA expression vectors. Images show the cFaUL (A), cUGL (B), and mCbpA (C) genes. mCbpA contains a carbohydrate-binding module (CBM) and two cohesin domains. In addition, cFaUL and cUGL contain EngB dockerin (PelB, signal peptide; Doc, dockerin domain).

3.2. Protein subunits for the construction of ulvan-degrading complexes

The expression of cFaUL protein derived from the recombinant strain *E. coli* (pColdII FaUL) containing the Ulvan lyase FaUL plasmid was confirmed by SDS PAGE and Western blotting. (Fig. 4.)

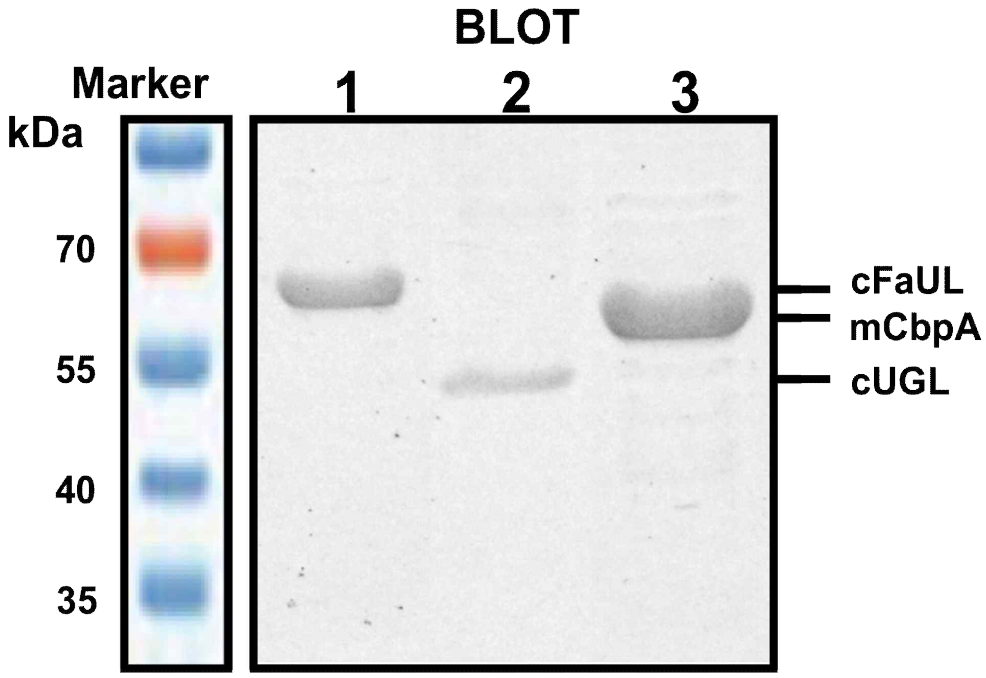

Fig. 4. Individual protein expression was confirmed using SDS PAGE and Western blot of cFaUL, cUGL, and mCbpA. lanes: 1, purified cFaUL; 2, purified cUGL; 3, mCbpA.

The calculated molecular weight of cFaUL was 61.3 kDa (54.7 kDa FaUL + 6.6 kDa residue containing flexible linker, dockerin module, and His tag). A single 61 kDa band identified by SDS PAGE and Western blot was observed to correspond to the molecular size of cFaUL (61.3 kDa) predicted by the nucleotide sequence. Expression of cUGL protein was also confirmed in the same way as above. The calculated molecular weight of the cUGL was 50.1 kDa (43.457 kDa UGL + a 6.667 kDa residue containing a flexible linker, dockerin module, and His tag). A single 50 kDa band identified by SDS PAGE and Western blot was observed to correspond to the molecular size of cUGL (50.1 kDa) predicted by the nucleotide sequence. These successful results of protein expression confirmed that cFaUL and cUGL were expressed in active form and that the dockerin domain at the C-terminus of FaUL and UGL reconstructed for enzymatic digestion does not interfere with the activity of ulvan-degrading enzymes. In addition, as a result of SDS PAGE and Western blot of the recombinant scaffoldin mCbpA protein based on the scaffoldin CbpA of C. cellulovorans, the size was approximately 58 kDa. This protein's molecular size was consistent with that predicted by the nucleotide sequence.

3.3. Confirmation of assembly of ulvan enzyme complex using CBM purification

After confirming the protein expression for constructing the ulvan degrading enzyme complex, the ulvan degrading enzyme complex was assembled, and purification by a CBM-based enzyme regeneration method was performed to confirm the formation of the ulvan degrading enzyme complex. SDS PAGE and Western blot analysis confirmed the formation of the enzyme complex. The mCbpA protein contains two cohesin modules that enable the complex formation and CBM (CBM-Coh1-Coh2) which enables simple recycling. Because mCbpA is designed to have two cohesin modules, the mixture can form a complex with both enzymes in a 1:1:1 ratio. Three bands corresponding to complex subunits were identified by CBM purification. To confirm the formation of a single mCbpA subunit with two enzymes, cFaUL, cUGL, and purified mCbpA were mixed in a 1:1:1 molar ratio. After mixing, as a result of SDS PAGE and Western blot, the cFaUL band, cUGL band, and mCbpA band were mixed and confirmed in one lane. This band indicates that the ulvan-degrading enzyme complex was formed by mixing two chimeric ulvan enzymes (cFaUL and cUGL) and mCbpA. These results indicate that cFaUL, cUGL, and mCbpA are correctly folded and that their high-affinity interaction makes them suitable for the direct assembly of ulvanhydrolase complexes. (Fig. 5.)

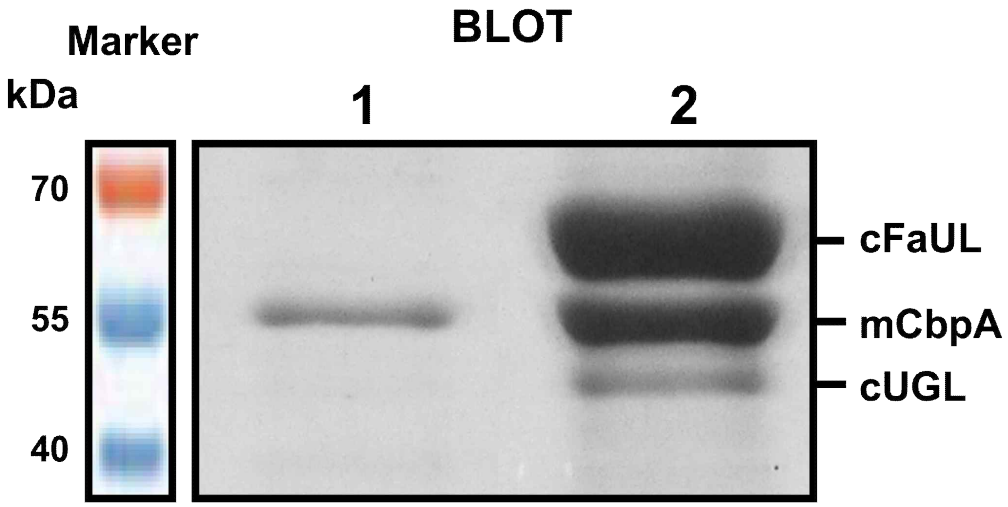

Fig. 5. Ulvan depolymerase complex formed by purification using CBM. The assembly of the ulvan depolymerase complex was confirmed by SDS PAGE and Western blot. lane: 1, mCbpA only; 2, Subunit of ulvan-degrading enzyme complex purified by CBM-based enzyme recycling method.

3.4. Effect of ulvan degrading enzyme complex

To confirm the effect of the enzyme complex, the enzyme activity was analyzed. The concentration of the reducing sugar terminal formed by enzymatic depolymerization was measured by using a nanodrop set with an absorbance of 232 nm. Previous studies have shown that when ulvan is depolymerized with ulvan lyase, the absorbance increases at 232 nm. This is because ulvan is degraded by ulvan lyase to release oligosaccharides containing unsaturated 4-deoxy-L-threo-hex-4-enopyranosiduronic acid via a β-elimination mechanism (Konasani et al., 2018b). This suggests that ulvan lyase exhibits an increase in absorption at 232 nm when it undergoes normal degradation. As a result of observing the enzymatic activity of ulvan lyase every 10 minutes using the reconstructed chimeric ulvan lyase, it can be seen that the absorption increases at an absorbance of 232 nm. (Fig. 6.)

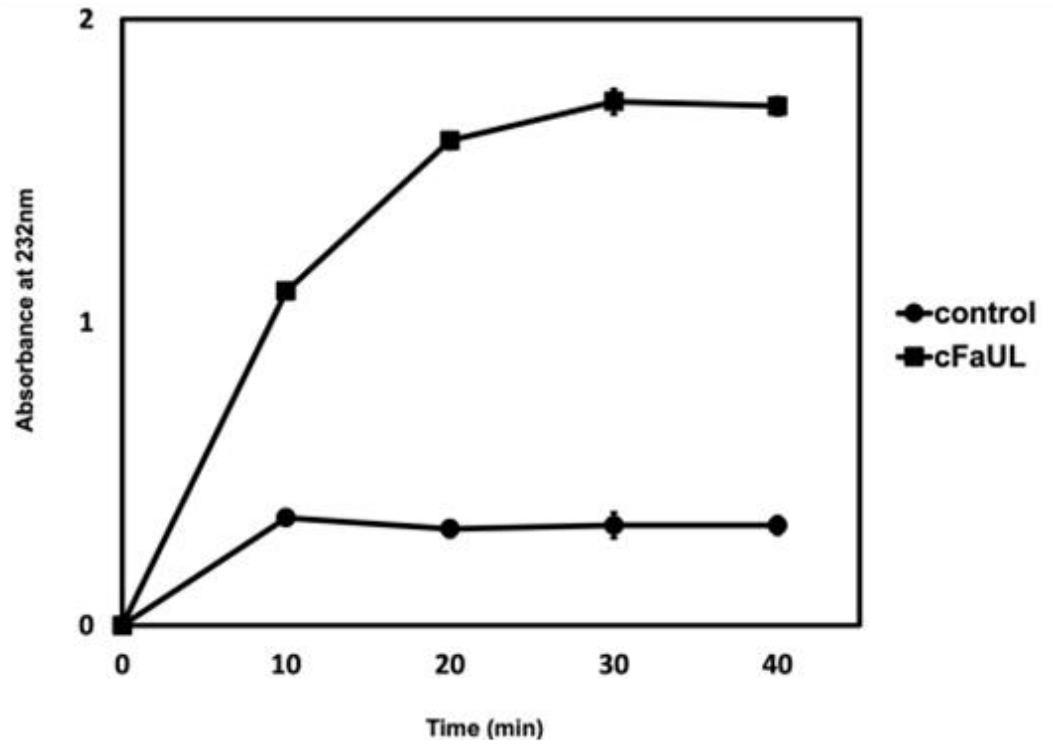

Fig. 6. Decomposition of ulvan extracted from Ulva powder using the constructed Ulvan lyase enzyme; (A) control (B) cFaUL Data show the mean of triplicate reactions. Error bars represent standard deviations.

These results show that the dockerin domain at the C-terminus of the reconstructed chimeric FaUL functions normally without interfering with the activity of the ulvan-degrading enzyme. ulvan degradation releases oligosaccharides containing unsaturated 4-deoxy-L-threo-hex-4-enopyranosiduronic acid through a β-elimination mechanism. Next, the mCbpA-cFaUL-cUGL enzyme complex showed a decrease in absorbance at an absorbance of 232 nm. (Fig. 7.)

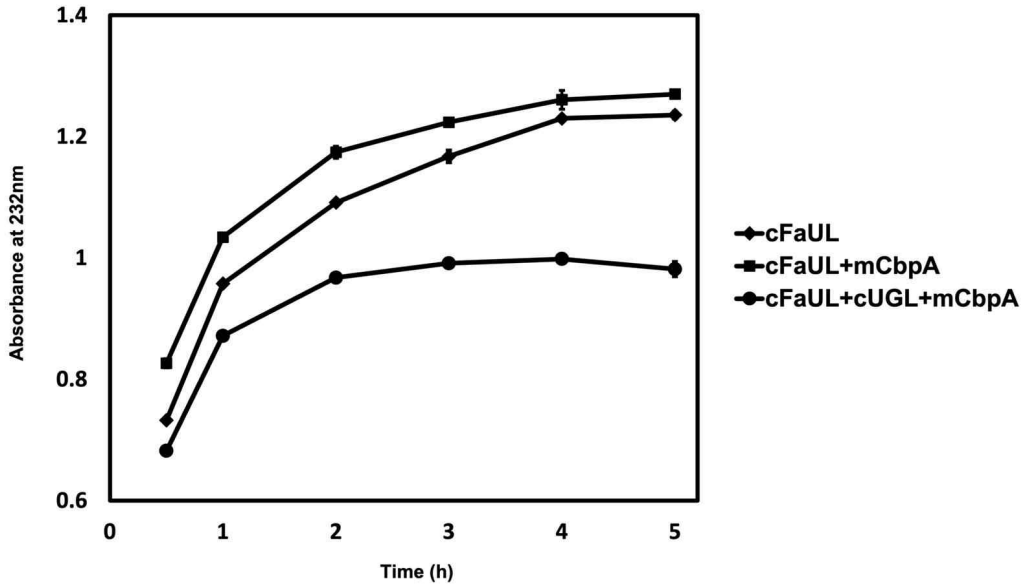

Fig. 7. Decomposition of ulvan extracted from Ulva biomass using the constructed Ulvan degrading enzyme; (A) cFaUL, (B) cFaUL+mCbpA, and (C) cFaUL+cUGL+mCbpA. Data show the mean of triplicate reactions. Error bars represent standard deviations.

The mixture of unsaturated oligosaccharides obtained from degradation by ulvan lyase is released. Then, the ring form is spontaneously rearranged to linear 4-deoxy-L-threo-5-hexosulose uronate by the action of UGL (Collén et al., 2014). Therefore, this residue loses its ability to absorb at 232 nm, which may explain the decrease in absorbency after oligo-ulvan degradation. When comparing the ulvan hydrolysis of the FaUL-only enzyme and the mCbpA-cFaUL enzyme complex mixture, it was confirmed that the hydrolytic activity of the cFaUL enzyme complex mixture combined with mCbpA was higher than that of the FaUL-only enzyme. This shows that the enzyme complex assembled through the interaction of dockerin and cohesin, designed to increase ulvan degradation performance, is more effective in ulvan degradation. As a result of confirming the activity of the chimeric UGL enzyme complex at an absorbance of 232 nm, the absorbance decreased compared to the FaUL alone enzyme and the mCbpA-cFaUL enzyme complex mixture. These results show that the dockerin domain at the C-terminus of the reconstructed chimeric UGL functions normally without interfering with the activity of the ulvan-degrading enzyme, and the cFaUL-cUGL enzyme complex bound to mCbpA is activated by ulvan lyase, which is the theoretical role of UGL. This indicates successful further degradation of the degraded oligo-ulvan. In addition, this result shows that the newly constructed cUGL acts without any problems with the activity of the enzyme.

3.5. Effect of ulvan degradation products on the growth of *L. casei*

The potential prebiotic availability of step-by-step ulvan degradation products using the Ulvan lyase enzyme complex was confirmed using growth profiling of the probiotic strain *L. casei*. To investigate the prebiotic availability of ulvan degradation products through the growth of the *L. casei*. comparison group with the control group not supplemented with ulvan, two groups were set and compared: ulvan depolymerized by the cFaUL-mCbpA enzyme complex and ulvan depolymerized by the enzyme complex cFaUL-cUGL-mCbpA. As a result, *L. casei* showed a significantly higher growth rate than the other comparison groups when supplemented with ulvan degradation products depolymerized by the cFaUL-mCbpA-cUGL enzyme complex. The ulvan degradation product depolymerized by the cFaUL-mCbpA-cUGL enzyme complex shows approximately 2-3 times higher activity when growing the probiotic strain *L. casei* compared to undigested ulvan. Additionally, compared to the group supplemented with the ulvan degradation product depolymerized by the cFaUL-mCbpA enzyme complex, the ulvan degradation product depolymerized by the enzyme complex with cUGL showed higher growth activity of *L. casei*. This means that the further decomposition of ulvans through cUGL increases the usability of ulvan-derived oligosaccharides. This is because the low molecular weight ulvan degraded through cUGL has a more significant effect on the growth of the probiotic strain *L. casei* than the high molecular weight ulvan, showing its applicability as a prebiotic. The degradation of ulvan by the enzyme complex increases the

bioavailability of ulvan. (Fig. 8.)

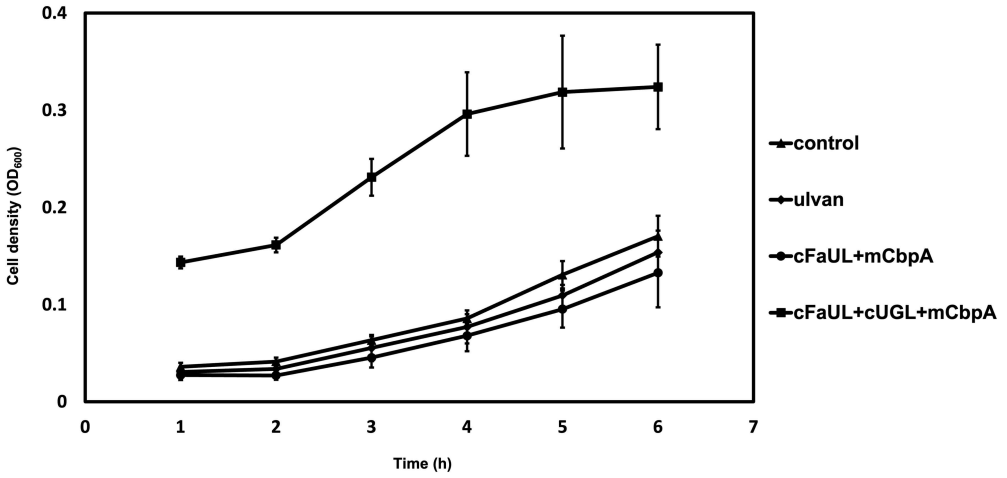

Fig. 8. *L. casei* growth using ulvan degradation products of the constructed enzyme complex; (A) control, (B) ulvan, (C) cFaUL+mCbpA, and (D) cFaUL+mCbpA+cUGL. Data show the means of duplicate reactions. Error bars represent standard deviations.

Ⅳ. CONCLUSION

In this study, a high-activity enzyme complex was constructed by assembling the enzymes cFaUL, cUGL, and mCbpA that depolymerize ulvan. The constructed ulvan depolymerase complex showed higher ulvan substrate depolymerization activity than the single subunit enzyme. These results indicate that mCbpA, cFaUL, and cUGL enzymes form a complex and that the dockerin fusion enzyme of mCbpA exhibits a synergistic action in ulvan degradation. The low-molecular-weight ulvan lysate using the enzyme complex showed higher prebiotic activity than the ulvan lysate of the single subunit enzyme. This study confirmed the bioavailability of green algae biomass ulvan using the ulvan depolymerase complex. As the potential of algal biomass is highlighted, industrial applications such as food additives, biofuels, biofertilizers, pharmaceuticals, and health care products are expected. Therefore, research on the utilization of enzyme complexes for more efficient conversion of algal biomass will lead to the development of the algal biomass industry.

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ABSTRACT IN KOREAN (국문 요약)

조류 바이오매스의 다양한 구조적 및 기능적 특성은 생물학적 연구의 잠재력을 제공합니다. 조류 바이오매스를 효과적으로 활용하기 위해서는 저분자량 조류 올리고당으로의 해중합이 필요합니다. 조류 바이오매스의 해중합을 확인하기 위해 녹조류 울반을 실험에 사용했습니다. *C. cellulovorans*에 의해 생성된 복잡한 효소 시스템 중 하나인 셀룰로좀은 도커린-코헤신 상호 작용을 통해 비효소 스캐폴딘 씨비피에이에 결합된 다양한 셀룰로오스 분해 소단위를 가지고 있습니다. 도커린-코헤신 상호작용은 고친화성 단백질-단백질 상호작용입니다. 이전 연구에서는 CBM이 결합 시 효소를 기질에 표적화함으로써 기질에 대한 효소의 근접성을 증가시키고 다당류를 분해하는 탄수화물 활성 효소(CAZymes)의 촉매 기능을 향상시킨다고 보고했습니다. 이 연구에서는 도커린-코헤신 상호작용을 사용하여 저분자량 올리고당으로 해중합할 수 있는 고활성 효소를 생성했습니다. 키메라 효소는 효소의 C-말단에 도커린을 부착하여 생성하였으며, 씨비엠과 2개의 코헤신을 포함하는 스캐폴딩 단백질을 사용했습니다. 그런 다음, 효소 복합체를 바이오매스에서 추출한 다당류와 반응시켜 기질 해중합 효과를 확인했습니다. 그 결과, 도커린-코헤신 상호작용에 의해 구축된 효소복합체의 우수한 울반 해중합 효율을 확인할 수 있었습니다. 셀룰로오스 도커린-코헤신 상호작용을 이용한 해조 다당류 탈중합효소 복합체는 효율적으로 작용하여 기능성 올리고당 생산을 위한 생명공학 연구의 발전으로 이어질 것입니다.

감사의 글

이제 마지막이 될 인사네요. 안녕하세요, 성신여자대학교 석사과정 미래응용과학학과 식품미생물 전공 조예원입니다.

대학원생의 부푼 꿈을 안고 연구실에 들어왔던 게 엊그제 같은데 벌써 2년 6개월의 시간이 흘러 졸업이 다가왔습니다. 연구실에서의 생활을 돌이켜보면 초반에는 항상 긴장되고 어색하기만 했는데 어느샌가 참 많은 추억이 쌓여 저의 일상이 되어있네요. 어렵고도 즐거웠던 연구실 생활을 마치며 감사했던 분들께 인사를 전하고자 합니다.

먼저 저의 지도 교수님이신 현정은 교수님의 아낌없는 지도에 깊은 감사의 말씀드립니다. 교수님의 제자로 석사과정을 보낼 수 있어 영광이었습니다. 교수님, 항상 존경합니다. 교수님의 가르침 잊지 않고 사회에서도 교수님의 제자 조예원으로서 나아가겠습니다.

그리고 저의 졸업 논문을 심사해 주신 고병준 교수님, 장혜원 교수님께 감사 인사를 드립니다. 바쁘신 와중에도 따뜻한 격려와 조언해 주셔서 감사합니다. 논문 심사 외에도 평소 해주셨던 말씀들 잊지 않고 기억하겠습니다.

잊지 못할 추억을 남겨준 우리 C614 Microbe lab 친구들!
언제나 소중한 주희언니, 언니와 함께한 그 무엇보다 값진 성신여대에서의 7년이 너무 소중해. 언니가 있었기에 대학원 생활이 행복했어. 든든한 나의 친구이자 언니가 되어줘서 고마워. 졸업 동기 하빈이, 처음 만났을 때 기억이 생생한데 함께 졸업하네요. 새로운 곳에서도 적응 잘하고, 멋진 앞날

응원해요! 인생을 즐기며 사는 유경이, 학회에서 함께한 행복했던 많은 추억들 간직할게요. 좋은 일 가득하길 바라요! 옆자리 메이트 운정이, 운정이의 밝은 에너지 덕분에 많이 웃었어요. 앞으로 행복이 가득하길 바랄게요! 충청도 메이트 은서, 은서랑은 통하는 점이 많아 항상 든든했어요. 똑순이 은서 어디서든 잘 할 수 있을 거라고 믿고 응원할게요! 연구실 막내 수민이, 덕분에 걱정 많던 마지막 한학기가 행복했어요. 앞으로 연구실 왕언니가 될 수민이 응원해요! 연구실 막내 지현이, 식품영양학과 후배를 만나서 정말 반가웠어요. 함께 대화하며 힐링하던 순간 잊지 않을게요. 언제나 웃음이 가득하길 바랄게요!

마지막으로 세상에서 가장 사랑하는 가족들에게 감사의 인사를 전합니다. 제가 어떤 선택을 하던 믿고 지지해 주셔서 감사합니다. 부모님과 오빠의 지지가 있었기에 어떤 선택도 두렵지 않았습니다. 언제나 부모님께 기쁨과 자랑이 될 수 있도록 열심히 노력하는 딸이 되겠습니다.

저는 지금 석사 과정을 마치지만, 끝이 아닌 새로운 시작이라고 생각합니다. 스스로에게 부끄럽지 않은 사람이 되기 위해 항상 노력하고 고민하여 앞으로의 미래를 향해 달려 나가겠습니다. 모두 감사합니다!