creative commons
COMMONS DEED

저작자표시-비영리-변경금지 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.

변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

The Increased Efficiency of Porphyran Hydrolysis by Constructing a Multifunctional Enzyme Complex from Marine Microorganisms

JooHee Han

Department of Next Generation Applied Sciences

The Graduate School of Sungshin University

The Increased Efficiency of Porphyran Hydrolysis by Constructing a Multifunctional Enzyme Complex from Marine Microorganisms

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

inpartial fulfillment of the requirements
for the degree of Master of Food Science

[JooHee Han]

[05, 2022]

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

Approved as to style and content:

Thesis Advisor	현 정 은	.....
Committee Chairman	장 혜 원	.....
Committee Member	고 병 준	.....
Committee Member	현 정 은	.....

The Graduate School of Sungshin University

ABSTRACT

The Increased Efficiency of Porphyran Hydrolysis by Constructing a Multifunctional Enzyme Complex from Marine Microorganisms

JooHee Han
Department of Next Generation Applied Sciences
The Graduate School of
Sungshin University

Porphyran, a polysaccharide composed of red algae, is a source of a multifunctional oligosaccharide material and new biomass raw materials with various physiological activities. The glycolysis of porphyrans into oligosaccharides through various porphyranases is a process to obtain excellent and promising alternative resources. In this study, porphyran was extracted from *Porphyra yezoensis*, and it was used as a research substrate. We also established an efficient hydrolysis method using an enzymatic complex obtained through cohesin-dockerin interactions that degrade natural polysaccharides. The cohesion-dockerin interaction is designed to genetically binding dockerin module to the end of an existing enzyme and then attaching cohesin module to obtain a protein complex. The designed protein complex has been shown to further increase the

activity on the substrate, which can be considered as a useful method to obtain efficient oligosaccharides or monosaccharides through hydrolysis of red algae as bioresources.

CONTENTS

List of Tables

List of Figures

Ⅰ. INTRODUCTION

Porphyran is a sulfated carbohydrate produced from the Porphyra genus of red algae. It's a complex carbohydrate with a linear backbone made up of 3-linked beta-D-galactosyl units alternating with either 3,6-anhydro-alpha-L-galactosyl or 4-linked alpha-L-galactosyl 6-sulfate units. L-galactose, 3,6-anhydro-L-galactose, 6-O-methylated D-galactose, 6-O-sulfated L-galactose, ester sulfate, and 6-O-methyl D-galactose are among the ingredients. 1-4-linked L-galactose 6-sulfate makes up a portion of the ester. Porphyra yezoensis is a significant alga that is mostly grown in China, Japan, and Korea. (Chopin et al., 1999). *P. yezoensis* has a faster growth rate than other red algae and is less susceptible to disease.

P. yezoensis is high in proteins (25 - 50%) and polysaccharides (20 - 40%) (Lahaye & Jegou, 1993), and contains therapeutic properties such as tumor inhibition, anti-viral activity, and anti-ulcer activity, which are linked to bioactive polysaccharides (Huheihel et al., 2002; Nishioka et al., 1999; Yoshizawa et al., 1995). The *P. yezoensis* polysaccharide is a low-cost, high-sulfuric acid-group-rich porphyran with strong antioxidant potential.

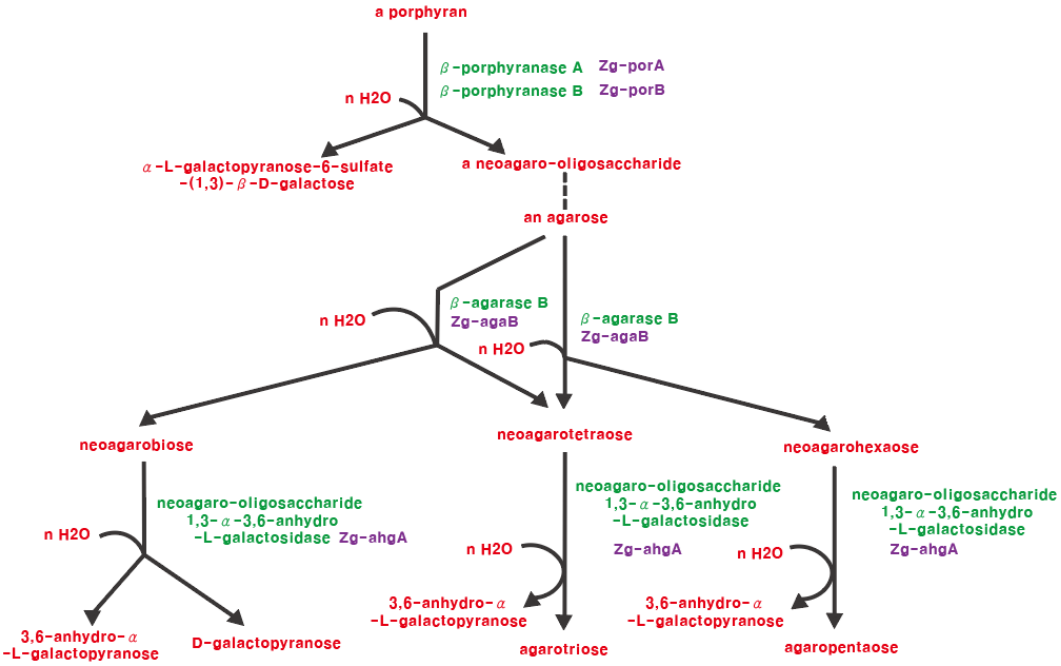

Fig. 1. Schematic diagram of enzymatic decomposition of porphyran

Porphyran is a polysaccharide of red algae. Enzymes that degrade the polysaccharide porphyran include ZgPorB (beta-porphyranase) and ZgAgaB (beta-agarase). Then, porphyran is hydrolyzed with β-porphyranase B to obtain a neoagaro oligosaccharide and α-L-galactopyranose-6-sulfate-(1,3)-β-D-galactose. Subsequently, a neoagaro oligosaccharide is hydrolyzed with β-agarase B enzyme to obtain neoagarobiose and neoagarotetraose.

The molecular weights of polysaccharides determine many of their characteristics. This includes both physical features like solution viscosity as well as more sophisticated properties like bioactivity. Polysaccharides from *P. yezoensis* digested with -agarase were shown to have greater macrophage stimulating activity and solubility (Zhou & Ma, 2006). Antithrombotic and growth-stimulating properties of plants are also influenced by their molecular weight. (Olanrewaju et al., 2017). Therefore, the study of how to effectively degrade porphyran is a promising field.

There are various methods for glycolysis of porphyran polysaccharides. Porphyran polysaccharide decomposition methods include chemical decomposition methods such as acid or alkali hydrolysis and ultrasonic decomposition methods. Chemical treatment, such as alkali or acid hydrolysis, is frequently utilized, although it consumes a large amount of solvents and energy, and chemical waste is typically unavoidable. And the sonication process is characterized by high cost (Jönsson et al., 2020).

On the other hand, the microbial enzymatic decomposition method is an environmentally friendly and efficient method of decomposing porphyran. The decomposition of porphyran polysaccharides by the enzymatic decomposition method can be introduced as a method that can compensate for the disadvantages of environmental pollution caused by chemical decomposition methods such as acid or alkali hydrolysis, which were mainly used in the past (Lee et al., 2009).

Representative enzymes that degrade porphyrans include porphyranase and agarase. These enzymes are a natural enzyme and are responsible for breaking down the polysaccharide porphyran into various molecules. (Schultz-Johansen et al., 2018). However, natural enzymes have limitations in degrading substrate polysaccharides. Biological processes using enzymes have not yet been representatively used as a practical method due to the slow rate of enzymatic degradation (Liu & Qu, 2021). To enable the broad application of biodegradation, new enzymes that are both efficient and cost-effective must be developed. Developing complex enzymatic systems is one strategy to overcome this problem, providing significantly greater degradation potential through highly ordered structural organizations that enable enzyme proximity synergies. Such complex systems can provide a faster disassembly system that can be used more efficiently (Tamoor et al., 2021).

Cellulosomes, one of the complex enzymatic systems produced by anaerobic bacteria such as *Clostridium cellulovorans*, have various cellulolytic subunits bound to the non-enzymatic scaffoldin CbpA via cohesin-dockerin interactions (Doi et al., 2003). The dockerin-cohesin interaction is a protein-protein interaction with high affinity that occurs between an enzyme containing overlapping sequences (dockerin domain) and a non-catalytic scaffold containing repeating sequences (cohesin domain) (Gad & Ayakar, 2021). To construct a complex enzymatic system, a chimeric enzyme with a dockerin domain and splicing scaffoldin (such as CbpA from *C. cellulovorans*) to a small recombinant scaffoldin such as mini-CbpA was also required (Hyeon et al., 2010).

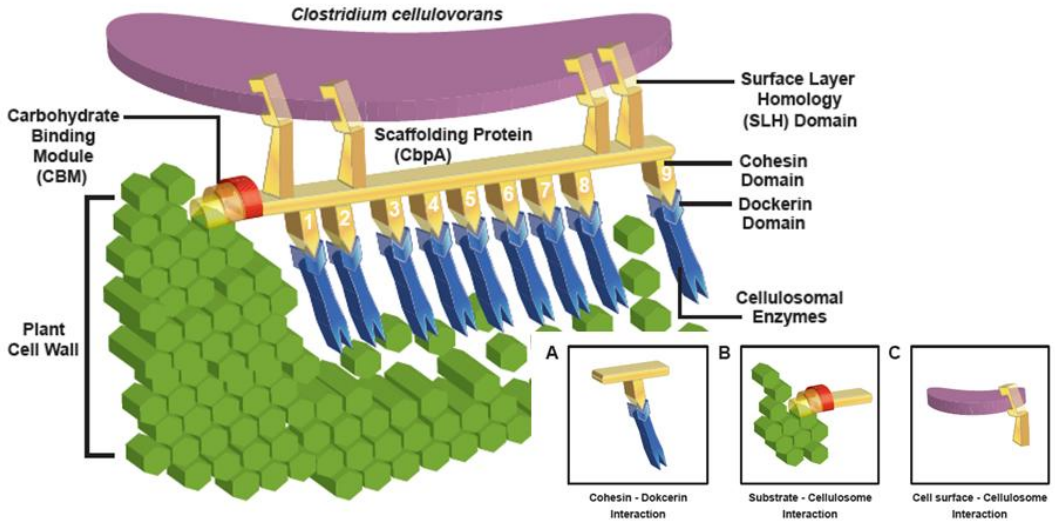

Fig. 2. Design of the degradation of porphyran by porphyranolyric enzyme complex. (Hyeon et al., 2010)

Schematic illustrations of a *C. cellulovorans* cellulosome. The cellulosome structure is along with the protein components and cellulosome system modules. CbpA has nine cohesin domains, carbohydrate binding module (CBM), and four surface layer homology (SLH) domains. The cohesin domain of cellulosomal enzymes binds to the dockerin domain, that leads to the assembly of the cellulosome. (A) The cohesin-dockerin interaction has a high affinity, sensitivity, and selectivity. (B) The CBM in a scaffolding protein mediates the substrate-cellulosome interaction. (C) The proximity effect is used for the cell surface-cellulosome interaction.

The porphyran enzymes are more effective at hydrolyzing crystalline porphyran when assembled in complex with mini-CbpA than when isolated. In this study, for efficient enzymatic degradation of marine biomass polysaccharides, dockerin-fused chimeric *Zobellia galactanivorans* agarase ZgAgaB and recombinant mini-CbpA were used to construct porphyranolytc enzyme complexes through cohesin-dockerin interactions. As a result of enzymatic digestion of porphyran polysaccharide, a substrate, with the porphyran enzyme complex, it was confirmed that the polysaccharide decomposition efficiency was higher than that of the natural enzyme.

Ⅱ. MATERIALS AND METHODS

2.1. Bacterial Strains, Plasmids, and Media

Table 1 lists the microbial strains employed in this study. The *Escherhichia coli* DH5α was used for DNA manipulation and The *E. coli* BL21 (DE3) was used for proteins manipulation, respectively. Plasmid for protein expression was a pCold II vector. Genomic DNA of *C. cellulovorans* ATCC 35296, *Z. galactanivorans*, and *Bacteroides plebeius* was used as the templates for target genes. *E coli* was cultured in Luria-Bertani (LB) medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} sodium chloride) containing 50 μg mL^{-1} ampicillin at 37 °C. *C. cellulovorans* was cultivated anaerobically in round-bottomed flasks at 37 °C in a previously reported medium (Doi et al., 2003).

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*, *relA*, *supE44*, *thi-1*, Δ(*lacZYA- argF*)*U169*, (Phi80*lacZ*delM15)	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]
Bacteroides plebeius	WT strain KCTC 5793	KCTC [b]
Zobellia galactanivorans	WT strain KCTC 12921	KCTC [b]
Transformants		
E. coli BL21 (pET22b (+) Control)	[$T7_p$-*pelB*-$T7_t$]	This study
E. coli BL21 (pET22b (+) mCbpA)	[$T7_p$-*pelB-minicbpA*-$T7_t$]	This study
E. coli Rosetta (DE3) (pColdII BpPorB-Doc)	[$CspA_p$-*TEE-BpPorB-docB*-$CspA_t$]	This study
E. coli Rosetta (DE3) (pColdII ZgAgaB-Doc)	[$CspA_p$-*TEE-ZgAgaB-docB*-$CspA_t$]	This study
E. coli Rosetta (DE3) (pColdII ZgPorB-Doc)	[$CspA_p$-*TEE-ZgPorB-docB*-$CspA_t$]	This study
Plasmids		
pET22b (+)	$T7_p$-*pelB*-$T7_t$	Novagen (San Diego, CA, USA)
pET22b (+) mCbpA	$T7_p$-*pelB-miniCbpA*-$T7_t$	This study
pColdII BpPorB-Doc	$CspA_p$-*TEE-BpPorB-docB*-$CspA_t$	This study
pColdII ZgAgaB-Doc	$CspA_p$-*TEE-ZgAgaB-docB*-$CspA_t$	This study
pColdII ZgPorB-Doc	$CspA_p$-*TEE-ZgPorB-docB*-$CspA_t$	This study

[a]ATCC, American Type Culture Collection.

[b]KCTC, Korean Collection for Type Cultures.

2.2. DNA Manipulations

All genetic changes were carried out using standard molecular biology procedures. (Sambrook and Russell 2006). Commercial enzymes such as EZ-FusionTM HT Cloning Kit were purchased from Enzynomics and restriction enzymes were purchased from Takara Bio, Korea. GenEx TM Cell genomic DNA purification kit (Gene All, Korea) was used for isolation of genomic DNA of *Z.* galactanivorans, *B. plebeius* and *C. cellulovorans* by the manufacturer's instructions. The plasmid was purified by the LaboPassTM Plasmid Purification Kit Mini (Cosmogenetech, Korea) in accordance with the manufacturer's recommendations.

2.3. Overlap PCR and Plasmid Construction for Protein Expression

Multistep overlap PCR strategy was implemented separately for two types of chimeric enzymes. Multistep overlap PCR technique was utilized for construction of the chimeric ZbPorB or BpPorB gene that contains the dockerin module of *C. cellulovorans* endoglucanase EngB at its 3′ terminus by the ZgPorBdoc-P1, ZgPorBdoc-P2, ZgPorBdoc-P3, and ZgPorBdoc-P4 or BpPorBdoc-P1, BpPorBdoc-P2, BpPorBdoc-P3, and BpPorBdoc -P4 overlapping primers. (Table 2.)

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

Primer	Sequence[a]
ZgPorBdoc-P1	CATCATCATATG*GAGCTC*ATGAAGCTTTCCAACCAATT
ZgPorBdoc-P2	CAGCGGATCCATTCTTTGAATCAACCAATTGCC
ZgPorBdoc-P3	TTCAAA GAATGGATCCGCTGGCTCC
ZgPorBdoc-P4	TCTAGACTGCAG*GTCGAC*TCATAAAAGCATTTTTTTAAGA
BpPorBdoc-P1	CATATGGAGCTC*GGTACC*AATGCGCAAGACCGTACTGTA
BpPorBdoc-P2	CAGCGAGCCAGCGGATCCTTCCTCGATGGGAACCAA
BpPorBdoc-P3	CATCGAGGAAGGATCCGCTGGCTCC
BpPorBdoc-P4	TCTAGACTGCAG*GTCGAC*TCATAAAAGCATTTTTTTAAGA
mCbpA-f	CATCATCATATG*GAGCTC*GCAGCGACATCATCAATGTC
mCbpA-r	TCTAGACTGCAG*GTCGAC*CTATATAGGATCTCCAATATTTATT

[a] The sites of restriction enzyme are italicized, and the 5-nucleotide extension is highlighted.

The restriction enzyme sites and the regions of 10-nucleotide-long 5′ extensions are underlined and italicized, respectively. At 5′ extension, overlapping primers (ZgPorBdoc-P2, ZgPorBdoc-P3 or BpPorBdoc-P2, BpPorBdoc-P3) had a 10-nucleotide complementary sequence to the neighboring segment terminal. Furthermore, the dockerin module is linked to the N-terminus of expansin protein by a flexible synthetic 12 amino acid linker sequence (GSAGSAAGSGEF). The flexible linker, GSAGSAAGSGEF, reduced the amounts of homologous repetitions in the DNA coding sequence and has been used to create a variety of additional fusion proteins. Restriction enzyme sites Sac I and Sal I were inserted into the ZgPorBdoc-P1 and ZgPorBdoc-P4 or BpPorBdoc-P1, BpPorBdoc-P4 primers at their 5′ and 3′ termini, respectively. Second PCR step caused the fusion of purified 879 Bp and 198 Bp fragments. The overlapped 1.0 kb fragment was amplified and separated for ligation in the pColdⅡ (+) plasmid at the Kpn I -Sal I sites, resulting in the pColdⅡ (+) ZgPorB-Doc or BpPorB-Doc- plasmid. The constructed plasmids are introduced into *E. coli* BL21. After Isopropyl thio-β -D-galactose (IPTG) adding for the production of complex components in the culture medium, Ni-affinity chromatography was used to separate recombinant components from lysed cell samples.

A recombinant scaffoldin(miniCbpA) was designed from the scaffoldin CbpA from *C. cellulovorans* for the construction of cellulosome-based protein complexes (Hyeon et al., 2013). Multistep overlap PCR strategy was also performed to construct the chimeric AgaB gene as previously described. The chimeric β-agarase (ZgAgaB)

was constructed by the C-terminal fusion between the catalytic domain of AgaB and dockerin module (*docB*) in a previous study (Hyeon et al., 2012).

2.4. Protein Expression from the Recombinant Strains.

LB medium with 50 μg mL^{-1} ampicillin was utilized as the cultivation medium of *E. coli* strains in 15 mL conical tube for culture preparations and in 200 mL shake flasks for enzyme expression at 37 °C by shaking at 200 rpm. IPTG was used as a T7 promoter inducer at 1 mM in a chilled medium with a 0.6 optical density value (OD600). The extracted ice-cold cells were suspended in 10 mL lysis buffer (10 mM imidazole, 400 mM NaCl, and 50 mM Na_2HPO_4, pH 8.0) after an overnight induction at 16 °C. The ruptured cells were then collected by centrifugation at 4,000 rpm for 30 minutes. A Ni-NTA column (Qiagen, USA) was used to bind the recombinant protein in the supernatants of the cell extract by its His-tag for protein purification. Elution buffer (50 mM Na_2HPO_4, 400 mM NaCl and 250 mM imidazole, pH 8.0) and Wash buffer (50 mM NaH_2PO_4, 400 mM NaCl and 50 mM imidazole, pH 8.0) were used for elution and wash of the target enzymes, respectively. The isolated proteins were dialyzed and concentrated against 20 mM Tris-HCI buffer (pH 7.0) by an Ultrafree Biomax centrifugal filter unit (Millipore, USA) with a 10 kDa cut-off membrane. The protein content was determined using the Bradford assay by bovine serum albumin (BSA) standard.

2.5. Oligosaccharide production by hydrolysis from porphyran by enzyme complex

First, in order to use Porphyran as a substrate, Porphyran is extracted from *P. yezoensis*. Grind 100 g of dried *P. yezoensis* into a powder. Immerse in 1000 mL ethyl alcohol and reflux in hot water bath. Then, the solids are collected separately and filtered with water through gauze. The extracted mixture is stirred and sterilized at 100 °C. When centrifuged at 8000 rpm and 30 min, the supernatant is concentrated to a capacity of 1/10-1/20. 95% ethyl alcohol is added to the concentrated supernatant, stirred until the final concentration is 80%, left for 48 hours, and centrifuged. The pellet is collected and redissolved in 100-200 mL of DDW. The insoluble part is removed by centrifugation. For extraction, 3Vol ethyl alcohol is added and porphyran is obtained by freeze-drying.

The PAHBAH reaction method is to measure the amount of oligosaccharides produced by enzymatic hydrolysis with an enzyme complex using porphyran as a substrate. The corresponding working solution for the PAHBAH reaction was made just before the test by combining one part reagent A and nine parts reagent B. The working reagent is only stable for a few hours. For reagent A, 30 mL H_2O (dist.), 5 mL HCl (37%) and 5 g 4-hydroxybenzoic acid hydrazide are mixed and filled up to 100 mL with H_2O. For reagent B, 1.1 g $CaCl_2$, 20 g NaOH and 12.5 g trisodium citrate are dissolved in 500 mL H2O (dist.) and filled up to 1 L with H_2O. Both reagents have a shelf life of 3-4 weeks. In a 2 mL tube, 75 L sample was combined with 150 µL working reagent and incubated for 10 minutes at 900 rpm and 100 °C on

a thermo mixer. After cooling, 100 µL were transferred to a flat-bottom plate and the absorbance was measured at 410 nm.

Ⅲ. RESULTS AND DISCUSSION

3.1. Design of chimeric ZgPorB-Doc and BpPorB-Doc for complex assembly.

The genes encoding the ZgPorB and BpPorB have an apparent of approximately 879 bp and 963 bp, respectively. Previous research has showed that these enzymes has a high catalytic activity for degrading porphyran., a red algae polysaccharide, allowing D-galactose bioconversion, which is useful for commercial polysaccharide oligosaccharide production. β-Porphyranases (ZgPorB-Doc and BpPorB-Doc) containing the dockerin module docB at the C-terminus were required to assemble the enzymes into a complex. ZgPorB-Doc and BpPorB-Doc proteins were expressed from recombinant strains containing pCold II ZgPorB-Doc and pCold II BpPorB-Doc plasmid,s respectively. (Fig. 3).

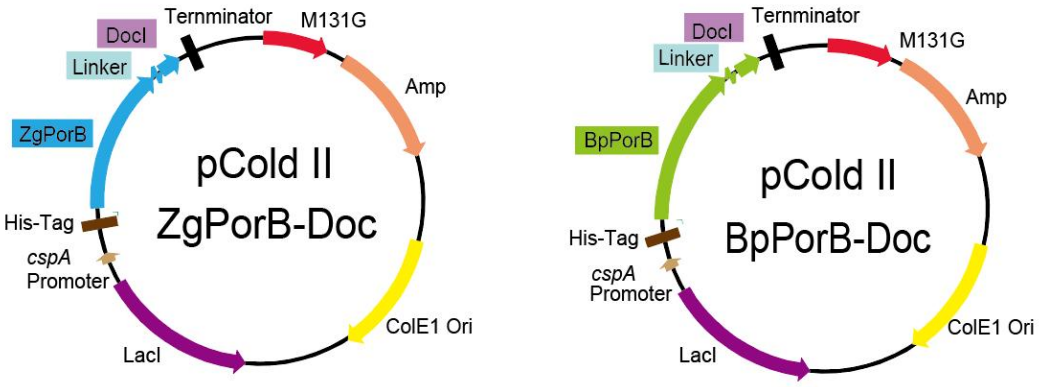

Fig. 3. Construction of plasmids to express the chimeric ZgPorB-Doc and BpPorB-Doc genes

β-Porphyranase (ZgPorB-Doc and BpPorB-Doc) protein complexes were identified by protein Western Blot (Fig. 4). The molecular masses of ZgPorB-Doc and BpPorB-Doc are 40 kDa (35 kDa ZgPorB 5.7 kDa BpPorB additional residues (e.g. His-tag, dockerin module and flexible linker) and 44 kDa (38 kDa LsAraA 5.7 kDa additional residues (e.g. dockerin module; flexible linkers and His-tags) by nucleotide sequence prediction. They were equivalent to the single-band of the gel molecular mass (approximately ZgPorB-Doc: 40.3 kDa and BpPorB-Doc: 43.9 kDa).

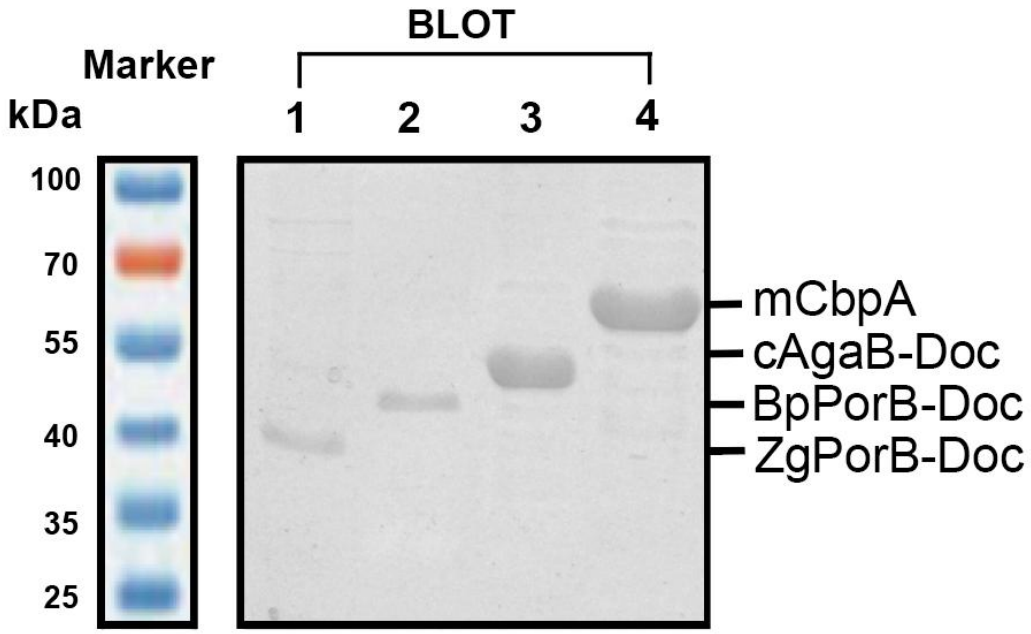

Fig. 4. Expression of dockerin fused enzymes and mCbpA.

Porphyranase confirmed by Western blotting: ZgPorB-Doc, BpPorB-Doc, ZgAgaB-Doc, and mCbpA enzymes. ZgPorB-Doc is 40.3 kDa, BpPorB-Doc is 43.9 kDa, ZgAgaB-Doc is 47.3 kDa, and mCbpA is 56.7 kDa. It is confirmed that each has an appropriate size.

A batch binding and elution procedure was employed in this purification process to purify a target dockerin-fused protein by the high specificity and affinity of scaffoldin CBM for cellulose. (Fig. 5) CBM module is distinguished by a continuous amino acid sequence including carbohydrate activating enzymes with unique folds and carbohydrate binding ability. Certain aromatic amino acids on the hydrophobic surface can bind carbohydrates. Therefore, such single-step purification of target enzymes supported by CBM will greatly improve the purification cost-effectiveness of enzyme. SDS PAGE was used to examine the protein of the cellulose binding fraction following CBM assisted affinity purification method, suggesting that the ZgPorB-Doc, BpPorB-Doc enzyme complex was well developed.

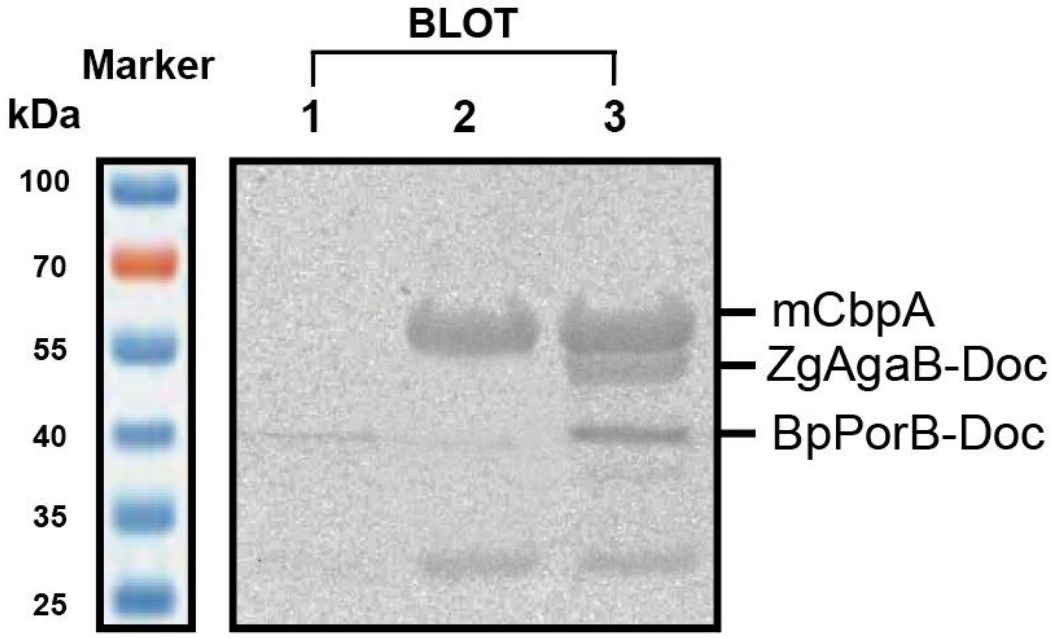

Fig. 5. Design of purification of assembled porphyranolytic complex by CBM-utilizing purification. Western blotting property of binding fractions.

1: BpPorB-Doc only, 2: mCbpA only, 3: BpPorB-Doc, ZgPorB-Doc and mCbpA constituting the porphyranolytic complex. It can be confirmed that the porphyranolytic complex was successfully bound and expressed through CBM-utilizing purification.

3.2. Synergistic Effect of the Porphyranolytic Complex Containing ZgPorB-Doc, BpPorB-Doc.

Following the effective expression of complex components, the effect of ZgPorB-Doc, BpPorB-Doc on D-galactose production using porphyran substrates was examined. *Z. galactanivorans*, *B. plebeius*, is a marine microorganism capable of metabolizing and hydrolyzing porphyrans as energy and carbon sources. *Z. galactanivorans* and *B. plebeius* are porphyranolytic bacteria often generate porphyranase, which catalyzes porphyran degradation. Hydrolysis of porphyran by enzymes provides α-L-galactopyranose-6-sulfate-(1,3)-β-D-galactose, a neoagaro-oligosaccharide, neoagarobiose and neoagarotetraose as the monomeric sugars for biorefining or developing biofuels. Among these, D-galactose can be used as a substrate for the chemical or enzymatic production of D-tagatose.The enzyme complexes formed with cAgaB have previously been described.

In this study, the results of the amount of sugar decomposed by hydrolysis using porphyran as a substrate are presented. First, comparing the average of the amount of sugar hydrolysis of the ZgPorB-Doc (0.29 g/L) and BpPorB-Doc (0.38 g/L) enzymes over time, it can be seen that the hydrolysis efficiency of BpPorB-Doc is about 1.3 times higher than that of ZgPorB-Doc. (Fig. 6)

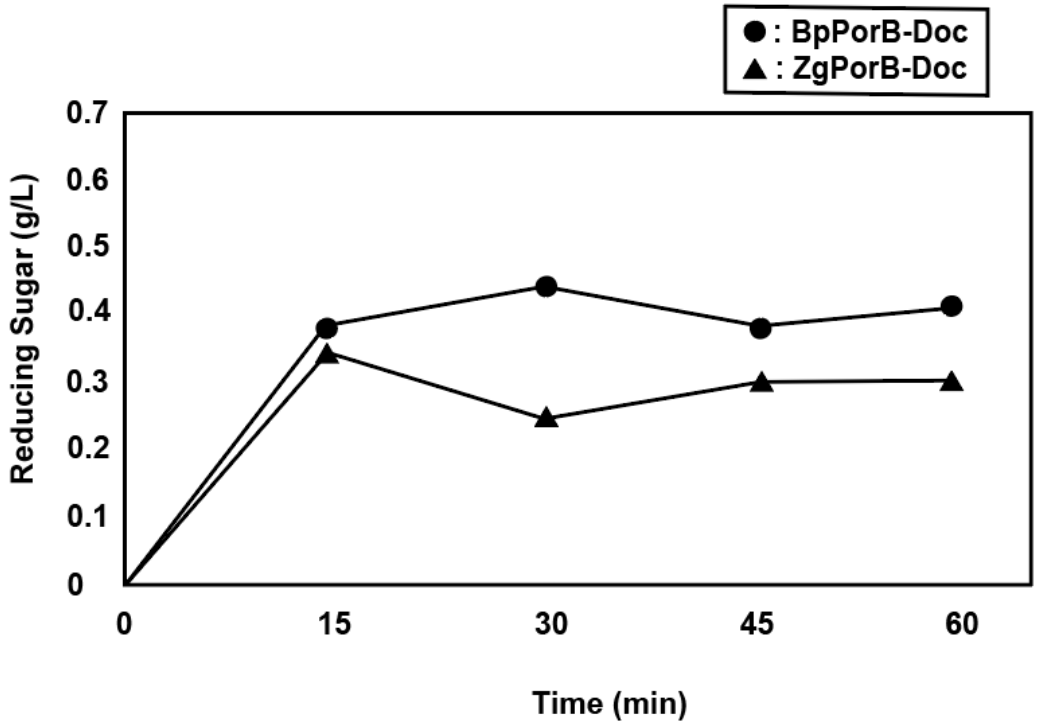

Fig. 6. Determine of porphyranolytic activity by two porphyranase on the pure porphyran substrate.

Comparing the average of the amount of sugar hydrolysis of the ZgPorB-Doc (0.29 g/L) and BpPorB-Doc (0.38 g/L) enzymes over time, it can be seen that the hydrolysis efficiency of BpPorB-Doc is about 1.3 times higher than that of ZgPorB-Doc.

That is, it suggests that BpPorB-Doc has superior enzymatic ability to hydrolyze the porphyran substrate into polysaccharides than ZgPorB-Doc. Therefore, we selected BpPorB-Doc as a porphyranase and assembled an enzyme complex with ZgAgaB-Doc. We then evaluated the glycolytic activity of the complex system using a porphyran matrix assay to measure the synergistic effect of the BpPorB-Doc + ZgAgaB-Doc complex. As a result of comparing the glycolytic ability of the BpPorB-Doc and BpPorB-Doc + ZgAgaB-Doc complexes, the average degree of glycolysis of BpPorB-Doc was 0.38 (g/L), and BpPorB-Doc + ZgAgaB-Doc The average degree of sugar hydrolysis was 0.54 (g/L). These results indicate that the dockerin fusion chimeric enzyme of the successfully assembled complex to scaffoldin glycosylates the pure porphyran substrate. The activity on the pure porphyran substrate was increased by 1.4-fold compared to the uncomplex system (BpPorB-Doc) (Fig 7.).

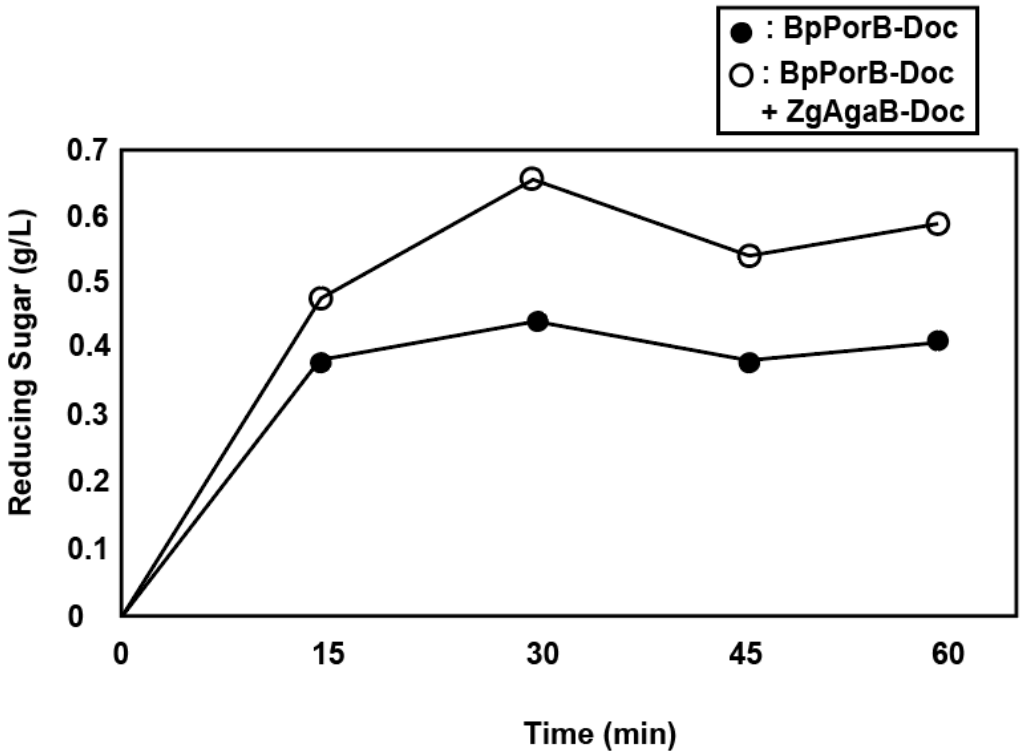

Fig. 7. Determine of synergistic activity by porphyranase and agarase on the pure porphyran substrate.

The average degree of glycolysis of BpPorB-Doc was 0.38 (g/L), and BpPorB-Doc + ZgAgaB-Doc The average degree of sugar hydrolysis was 0.54 (g/L). The activity on the pure porphyran substrate was increased by 1.4-fold compared to the uncomplex system (BpPorB-Doc)

3.3. Improved performance of porphyranolytic complexes containing the artificial protein Mini-CbpA.

The recombinant scaffolding protein mini-CbpA was engineered based on the *C. cellulovorans* scaffolding protein CbpA. Another report reported that minicellulosomes are successfully formed by the interaction between the cellular cellulase gene and cohesin containing miniCbpA and dockerin. So, in this study, we constructed a functional multi-Porphyranolytic complex enzyme by assembling mini-CbpA into the BpPorB-Doc + ZgAgaB-Doc enzyme. Afterwards, the successful expression of functional multi-porphyranolytic complex enzymes was verified and the effect of glycolysis on porphyran substrates was investigated. As a result, compared to the single subunit (B) enzyme, the (BAM) enzyme in which mini-CbpA was assembled with the (BA) enzyme showed a higher glycolytic ability. These results indicate that mini-CbpA and BpPorB-Doc + ZgAgaB-Doc enzymes form a complex, and dockerin-fused enzymes in the miniCbpA exhibit synergistic action in porphyran degradation. The observed activity enhancement was 3.73-fold higher (Fig. 8). This enzyme complex will be useful for bioconversion of a neoagaro oligosaccharide, α-L-galactopyranose-6-sulfate-(1,3)-β-D-galactose, neoagarobiose and neoagarotetraose from the porphyran substrate of red algae by enzymatic conversion with a one-step process. The construction of multi-porphyranolytic complex enzymes can be an important strategy to reduce the overall process time for glycolysis of porphyran.

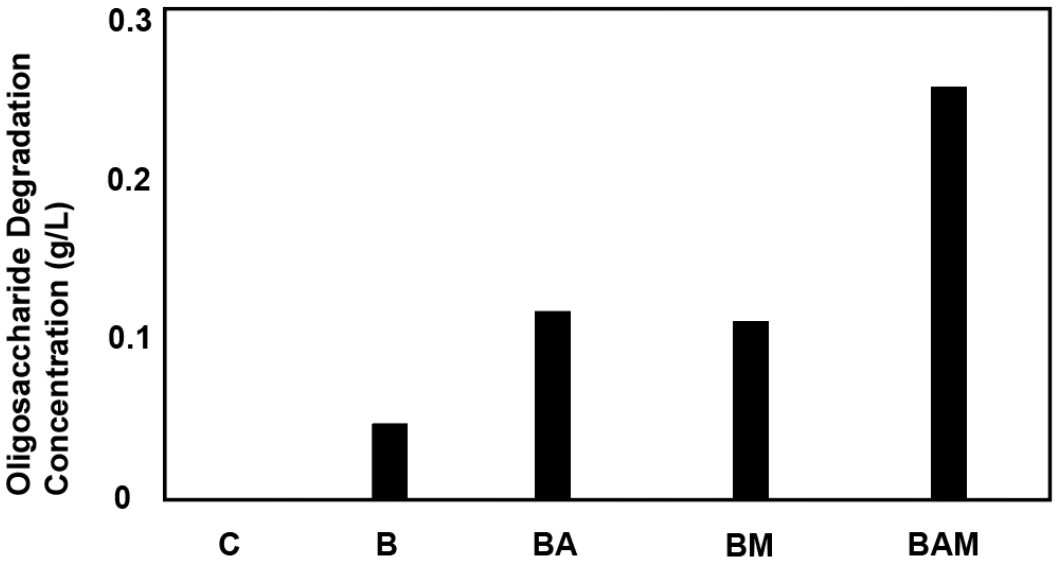

Fig. 8. Determine of synergistic activity by porphyranolytic complex on the pure porphyran substrate.

Compared with single subunit (B) enzyme, miniCbpA (BA) and assembled (BAM) enzyme showed higher porphyran oligosaccharide production capacity. The porphyran enzyme hydrolysis efficiency of the (BAM) enzyme was about 3.73-fold higher than the single subunit (B) enzyme hydrolysis efficiency.

3.4. Glycolysis of natural substrates extracted from red algae.

In chemical methods, the production of a neoagaro-oligosaccharide, α-L-galactopyranose-6-sulfate-(1,3)-β-D-galactose, neoagarobiose and neoagarotetraose can be obtained under basic or acidic conditions. However, chemical methods is uneconomical, the complexity of purification, the complexity of purification, high waste disposal cost and high by-product yield. On the other hand, the enzymatic approach has the advantage of being eco-friendly, and has aroused a lot of interest in research. Here, we tried to directly utilize natural red algal biomass. *P. yezoensis* is economically important species of red algae, commonly harvested in the seas surrounding East Asian countries, including South Korea and China. *P. yezoensis* is a major food resource in East Asian countries, and it is used in representative red algae culture because of its rapid growth rate.

In this experiment, we selected the red alga, *P. yezoensis*, as a natural substrate and treated it as a suitable substrate for use in the experiment through grinding, drying and extraction processes. In order to have the same conditions as in the experiment of the enzyme sugar hydrolysis reaction with the existing powdered porphyran substrate, the concentration of sugar extracted from the natural substrate *P. yezoensis* was adjusted to 0.5%. After that, We measured the glycolytic activity of three enzymes: BpPorB-Doc, BpPorB-Doc + ZgAgaB-Doc, and BpPorB-Doc + ZgAgaB-Doc + mini-CbpA. The glycolysis of the enzymatic complex composed of BpPorB-Doc, BpPorB-Doc + ZgAgaB-Doc and BpPorB-Doc + ZgAgaB-Doc + mini-CbpA was carried out at 50 °C

for substrate enzymatic digestion. non-complex enzymes and complex enzymes successfully degraded the natural substrate. The results of sugar hydrolysis of uncomplexed and complex enzymes showed relative ratio values of 1 (B), 1.1 (BM) and 1.2 (BAM) after 2 hours. That is, the complex enzyme showed the result that hydrolysis of porphyran sugar was more efficient than the non-complex enzyme. Large-scale a neoagaro-oligosaccharide, α-L- galactopyranose-6-sulfate-(1,3)-β -D-galactose, neoagarobiose and neoagarotetraose production from marine bioresources. Enzymatic process can be commercialized as oligosaccharide production for further study. Nevertheless, enzymatic technique may be difficult to utilize with the natural red algae substrate, *P. yezoensis*. This is because there are disadvantages due to impurities in the process of refining natural *P. yezoensis* into porphyran. This can be seen from this study report that the glycolysis efficiency using the neatly purified porphyran powder substrate (Fig. 9) was relatively higher than the glycolysis efficiency using the purified Porphyra yezoensis, a natural substrate. Therefore, for practical commercialization, the purification process of porphyran from Porphyra yezoensis is an area for further study.

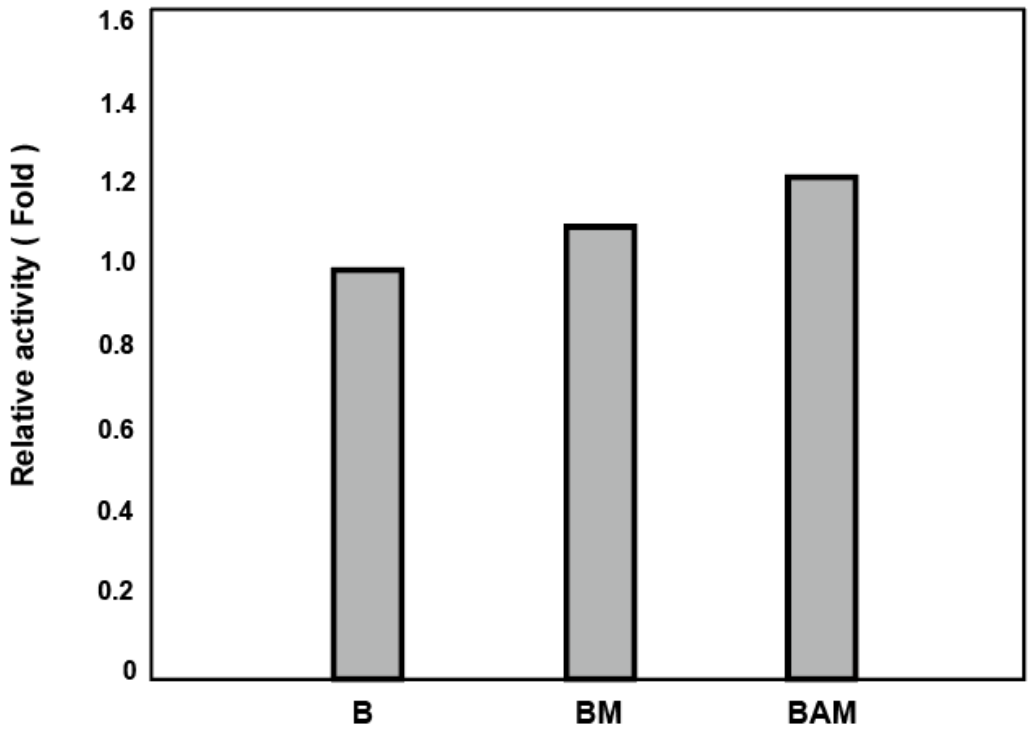

Fig. 9. Synergistic effect of porphyranolytic complex activity on red algae degradation.

porphyran hydrolysis of uncomplexed and complex enzymes showed relative ratio values of 1 (B), 1.1 (BM) and 1.2 (BAM) after 2 hours.

Ⅳ. CONCLUSION

By hydrolyzing *P. yezoensis* with a porphyranolytic compex enzyme, a greater amount of polysaccharide could be obtained than the conventional hydrolysis result using a single enzyme. The porphyranolytic compex enzyme was constructed as a complex enzyme with BpPorB-Doc + ZgAgaB-Doc + mini-CbpA (BAM) and has the ability to enzymatically degrade porphyran. The hydrolyzate obtained by enzymatic digestion of porphyran with porphyranolytic compex enzyme is a neoagaro-oligosaccharide, α-L-galactopyranose-6-sulfate-(1,3)-β-D-galactose, neoagarobiose and neoagarotetraose. The result of oligosaccharide degradation concentration (g/L) obtained by enzymatic hydrolysis of porphyran showed that BpPorB-Doc + ZgAgaB-Doc + mCbpA (BAM) was about 3.73 times higher than that of BpPorB-Doc only (B). The optimal conditions for enzymatic hydrolysis are 0.5% substrate, temperature 50 °C, and hydrolysis time 60-120 minutes.

We succeeded in purifying porphyran from *P. yezoensis* and performing enzymatic digestion. As a result of hydrolysis of non-complex enzymes and complex enzymes in natural porphyran extracted from *P. yezoensis*, the relative ratio values after 2 hours were about 1 (B), 1.1 (BM), and 1.2 (BAM). This is a result that can prove the enzymatic degradation effect of The porphyranolytic compex on natural substrates, and suggests the possibility of utilizing natural substrates as biomass.

REFERENCES

Chopin, T., Yarish, C., Wilkes, R., Belyea, E., Lu, S., & Mathieson, A. (1999). Developing Porphyra/salmon integrated aquaculture for bioremediation and diversification of the aquaculture industry. Journal of Applied Phycology, 11(5), 463. https://doi.org/10.1023/A:1008114112852

Doi, R. H., Kosugi, A., Murashima, K., Tamaru, Y., & Han, S. O. (2003). Cellulosomes from Mesophilic Bacteria. Journal of Bacteriology, 185(20), 5907. https://doi.org/10.1128/JB.185.20.5907-5914.2003

Gad, S., & Ayakar, S. (2021). Protein scaffolds: A tool for multi-enzyme assembly. Biotechnology Reports, 32, 670. https://doi.org/10.1016/J.BTRE.2021.E00670

Huheihel, M., Ishanu, V., Tal, J., & Arad, S. (2002). Activity of Porphyridium sp. polysaccharide against herpes simplex viruses in vitro and in vivo. Journal of Biochemical and Biophysical Methods, 50(2–3), 189–200. https://doi.org/10.1016/S0165-022X(01)00186-5

Hyeon, J. E., Jeon, S. D., & Han, S. O. (2013). Cellulosome-based, Clostridium-derived multi-functional enzyme complexes for advanced biotechnology tool development: advances and applications. Biotechnology Advances, 31(6), 936–944. https://doi.org/10.1016/J.BIOTECHADV.2013.03.009

Hyeon, J. E., Kang, D. H., Kim, Y. I., Jeon, S. D., You, S. K., Kim, K. Y., Kim, S. W., & Han, S. O. (2012). Production of functional agarolytic nano-complex for the synergistic hydrolysis of marine biomass and its potential application in carbohydrate-binding module-utilizing one-step purification. Process Biochemistry, 47(5), 877–881. https://doi.org/10.1016/J.PROCBIO.2012.01.009

Hyeon, J. E., Yu, K. O., Suh, D. J., Suh, Y. W., Lee, S. E., Lee, J., & Han, S. O. (2010). Production of minicellulosomes from Clostridium cellulovorans for the fermentation of cellulosic ethanol using engineered recombinant Saccharomyces cerevisiae. FEMS Microbiology Letters, 310(1), 39–47. https://doi.org/10.1111/J.1574-6968.2010.02035.X

Jönsson, M., Allahgholi, L., Sardari, R. R. R., Hreggviosson, G. O., & Karlsson, E. N. (2020). Extraction and Modification of Macroalgal Polysaccharides for Current and Next-Generation Applications. Molecules, 25(4). https://doi.org/10.3390/MOLECULES25040930

Lahaye, M., & Jegou, D. (1993). Chemical and physical-chemical characteristics of dietary fibres from Ulva lactuca (L.) Thuret and Enteromorpha compressa (L.) Grev. Journal of Applied Phycology 1993 5:2, 5(2), 195–200. https://doi.org/10.1007/BF00004017

Lee, Y. C., Lo, S. L., Chiueh, P. Te, & Chang, D. G. (2009). Efficient decomposition of perfluorocarboxylic acids in aqueous solution using microwave-induced persulfate. Water Research, 43(11), 2811–2816. https://doi.org/10.1016/J.WATRES.2009.03.052

Liu, G., & Qu, Y. (2021). Integrated engineering of enzymes and microorganisms for improving the efficiency of industrial lignocellulose deconstruction. Engineering Microbiology, 1, 100005. https://doi.org/10.1016/J.ENGMIC.2021.100005

Nishioka, T., Shirato, H., Fukuda, S., Arimoto, T., Kamada, T., Furuta, Y., Nishino, S., Hosokawa, Y., Kitahara, T., Kagei, K., Inuyama, Y., & Miyasaka, K. (1999). A phase II study of concomitant chemoradiotherapy for laryngeal carcinoma using carboplatin. Oncology, 56(1), 36–42. https://doi.org/10.1159/000011927

Olanrewaju, O. S., Glick, B. R., & Babalola, O. O. (2017). Mechanisms of action of plant growth promoting bacteria. World Journal of Microbiology & Biotechnology, 33(11), 197. https://doi.org/10.1007/S11274-017-2364-9

Schultz-Johansen, M., Bech, P. K., Hennessy, R. C., Glaring, M. A., Barbeyron, T., Czjzek, M., & Stougaard, P. (2018). A Novel Enzyme portfolio for red algal polysaccharide degradation in the marine bacterium Paraglaciecola hydrolytica S66T encoded in a sizeable polysaccharide utilization locus. Frontiers in Microbiology, 9(MAY), 839. https://doi.org/10.3389/FMICB.2018.00839/BIBTEX

Tamoor, M., Samak, N. A., Jia, Y., Mushtaq, M. U., Sher, H., Bibi, M., & Xing, J. (2021). Potential Use of Microbial Enzymes for the Conversion of Plastic Waste Into Value-Added Products: A Viable Solution. Frontiers in Microbiology, 12, 3632. https://doi.org/10.3389/FMICB.2021.777727/BIBTEX

Yoshizawa, Y., Tsunehiro, J., Fukui, F., Ametani, A., Kaminogawa, S., Nomura, K., & Itoh, M. (1995). Macrophage stimulation activity of the polysaccharide fraction from a marine alga (Porphyra yezoensis): structure-function relationships and improved solubility. Bioscience, Biotechnology, and Biochemistry, 59(10), 1933–1937. https://doi.org/10.1271/BBB.59.1933

Zhou, C., & Ma, H. (2006). Ultrasonic Degradation of Polysaccharide from a Red Algae (Porphyra yezoensis). Journal of Agricultural and Food Chemistry, 54(6), 2223–2228. https://doi.org/10.1021/JF052763H

ABSTRACT IN KOREAN (국문 요약)

포피란은 홍조류의 다당류로, 다당류로 이루어져있는 유망한 바이오매스입니다. 포피란으로 이루어져있는 대표적인 홍조류는 포피라 에조엔시스가있습니다. 포피라에조엔시스는 대한민국, 중국 등 동남아시아 해안지역에 광범위하게 분포되어 채취되고 식용으로 사용되고 있습니다. 포피라 에조엔시스는 성장속도가 빠르며, 질병에 강한 것이 특징입니다. 포피란을 가수분해하여 얻을 수 있는 당으로는 알파-L-갈락토피라노스-6-설페이트-(1,3)-베타-디-갈락토스, 네오아가로-올리고사카라이드, 네오아가로비오스 그리고 네오아가로테트라오스가 있습니다. 포피란을 분해하는 방법으로는 화학적 분해방법이 가장 많이 사용되나, 이는 환경적 문제를 야기합니다. 그렇기에 친환경적인 포피란 가수분해방법 개발의 필요성이 점점 증가하는 추세이고 포피란을 가수분해할 수 있는 친환경적인 분해방법으로는 미생물 효소를 통한 분해방법이있습니다.

이 연구에서는 포피란을 효율적이고 친환경적으로 가수분해하고자 포피란 복합체 효소를 구축하였으며 복합체 효소 구축을 통한 포피란 가수분해는 단일 효소를 사용한 기존의 가수분해 결과보다 더 많은 양의 다당류를 얻을 수 있다는 결과 얻었습니다.

포피란 복합체 효소는 박테로이데스 플레베이우스 포르비-도커린 + 조벨리아 갈락토니보란스 아가비-도커린 + 미니씨비피에이 (비에이엠) 복합 효소로 구성되었으며 가수분해의 최적 조건은 기질 0.5%, 온도 40℃, 가수분해 시간 60~120분이었습니다.

포피란을 효소 가수분해하여 얻은 올리고당 분해 농도를 실험한 결과 플레베이우스 포르비-도커린 + 조벨리아 갈락토니보란스 아가비-도커린 + 미니씨비피에이 (비에이엠)이 박테로이데스 플레베이우스 포르비-도커린(비) 단일 효소에 비해 약 3.73배 높은 유의미한 포피란 가수분해 효율을 얻었습니다.

다음으로, 천연 기질에 대한 포피란 복합체 효소의 가수분해 효율을 확인

하기 위해 실험을 진행하였으며, 포피라 에조엔시스에서 포피란을 정제하고 효소 가수분해에 대해 성공적인 결과를 얻었습니다. 포피라 에조엔시스에서 추출한 천연 포피란을 기질로 비복합체 효소와 복합체 효소를 가수분해한 결과, 2시간 후의 상대비율 값은 약 1(비), 1.1(비엠), 1.2(비에이엠)이었습니다.

이는 포피란 복합체 효소가 천연 기질에 미치는 효소적 분해 효과가 효율적이라는 것을 입증할 수 있는 결과이며, 더 나아가 인공 복합체 효소 구축으로 가수분해효율을 높여, 천연 기질인 홍조류를 바이오매스로 활용할 수 있는 유망한 기술개발에 기여합니다.