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Efficient enzymatic degradation process for hydrolysis activity of the Carrageenan from red algae in marine biomass

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ABSTRACT

Carrageenan is a generic name for a family of polysaccharides obtained from certain species of red algae. New methods to produce useful cost-efficiently materials from red algae are needed to convert enzymatic processes into fermentable sugars. In this study, we constructed chimeric genes *cCgkA* and *cCglA* containing the catalytic domain of κ-carrageenase *CgkA* and λ-carrageenase *CglA* from *Pseudoalteromonas carrageenovora* fused with a dockerin domain. Recombinant strains expressing the chimeric carrageenase resulted in a halo formation on the carrageenan plate by alcian blue staining. The recombinant cCgkA and cCglA were assembled with scaffoldin miniCbpA via cohesin and dockerin interaction. Carbohydrate binding module (CBM) in scaffoldin was used as a tag for cellulose affinity purification using cellulose as a support. The hydrolysis process was monitored by the amount of reducing sugar released from carrageenan. Interestingly, these results indicated that miniCbpA, cCgkA and cCglA assembled into a complex and that the dockerin-fused enzymes on the scaffoldin had synergistic activity in the degradation of carrageenan. The observed enhancement of activity by carrageenolytic complex was 3.1-fold-higher compared with the corresponding enzymes alone. Thus, the assemblies of advancement of active enzyme complexes will facilitate the commercial production of useful products from red algae biomass which represents inexpensive and sustainable feed-stocks.

1. Introduction

Carrageenan is a component of red algae species in various marine biomasses. Carrageenan is an important carbon sources for heterotrophic marine bacteria, which degrade the cell walls of marine red algae by secreting specific glycoside hydrolases (GHs), referred to as carrageenases. Additionally, carrageenan is used commercially for various products, such as stabilizers, thickeners, emulsifiers, food additive, feed and beverages (Flores et al., 2007; McKim, 2014; Weiner, 2014). Their microbial degradation from red algae is a necessary process for the development of practical life. Thus, efficient microbial degradation of these algal carbohydrates is of increasing interest for various industrial applications (Hehemann et al., 2012). Carrageenans are sulfated linear polysaccharides of D-galactose extracted from certain red seaweeds, with repeating disaccharide sequences of alternating 3-linked β-D-galactopyranose (β-Gal, G-unit) and 4-linked α-D-galactopyranose (α-Gal, D-unit) (Popper et al., 2011). Carrageenans are classified further according to the number and position of sulfate esters (S) and by the occurrence of 3, 6-anhydro-bridges in the α-linked residues (DA unit) found in gelling carrageenans. For example, κ-(DA-G4S), ι-(DA2SG4S) and λ-(D2S6S-G2S) carrageenans are distinguished by the presence of one, two or three ester sulfate groups per repeating disaccharide unit, respectively (Lemoine et al., 2009). Specific cleavage of linkages in the backbones of complex carrageenan polymers without the risk of modification of the native structure requires specific enzymes for the respective structures. Only a small number of enzymes related to carrageenan degradation have been identified and characterized to date. The enzymatic properties and gene sequences of a few κ-carrageenases, ι-carrageenases and λ-carrageenases have been reported (Barbeyron et al., 2000; Guibet et al., 2007). Based on their amino acid sequences, κ-carrageenases are classified into the GH family 16 in the carbohydrate-active enzymes. The λ-carrageenases have not been classified in the CAZy database to date. The κ-carrageenans are gel-forming, sulfated

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1,3-α-1,4-β-galactans, which are degraded by the κ-carrageenase, both in solution and in solid state by an endoprocessive mechanism. These enzymes role in degradation is of the β-1,4 glycosidic linkages with retention of the anomeric configuration. Conversely, the λ-carrageenan is the most sulfated carrageenan and carries at least three sulfates per disaccharide unit. The degradation process occurs in on three separate reactions by different combinations of enzyme complexes. This conception applies to the marine area from the cellulosome system of anaerobic bacteria such as *Clostridium cellulovorans* (Jeon et al., 2013). These bacteria produce an extracellular enzyme complex (called a cellulosome) containing a variety of cellulolytic subunits attached to the non-enzymatic scaffoldin CbpA via the interaction between the cohesin modules of the scaffoldins and the dockerins of the enzymes (Jeon et al., 2012a,b). Additionally, a carbohydrate-binding module (CBM) in scaffoldin can be used as a fusion affinity tag for both the purification and immobilization of proteins onto cellulose carriers because cellulose is a safe and inert macromolecule with excellent physical properties (Linder et al., 1998; Shoseyov and Doi, 1990). The most important aspects of this study are (1), the suggestion of an efficient enzymatic degradation process for enhanced hydrolysis activity of carrageenan from red algae in marine biomass and (2) the initial successful assembly of a functional carrageenolytic complex and the demonstration of its synergistic hydrolysis activity.

The functional carrageenolytic complex, which is a designer enzyme complex containing only a small number of the nine cohesins in CbpA, has enhanced activity against the carrageenan substrate compared with that of the free carrageenase enzymes. In addition, it was purified by affinity chromatography using cellulose as a support. These advantages demonstrated the feasibility of developing a more efficient carrageenolytic complex using cohesion-dockerin interactions from the cellulosome system to utilize marine biomass better.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The bacterial strains used for cloning and expression of the recombinant protein are *Escherichia coli* DH5α and BL21 (DE3), which are used as the hosts for recombinant DNA manipulation and gene expression, respectively. Table 1 lists the strains used in this work. The plasmid pET22b (+) was used as the vector for cloning and expression (Guibet et al., 2007). *E. coli* strains was cultured in Luria-Burtani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L sodium chloride) or on LB agar supplemented with 50 μg/mL ampicillin at 37 °C when relevant.

2.2. DNA manipulation

All molecular methods used are standard molecular biology techniques. Restriction enzymes and T4 DNA ligase (Fermentas, Lithuania; Promega, USA; and Takara, Japan) were purchased. Genomic DNA from *Pseudoalteromonas carrageenovora* and *C. cellulovorans* was isolated using a genomic DNA purification kit (Promega) in accordance with manufacturer's instructions.

2.3. Construction of the expression plasmids

The coding region of the mature κ-type and λ-type carrageenases without the N-terminal signal peptide was amplified by polymerase chain reaction (PCR) using the oligonucleotide primers, GCGCGAATTCGGCATCTATGCAACCTC and GCGCCTCGAGATTCACCGCAATGGTTAATTTAT for *cgkA* and, GCGCGAATTCGTCTCAATCGGCTATTAAAAGTATT and GCGCCTCGAGCAATGTTGAACTTTGCATGTTT for *cglA*. The PCR product was digested with *BamH*I and *Nco*I and ligated into the corresponding site of pET22b (+) dockerin vector to generate pET22b (+) cCgkA and pET22b (+) cCglA (Hyeon et al., 2010). The 1.6-kb *BamH*I-*Kpn*I DNA fragment encoding the miniCbpA gene was prepared by PCR using the primer set, CCCGGATCCGGCAGCGACATCATCAATGTC and CCCGGTACCTCATATAGGATCTCC AATATTTA and the genomic DNA of *C. cellulovorans* as a template. The miniCbpA DNA fragment was introduced into the *BamH*I-*Kpn*I section of the pET22b (+) vector, resulting in the pET22b (+) miniCbpA plasmid.

2.4. Expression and purification of recombinant proteins

Transformed cells were grown on LB medium agar plates containing ampicillin (50 μg/mL) as the selection factor. A single colony of *E. coli* was cultured in 2 mL LB broth supplemented with 50 μg/mL ampicillin, shaking at 170 rpm in a 37 °C incubator overnight. The pre-culture was then transferred to LB broth with 50 μg/mL ampicillin and further cultured under the same conditions. For the expression of the recombinant proteins, isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added to a final concentration of 1.67 μM at an $O.D_{600}$ of 0.6 and the cells were further cultured for 90 min at 28 °C to induce the T7 promoter. After IPTG induction, cells were collected by centrifugation at 5000 × *g* for 10 min and suspended in 10 mL of ice-cold lysis buffer (50 mM Na_2HPO_4, 400 mM NaCl and 10 mM imidazole, pH 8.0). After cell disruption by sonication, the sample was centrifuged at 10,000 × *g* for 30 min. The supernatant of the crude cell extract was applied to the Ni-NTA column that bound the recombinant protein by its His-tag and the column was washed in wash buffer (50 mM NaH_2PO_4, 400 mM NaCl and 25 mM imidazole, pH 8.0). The bound

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− *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17 (rK−mK+), λ-	Invitrogen
Escherichia coli BL21 (DE3)	F–*ompT gal dcm lon hsd*S_B (r_B- m_B-) λ(DE3 [*lacI lac*UV5-T7 *gene 1 ind1 sam7 nin5*])	Invitrogen
Pseudoalteromonas carrageenovora	WT strain ATCC 43555	ATCC
Clostridium cellulovorans	WT strain ATCC 35296	ATCC
Plasmid		
pET22B(+)	$T7_p$-*pelB*-$T7_T$	Novagen
pET22B(+) cCgkA	$T7_p$-*pelB*-*CgkA*-*docB*-$T7_T$	This study
pET22B(+) cCglA	$T7_p$-*pelB*-*CglA*-*docB*-$T7_T$	This study
pET22B(+) miniCbpA	$T7_p$-*pelB*-*miniCbpA*-$T7_T$	This study

[a] ATCC, American Type Culture Collection.