Long-type ulvan lyase in *Alteromonas* sp. is a major enzyme involved in degradation of ulvan extracted from *Ulva ohnoi*



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ARTICLE INFO

Article history:
Received– 5 July, 2017
Revised– July 15, 2017
Accepted– 26 July, 2017
Available– 26 November, 2017
(online)

Keywords:
Ulvan lyase
Cloning
Alteromonas
Catalytic domain

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ABSTRACT

Ulvan is one of the key sulfated polysaccharide components constituting the cell walls of green algae, *Ulva*. A little study has been performed so far on the ulvan-degrading bacterial enzymes. In order to utilize ulvan effectively by breaking down to oligosaccharides and monosaccharide, we have studied ulvan-degrading bacterial enzymes. In this study, we firstly isolated a highly ulvan-degrading bacterium, *Alteromonas* sp. from feces of small marine animals. We identified an ulvan lyase with ca. 55 kDa in size from the secreted proteins of the *Alteromonas* sp. strain and cloned the gene for it. The predicted molecular weight of the long-type ulvan lyases is 110 kDa. The catalytic domain is located on the N-terminal half and the C-terminus was turned out to be deleted. Although the strain contains two ulvan lyases (long and short, It can be concluded that long ulvan lyase would be the major lyase for ulvan degradation.

1. Introduction

Among the three main divisions of macroalgae (red, brown, and green algae), green algae remain largely unexploited. In the past decade, marine eutrophication has promoted the proliferation of algal biomasses of *Ulva* sp. This is associated with an increase in human activity in coastal areas, which causes an increase in some nutrients in the aquatic environment, leading to growth of green algae, commonly known as algal blooms or green tide (Smetacek and Zingone 2013). As a consequence of the strong environmental impact of these green seaweeds, the interest in exploiting such natural resource has increased significantly, especially for its long chain polymer part, ulvan (Bobin-Dubigeon et al. 1997).

The main constituents of ulvan are 3-sulfated rhamnose (Rha3S), D-glucuronic acid (GlcA), L-iduronic acid (IdoA), and D-xylose (Xyl) (Lahaye and Robic 2007). The repetition moieties are disaccharides composed of Rha3S linked with GlcA or IdoA, or xylose in lower amounts. The physicochemical and biological features of ulvan have many potential applications in food, pharmaceutical, agricultural, and chemical industries (Lahaye and Robic 2007).

Polymers can be enzymatically broken down into fermentable oligomers. Discovery of new enzymes for ulvan degradation becomes a key step for better managing and utilizing this resource. Not many ulvan-degrading enzymes have been isolated. The first ulvan lyase activity is found in a marine bacterium in 1997 (Lahaye et al. 1997). A marine bacterium *Persicivirga ulvanivorans* sp. nov. (re-classified as *Nonlabens ulvanivorans*) PLR degrades ulvan (Barbeyron et al. 2011), and two ulvan lyases are purified from the culture supernatant of *N. ulvanivorans* (Nyvall Collén et al. 2011). Two ulvan lyases are 74% identical (Kopel et al. 2014). This is the first enzyme biochemically characterized. *N. ulvanivorans* ulvan lyase cleaves glycosidic bond between Rha3S and GlcA or IdoA via the β-elimination mechanism. The β-eliminative cleavage results in the formation of an unsaturated uronic acid (Δ, 4-deoxy-L-threohex-4-

enopyranosiduronic acid) on the non-reducing end. Recently two types, long and short, of ulvan lyase genes are identified from three *Alteromonadales* ulvan-degrading bacteria (Kopel et al. 2016). Three strains contain very similar short ulvan lyases. The long-type ulvan lyase is composed of two modules, catalytic module, which is similar to short-type ulvan lyase, and the C-terminal module. Both short- and long-types of ulvan lyases exhibit enzyme activity when expressed in *Escherichia coli*. Ulvan lyases of *N. ulvanivorans* and three *Alteromonadales* bacteria show no sequence similarity. We have isolated several ulvan-utilizing bacteria, which also contain two types of ulvan lyases.

The objective of the present study is to isolate ulval-degrading potential bacterial enzyme and to elucidate which type of bacterial ulvan lyase mainly works in the oligomerization of ulvan.

2. Materials and methods

2.1. Extraction of ulvan

Green alga *Ulva ohnoi* (Hiraoka et al. 2004) was collected at Uranouchi Bay, Tosa, Kochi, Japan. The washed and air-dried seaweeds were ground into fine pieces using a blender. Freshly ground seaweeds were soaked in 75% ethanol for 1 day and recovered by filtration with a 60 μm nylon mesh. Ulvan was extracted using hot water (Robic et al. 2008).

2.2. Screening and isolation of ulvan-utilizing bacteria

Mollusks and shrimps were fed with *U. ohnoi* in the laboratory using artificial seawater. Some of the small marine animals ate seaweed, and feces were used as sources of ulvan-utilizing bacteria. Feces were inoculated into half strength marine broth (1/2 MB, 18.7 g Difco Marine broth 2216 and 9.8 g NaCl in 1000 ml DW) containing 0.2 % ulvan and incubated at 25 °C overnight. Cell suspension was spread on ulvan agar media (0.2 % ulvan in three-quarter strength artificial seawater with 1.5% agar) and incubated 25 °C several days. Colonies were replicated onto both 1/2 MB agar and ulvan agar media. After cells grew, the ulvan agar media were flooded with 5% cetylpyridinium chloride (CPC). While ulvan reacted with CPC formed insoluble precipitates, clear haloes were visible when bacterial cells degraded ulvan to oligosaccharides. The bigger the halo is, the higher degradation activity cells shows (Ruijssenaars and Hartmans 2001).

2.3. Purification of crude ulvan-degrading enzymes

Ulvan-utilizing bacterial cells were grown in 4 l of fresh 1/2 MB containing 0.05% ulvan at 25 °C for 3 days. Bacterial culture supernatant was precipitated with 70% ammonium sulfate to extract secreted proteins. Proteins were loaded onto 10 ml of Q Sepharose Fast Flow (GE Healthcare) equilibrated with 20 mM Tris-HCl buffer (pH 8.0), and bound proteins were eluted with a 100-ml linear gradient of NaCl (0-0.5 M) in the same buffer. Proteins in each fraction were analyzed on SDS-PAGE, and active fractions containing ulvan-degrading enzymes were identified using ulvan-gel overlay assay indicated in section 2.4.

Active fractions were pooled, and ammonium sulfate was added at a final concentration of 2 M. This solution was loaded onto 10 ml of Phenyl Sepharose 6 Fast Flow (GE Healthcare) equilibrated with the buffer (20 mM Tris-HCl (pH 8.0) and 2 M ammonium sulfate). Bound proteins were eluted with a 100-ml linear gradient of ammonium sulfate (2-0 M) in 20 mM Tris-HCl (pH 8.0).

2.4. Activity staining overlaid on ulvan-containing agarose gel

We adopted the protocol for alginate lyase (Sawabe et al. 2001) with the following modifications. Proteins were separated on SDS-PAGE, and enzymes were renatured by incubating the gel three times in 100-ml of 20 mM Tris-HCl (pH 8.0) for 10 min. The polyacrylamide gel was overlaid onto an agarose gel (0.2 % ulvan in three-quarter strength artificial seawater with 1.0% agar) and incubated overnight at 30 °C. The agarose gel was immersed into 5% CPC, and ulvan-degrading activities were visualized as clear bands.

2.5. Assay for ulvan-degrading enzyme

The enzyme activity of ulvan lyase was measured as mentioned previously (He and Ohnishi 2017).

2.6. Cloning of ulvan lyase genes

Full length of long-type ulvan lyase gene (*ullA*) of KUL42 strain was PCR amplified with primers of 17ullAA1 and 42ullAB1 from chromosomal DNA of KUL42 using PrimeSTAR HS DNA polymerase (Takara Bio). C-terminal truncated versions of $ullA_{KUL42}$ were PCR amplified with primer pairs of 17ullAA2 and 42ullAB2 and 17ullAA2 and 42ullAB3. All the PCR products were cloned into pET21a using In-Fusion HD Cloning Kit (Takara Bio). Primer sequences and constructed plasmids were listed in supplementary Table 1. The nucleotide sequences of $ullA_{KUL42}$ have been deposited in DDBJ under accession number LC278383.

2.7. Purification of ulvan lyases

E. coli BL21(DE3) was transformed with plasmids and ulvan lyases were over expressed in the transformant cells by adding 1 mM IPTG as previously described (Yoshimochi et al. 2009). After IPTG addition, cells were incubated at 20 °C for 4 hr. Cells were harvested and disrupted by sonication. After removing cell debris, the supernatant was used for Ni^{2+} affinity column (Ni-NTA Agarose, Qiagen) purification.

3. Results and discussion

3.1. Isolation of ulvan-utilizing bacteria

We used feces of Ulva-eating small animals for screening ulvan-utilizing bacteria in this study. Plate assay for ulvan-degrading activity was in analogy with assay for alginate lyase activity (Sawabe et al. 2001). Ulvan-degrading activity was diverse judging from the size of halos among the isolated bacteria (Fig. 1). One strain KUL42 from shrimp

was isolated based on fastest growing and highest enzyme activity. Partial nucleotide sequences of 16S rRNA genes indicated that KUL42 strain belongs to *Alteromonas* sp.

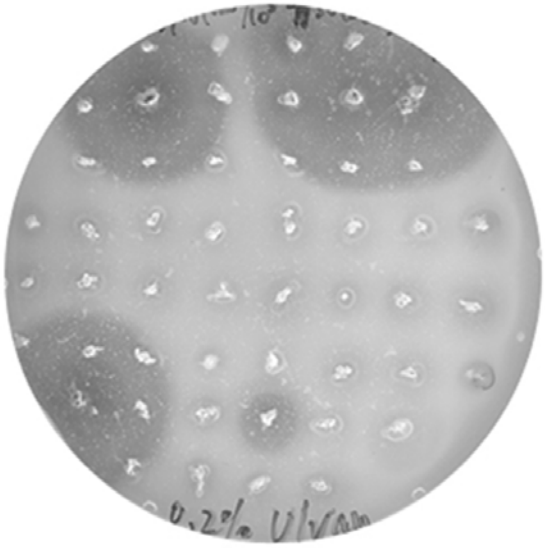


Fig. 1 Plate assay for ulvan-degrading activity. Bacterial strains were streaked on ulvan-containing agar media in artificial seawater. After cells grew, the plates were immersed by CPC to form clear halos

3.2. Identification of ulvan-degrading enzymes

Firstly, we tested if ulvan-degrading enzymes were inducible. KUL42 cells were cultivated in 1/2 MB with various concentration of ulvan. While almost no ulvan-degrading enzymes were detected without ulvan in the media, at least three bands were visible as proteins with enzyme activity in the presence of even 0.01% ulvan (Fig. 2). We used 0.05% ulvan for enzyme induction throughout this study.

Secreted proteins from KUL42 grown in 1/2 MB with 0.05% ulvan were precipitated with ammonium sulfate and subjected to two-steps of column chromatographies, strong anion exchange and hydrophobic interaction chromatographies, to partially purify ulvan-degrading enzymes. Although multiple active bands were observed in the secreted fractions (Fig. 2), we focused on the most active ca. 55 kDa protein in this study. After the second step of chromatography, fractions were analyzed on SDS-PAGE (Fig. 3A). At the same time, ulvan-degrading activity was assayed on ulvan-containing gel (Fig. 3B). The candidate of ulvan-degrading enzyme was shown by triangle.

Fractions from 30 to 42 of KUL42 proteins were also pooled. The N-terminal sequence of the KUL42 protein indicated by triangle (Fig. 3A) was "XVTLXQQVKI" and matched to the internal sequence of a fibronectin type III domain protein based on the draft genome sequence analysis of KUL42. This is 92% identical to a long ulvan lyase in *Alteromonas* sp. PLSV (Kopel et al. 2016). From this result, we identify the protein as the gene product of *ullA* (ulvan lyase A).

3.3. Cloning of *ullA* gene and expression in *E. coli*

Domain structure of KUL42 UllA ($UllA_{KUL42}$) is shown in Fig. 4A. It is composed of 999 amino acids. A 21 amino-acid signal peptide is predicted. Indeed, the determined N-terminal sequences of secreted UllAs matched to the sequences after cleavage sites. The catalytic domain is located on the N-terminal half and multiple repeats and dockerin domain are located on the C-terminal half.

We cloned the full length, but lacking the signal peptide, of *ullA* with His-tag on the N-terminus on the expression vector, pET21a, to construct p42ullA1 (Fig. 4A). While the full length of UllA was active (Fig. 4C), it was degraded very much (Fig. 4B, lane 2). Since His-tag was fused to N-terminus, degradation could occur on the C-terminus. Degraded UllA showed activity until they reached to the size of natural UllA secreted from original strains (Fig. 4C, lane 1 vs. 2). Judging from the size of secreted natural UllA, almost all the repeats and dockerin domain should be deleted. Several C-terminal truncated versions of UllA were designed (Fig. 4A). Truncated versions of UllA showed high enzyme activity (Fig. 4C, lanes 3 and 4). These results indicate that C-terminal half is dispensable for at least ulvan-degrading activity, which is consistent with data that the C-terminal half of the secreted natural UllA was also deleted.

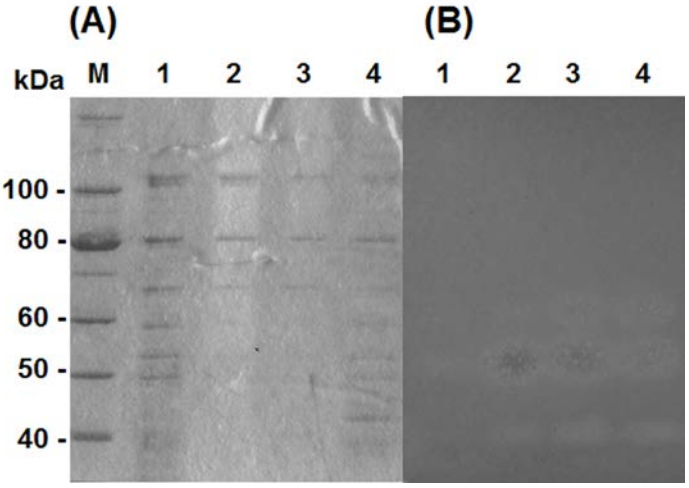


Fig. 2 Secreted proteins from KUL42. KUL42 cells were incubated in 1/2 MB media containing, lane 1; no ulvan, 2; 0.01% ulvan, 3; 0.05% ulvan, 4; 0.1% ulvan, at 25 °C for two days. Cell culture supernatant was prepared and concentrated for 10% SDS-PAGE. (A) Separated proteins were stained with CBB R-250. M indicated molecular weight marker with size in kDa on the left. (B) Activity staining using ulvan-containing agarose gel

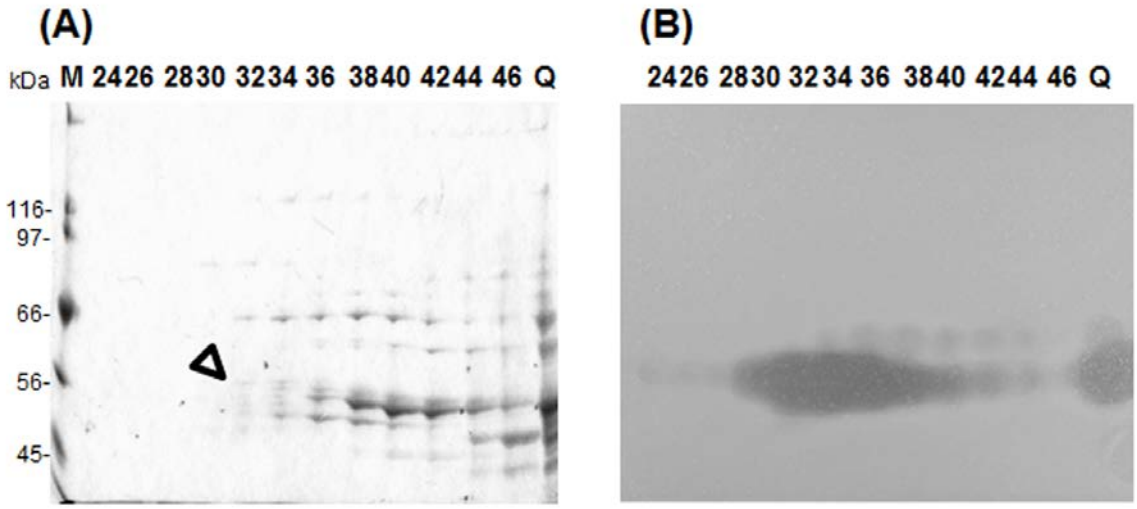


Fig. 3 SDS-PAGE analysis of proteins separated by phenyl sepharose chromatography. Active fractions after Q sepharose chromatographies of KUL42-secreted proteins were loaded on phenyl sepharose columns. Fractions were analyzed on 10% SDS-PAGE and stained by (A) CBB R-250and (B) activity staining using ulvan gel. Fraction numbers are shown on the top of each gel. Q indicates pooled fractions after Q sepharose. M is molecular weight markers. Molecular weights are shown on the left in kDa. Open triangle indicates the candidate of ulvan-degrading enzyme

3.4. Characterization of enzymes

The purified full length of UllA, $UllA1_{KUL42}$, was mixed with ulvan solution. When ulvan is degraded by an elimination reaction, a double bond is generated between C4 and C5 of the sugar ring at the newly formed non-reducing end, which can be monitored as a change in absorbance at 235 nm (Preiss and Ashwell 1962). As expected, the absorbance at 235 nm increased (data not shown), clearly demonstrating that both UllAs are polysaccharide lyases, not glycoside hydrolases. Since the full length of UllA was unstable, we used the truncated version UllA3 for further analysis and calculated the kinetic parameters by measuring the amount of newly formed reducing ends from ulvan reacted with $UllA3_{KUL42}$. The values of V_{max} and K_m for $UllA3_{KUL42}$ were 212 μmol min^{-1} mg $protein^{-1}$ and 0.83 % (w/v).

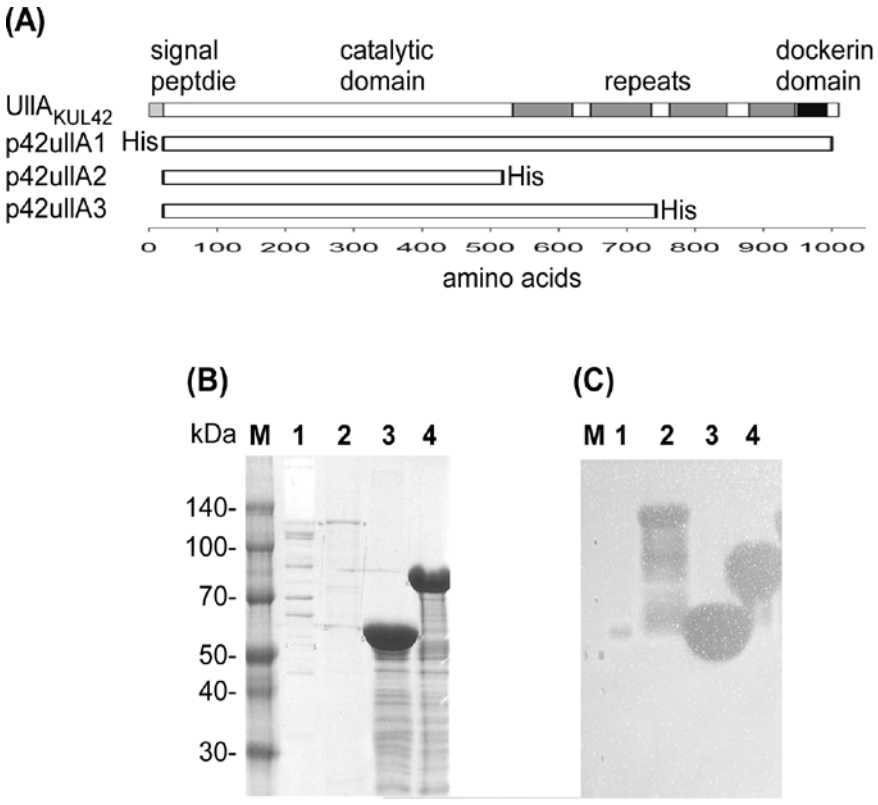


Fig. 4 Domain structure and purification of UllA. Domain structure of UllA from KUL42 is indicated in (A). Search for signal peptide, repeat sequence, and conserved domain is conducted with SignalP 4.1 Server ((Petersen et al. 2001), http://www.cbs.dtu.dk/ services/ SignalP/), RADAR ((Heger and Holm 2000), http://www.ebi.ac. uk/Tools /pfa/radar/), and Conserved Domain Search ((Marchler-Bauer and Bryant 2004), https://www.ncbi. nlm.nih.gov /Structure/cdd/wrpsb.cgi), respectively. Plasmids containing full length and C-terminal truncated version of *ullA*$_{KUL42}$ are listed in (A). All plasmids are lacking signal sequences. "His" indicates the addition of His-tag sequence. (B) CBB R-250 staining of 10% SDS-PAGE gel. Lane 1; secreted proteins from KUL42, lanes 2, 3, and 4; purified protein from BL21(DE3) containing p42ullA1, p42ullA2, and p42ullA3, lane M; molecular weight marker shown on the left in kDa. (C) Activity staining with ulvan-containing agarose gel. Lanes are same as in (B)

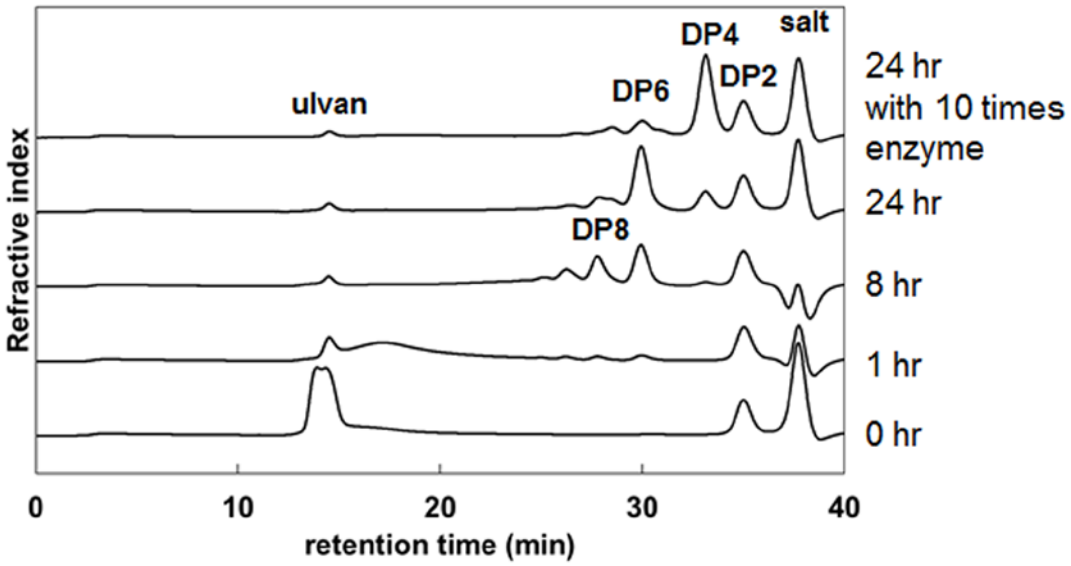


Fig. 5 Analysis of reaction products of ulvan by UllA3. 0.4% (w/v) ulvan was mixed with 0.2 μg of purified $UllA3_{KUL42}$ in the buffer (20 mM Tris-HCl (pH 8.0) and 500 mM NaCl) at 0 hr, 1 hr, 8 hr, and 24 hr. Enzyme reaction was stopped by boiling for 10 min and filtered. The products were analyzed on Superdex Peptide 10/300 GL column at flow rate of 0. 5 ml min^{-1}. Signal was detected by a RI detector. When 2 μg of the enzyme was used, chromatogram was indicated on the top. Ulvan showed the undigested ulvan and DP2, DP4, DP6, and DP8 were digested oligosaccharides

3.5. Identification of degradation products of ulvan

Ulvan was mixed with purified $UllA3_{KUL42}$ at different time and the reaction products were analyzed on size-exclusion chromatography. At the early reaction time, relatively large depolymerization products (DP) were generated. Products with lower molecular weight, such as DP6, DP4 and DP2, increased as time went on and most of the products were DP6 and DP4 at 24 hr in this reaction condition (Fig. 5). This indicated that UllA5 is an endo-ulvan lyase. When the higher amount of enzyme was used, almost all products became DP4.

4. Discussion

The long-type ulvan lyase was proved to be secreted from two different marine *Alteromonas* strains. While alginate can be imported across the cytoplasmic membrane using the ABC transporter (Maruyama et al. 2015), ulvan has never known to be incorporated. The ulvan lyase must be secreted to break down ulvan to oligosaccharides small enough to be imported with transporters. So far, 3 strains belonging to Alteromonadales order, *Alteromonas* sp. LTR, *Alteromonas* sp. LOR, and *Pseudoalteromonas* sp. PLSV, have been reported to contain two types of ulvan lyases (Kopel et al. 2016). We have isolated a ulvan-utilizing strain belonging to Alteromonadales order, KUL42, which also contains two types of ulvan lyases. When a long-type ulvan lyase is compared phylogenically, similarity among three enzymes from KUL42, LOR, and PLSV are more than 90%. In the secreted proteins from KUL42, we could not find short-type ulvan lyases. These data demonstrate that the long-type ulvan lyases are major ulvan lyases.

The long-type ulvan lyase is composed of two modules. The N-terminal half contains catalytic domain, which is similar to the short-type ulvan lyase (Kopel et al. 2016). We also demonstrated that the C-terminal truncated long ulvan lyase showed high enzyme activity. Although the full-size of the long-type ulvan lyase was also active, it was very much unstable, indicating that C-terminal half is a target for protein degradation. The size of secreted ulvan lyases are ca. 55 kDa, which exactly matches to the N-terminal catalytic domain. We do not have an answer to the question when the C-terminal is removed. The long-type ulvan lyase from *N. ulvanivorans* PLR, which is much smaller in 534 amino acids and has no similarity with Alteromonadales long ulvan lyases, is composed of two domains (Melcher et al. 2017). While the N-terminal contains catalytic domain, the C-terminus specifically binds to ulvan. The C-terminus of long-type ulvan lyase of KUL42 contains 3-4 repeats with ca. 100 amino acids each and dockerin domain. Although the function of repeats is unknown, ulvan could bind to these repeats. If this is the case, the long-type ulvan lyases are secreted with full-length and the C-terminus would be cleaved afterwards.

We detected at least three secreted proteins with ulvan-degrading activity from KUL42. One of them was long-type ulvan lyase. Since the activity staining with ulvan-containing agarose gel is based on precipitation of sulfated polysaccharide with CPC (Sawabe et al. 2001), cleavage of either the glycosidic linkages of the carbohydrate backbone or the sulfate ester groups can makes clear bands. Form the preliminary experimental data of N-terminal amino acid sequence analyses of many secreted protein form KUL42, one sulfatase was detected in the secreted proteins. This could be the protein with ulvan-degrading activity. κ-Carrageenan is one of the sulfated polysaccharide from red algae (Whyte et al. 1985). A carrageenan-sulfatase isolated from *Pseudoalteromonas atlantica* catalyzes desulfation on polymers instead of oligosaccharides as endo -acting enzyme (Préchoux et al . 2016). Secreted sulfatase could remove

sulfate group on polymer ulvan. Recently, the third ulvan lyase is identified from *Pseudoalteromonas* sp. PLSV (Ulaganathan et al. 2017). We have found a homologous gene in KUL42 (unpublished data), the third ulvan lyase could be detected in activity staining.

5. Conclusions

We have isolated an ulvan-utilizing *Alteromonas* strain of bactera. This strain secreted several proteins when the substrate ulvan was present. One of them was the ulvan lyase. Cloning the gene encoding the enzyme clearly indicated that the secreted enzyme is the long-type ulvan lyase, which is conserved among ulvan-utilizing *Alteromonas* strains all over the world. In further study, we need to elucidate the function of small-type ulvan lyase, which was not detected in the secreted proteins.

Acknowledgements

We thank Dr. Masanori Hiraoka for useful information about green algae *Ulva* sp. This work was supported in part by The Kochi University President's Discretionary Grant.

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