Journal of Bioscience and Bioengineering
VOL. 124 No. 3, 277–282, 2017

www.elsevier.com/locate/jbiosc

Gene identification and characterization of fucoidan deacetylase for potential application to fucoidan degradation and diversification

Tatsuhiko Nagao,[1,2] Ayako Kumabe,[1] Fumika Komatsu,[1] Hisashi Yagi,[3] Hirokazu Suzuki,[1] and Takashi Ohshiro[1,*]

Department of Chemistry and Biotechnology, Graduate School of Engineering, Tottori University, Tottori 680-8552, Japan,[1] FUKAEKASEI Co., Ltd., 2-2-7 Murotani, Nishi-ku, Kobe 651-2241, Japan,[2] and Center for Research on Green Sustainable Chemistry, Faculty of Engineering, Tottori University, Tottori 680-8552, Japan[3]

Received 30 January 2017; accepted 3 April 2017
Available online 22 April 2017

Fucoidan is an α-L-fucopyranosyl polymer found in seaweeds with forms that have acetyl and sulfuric modifications and derivatives that are lower and/or diversified, with modifications that have attracted interest as potential bioactive substances. We identified the gene for a fucoidan deacetylase that cleaves acetyl moieties from fucoidan and thereby contributes to fucoidan utilization in the marine bacterium *Luteolibacter algae* H18. Fucoidan deacetylase was purified to homogeneity from a cell-free extract of *L. algae* H18, and used to determine the internal amino acid sequence and identify the gene, *fud*, in a draft genome sequence of the H18 strain. The gene product was heterologously produced in *Escherichia coli* and was demonstrated to catalyze fucoidan deacetylation, but not desulfation, and degradation into lower forms. In addition to fucoidan deacetylation, the enzyme catalyzed the hydrolysis of *p*-nitrophenyl esters with organic acids, and *p*-nitrophenyl acetate was the best substrate among those tested. The present study provides a new tool for fucoidan degradation, potentially expanding investigations on fucoidan derivatives.

[**Keywords:** Fucoidan; Deacetylase; *Luteolibacter*; *Cladosiphon okamuranus* (Okinawamozuku); Esterase]

Brown algae produce unique polysaccharides, such as alginic acid, laminarin, and fucoidan. Many of these substances have been shown to possess a variety of useful biological functions, and have attracted considerable attention for their potential as ingredients in functional and healthy foods. Of these polysaccharides, fucoidans are hetero-sulfated polysaccharides with several potentially useful biophysical properties, including anticoagulant, antiviral, and antitumoral activities (1–3). While the structure of fucoidan in brown algae is species dependent (4), individual algal species may produce multiple fucoidans with different structures. Consequently, the relationship between the structure and function of fucoidans has not been investigated in detail. Although fucoidans are originally complexed macromolecule materials, the physiological properties of low-molecular weight compounds derived from fucoidans have been examined in some detail. Consequently, enzymes that act on fucoidans are considered to be useful tools for investigating the structural characteristics of this polysaccharide.

Several reports on the bacteria and enzymes involved in fucoidan degradation have been reported in the literature. The research group of Kloareg isolated the bacterium, *Flavobacteriaceae* SW5 strain (5), degrading fucoidan from *Pelvetia canaliculata*, purified the enzyme involved in fucoidan degradation, and cloned the corresponding gene, *fcnA* (6). Sakai et al. (7–9) investigated fucoidan-degrading enzymes from the three marine bacterial strains, *Fucobacter marina* SA-0082, *Fucophilus fucoidanolyticus* SI-1234, and *Alteromonas* sp. SN-1009. Moreover, *Sphingomonas paucimobilis* PF-1 and *Formosa algae* were also reported to produce intracellular enzymes involved in the degradation of fucoidan from *Undaria pinnatifida* and *Fucus evanescens*; these enzymes were purified and characterized (10–12). We isolated *Flavobacterium* sp. F31 (13) and *Luteolibacter algae* H18 (14), both of which are capable of degrading fucoidan from *Cladosiphon okamuranus* using intracellular enzymes. Furthermore, we found that the enzymatic deacetylation of fucoidan in strain H18 occurred before the reactions responsible for decreasing the molecular weight of fucoidan due to degradation (14). To our knowledge, the findings presented in this study are the first to demonstrate that multiple enzymes are involved in fucoidan degradation.

We purified an enzyme capable of catalyzing the deacetylation of fucoidan from cell-free extracts of strain H18, determined the amino acid sequences of the enzyme, and identified the corresponding gene by comparing the sequence of the gene against the genome of strain H18. We also expressed the gene in *E. coli* and confirmed the deacetylation function of the gene product.

MATERIALS AND METHODS

Purification of a deacetylase from *L. algae* H18 The high-molecular-weight fucoidan (SEA ALGA-F, Marine Products Kimuraya, Co., Ltd., Sakaiminato, Japan) used in this study was extracted and purified from *C. okamuranus* by the method of Kawamoto et al. (15). *L. algae* strain H18 was grown in medium F, a synthetic medium containing fucoidan as the sole carbon source at 30 °C for 48 h with reciprocal shaking at 100 strokes per min, and preparation of cell-free extracts was carried out as previously reported (13). The cell-free extracts prepared from

* Corresponding author. Tel./fax: +81 857 31 5269.
E-mail address: ohshiro@bio.tottori-u.ac.jp (T. Ohshiro).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2017.04.002

118 L culture broth were thoroughly dialyzed against 20 mM Tris–HCl buffer (pH 8.0), and applied to a DEAE-Sepharose (GE Healthcare, Little Chalfont, UK) column (6 × 40 cm) equilibrated with the same buffer. The chromatographic procedure was the same as previously reported (14). Fractions eluted with buffer containing 0.1 M NaCl were used as the partially purified fucoidan-degrading enzyme, and this preparation showed no deacetylase activity for fucoidan. To purify the deacetylase, fractions eluted with buffer containing 0.2 M NaCl were dialyzed against 20 mM Tris–HCl buffer (pH 8.0) with 2 M $(NH_4)_2SO_4$, and the resulting precipitant was removed by centrifugation (12,000 ×*g*, 30 min). The supernatant was applied to a Phenyl-Toyopearl (Tosoh, Tokyo, Japan) column (3 × 50 cm) equilibrated with the same buffer. The column was washed well with the same buffer, and the adsorbed proteins were eluted successively with the same buffer containing 1.5, 1, and 0.5 M $(NH_4)_2SO_4$ at a flow rate of 90 ml/h. Fractions eluted with the buffer containing 1 M $(NH_4)_2SO_4$ were dialyzed against 20 mM Tris–HCl buffer (pH 8.0) and applied to a Q-Sepharose (GE Healthcare) column (2.7 × 12 cm) equilibrated with the same buffer. After washing with the same buffer, the proteins were eluted successively with the same buffer containing 0.1, 0.2, and 0.3 M NaCl at a flow rate of 120 ml/h. Fractions eluted with the buffer containing 0.2 M NaCl were then dialyzed against 20 mM Tris–HCl buffer (pH 8.0) with 2 M $(NH_4)_2SO_4$ and applied to a Butyl-Toyopearl (Tosoh) column (1 × 8 cm) equilibrated with the same buffer. The column was washed well with the same buffer and the adsorbed proteins were eluted successively with the same buffer containing 1.5 and 1 M $(NH_4)_2SO_4$ at a flow rate of 18 ml/h. Fractions eluted with the buffer containing 1 M $(NH_4)_2SO_4$ were concentrated and desalted by ultrafiltration and applied to a Mono Q 10/10 (GE Healthcare) column equilibrated with the same buffer. Chromatography was performed using a linear gradient of 0–0.5 M NaCl in the buffer with the flow rate of 0.3 ml/min controlled by an ÄKTA system (GE Healthcare). The active fractions were combined and concentrated by ultrafiltration. The enzyme solution was applied to a Superose HR 10/30 (GE Healthcare) equilibrated with the same buffer. Chromatography was performed at a flow rate of 0.2 ml/min and controlled by an ÄKTA system. The active fractions were pooled and concentrated by ultrafiltration.

Enzyme assay The enzyme reaction mixture contained 0.25% fucoidan, 100 mM Tris–HCl buffer (pH 8.0), and the enzyme, typically in a total volume of 600 µL. The reaction mixture was incubated at 30 °C, and heated at 80 °C for 5 min to stop the enzyme reaction. After centrifugation to remove the precipitant (12,000 ×*g*, 5 min), the activity of fucoidan deacetylation was assayed using an Acetic Acid Test Kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's recommendations and measuring the rate of increase in absorbance at 340 nm. Ion chromatography was also performed to determine the amounts of acetate, as reported previously (14). The average molecular weight of fucoidan was estimated using an HPLC equipped with an RID-10A refractive index detector (Shimadzu, Kyoto, Japan) and a size-exclusion column TSKgel $GMPW_{XL}$ (7.8 mm × 300 mm, Tosoh) as described previously (14). Another enzyme assay was performed using *p*-nitrophenyl esters as substrates. The reaction mixture contained 1 mM *p*-nitrophenyl ester, 100 mM Tris–HCl buffer (pH 7.0), and the enzyme. After incubation for 30 min at 30 °C, the activity was determined by measuring the absorbance of *p*-nitrophenol formed at 410 nm. In this case, one unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol ($\varepsilon_{410} = 18{,}500\ M^{-1}\ cm^{-1}$) per min.

Complete enzymatic deacetylation of fucoidan was performed in a reaction mixture containing 0.25% fucoidan, 0.05 mg/ml recombinant enzyme, and 100 mM Tris–HCl buffer (pH 7.0) at 30 °C for 48 h. The amounts of acetate and sulfate liberated were determined by ion chromatography. Determination of sulfate in fucoidan was performed by measuring the optical density due to the precipitation of barium sulfate as previously described (13) after the hydrolysis of fucoidan with hydrochloric acid. Protein concentrations were measured by the method of Bradford (16) using bovine serum albumin as the standard.

SDS-PAGE and internal amino acid sequences SDS-PAGE was carried out by the method of Laemmli (17) employing a 12.5% gel for separation. Protein bands were visualized by staining with Coomassie Brilliant Blue G-250 dissolved in 50% methanol-10% acetic acid, and destained in 30% methanol-10% acetic acid. Calibration proteins for SDS-PAGE were obtained from GE Healthcare. The purified enzyme was digested with V8 protease for 24 h at 37 °C. The digested and purified enzyme samples were separated by Tricine SDS-PAGE and transferred to a PVDF membrane. After staining the membranes with Coomassie Brilliant Blue and excising the objective bands, the pieces of membranes were subjected to protein sequence analysis (PPSQ-31A Protein Sequencer, Shimadzu).

Cloning and expression of the deacetylase gene The whole nucleotide sequence of the draft genome of *L. algae* H18 was determined (HiSeq 2500, Illumina Inc., CA, USA). Amino acid sequences obtained from the purified enzyme were assigned to coding regions on the draft genome sequence of *L. algae* H18 using the in silico molecular cloning software package (In Silico Biology, Inc., Yokohama, Japan). The objective open reading frame, *fud*, that codes the amino acid sequences was amplified and ligated to the expression vector, pCold TF (Takara Bio, Kyoto, Japan). For PCR amplification, the sequences of the forward and reverse primers were CCCTGG**CATATG**AAAATCCTCAAAATC (the *Nde*I restriction site is shown in bold and the ATG initiation codon is underlined) and AACTCT**GTCGAC**TTTGGCGATCCACCT (the *Sal*I restriction site is shown in bold), respectively. PCR was performed with PrimeSTAR Max DNA polymerase (Takara Bio), and the PCR mixture was heated at 94 °C for 2 min and then subjected to 30 cycles of amplification (98 °C for 10 s, 56 °C for 5 s, and 72 °C for 3 min). After the amplified fragment and plasmid vector pCold TF were digested by *Nde*I (Takara Bio) and *Sal*I (Takara Bio), they were ligated to construct pCold TF/*fud*. The resultant plasmid was introduced into *E. coli* BL21(DE3).

The recombinant *E. coli* strain was cultivated in a 2-L Sakaguchi flask containing 1-L of LB medium with 100 µg/ml ampicillin. The medium was agitated at 160 strokes per min for 5 h at 37 °C before being cooled to 15 °C. After the addition of 1 mM IPTG, the flasks were incubated at 15 °C for 24 h. The cells were then harvested by centrifugation (10,000 ×*g*, 30 min), suspended in 20 mM Tris–HCl buffer (pH 7.0), and disrupted with an ultrasonic oscillator at 20 kHz (Sonifier 450, Branson Instruments, Danbury, CT, USA). After the centrifugation (15,000 ×*g*, 30 min), the supernatant was analyzed by SDS-PAGE to determine whether gene expression had occurred.

Purification of deacetylase from the recombinant *E. coli* strain Cell-free extracts were prepared from 3.3 L culture broth, which was dialyzed against 50 mM Tris–HCl buffer (pH 7.0) containing 500 mM NaCl and 20 mM imidazole. The enzyme solution was applied to a Ni-Sepharose 6 Fast Flow column (GE Healthcare, 1.2 × 15 cm) equilibrated with the same buffer. The unbound proteins were thoroughly washed with the same buffer at a flow rate of 150 ml/h, and the objective protein was eluted with 50 mM Tris–HCl buffer (pH 7.0) containing 500 mM NaCl and 50 mM imidazole. The eluted fractions were combined, dialyzed against 20 mM Tris–HCl buffer (pH 7.0), and concentrated by ultrafiltration. The concentrated enzyme sample (26 mg-protein) was digested with HRV 3C protease (260 units, Takara Bio) for 24 h at 4 °C to cleave the trigger factor, and then applied to a Ni-Sepharose 6 Fast Flow column (1.2 × 5 cm) equilibrated with 50 mM Tris–HCl buffer (pH 7.0) containing 500 mM NaCl and 20 mM imidazole. The column was washed with the same buffer and the unbound fractions with objective proteins were collected and concentrated by ultrafiltration and desalting.

RESULTS

Enzyme purification from the wild strain and *N*-terminal and internal amino acid sequences The enzyme that catalyzed the deacetylation of fucoidan from *C. okamuranus* was purified by column chromatography of cell-free extracts of *L. algae* H18. The activity of the enzyme was estimated by measuring the amounts of acetate liberated from fucoidan with an Acetic Acid Test Kit during enzyme purification. SDS-PAGE showed that the enzyme was purified to almost homogeneity and that the molecular mass of the subunit was approximately 70 kDa (Fig. 1). Although the N-terminal amino acid sequence could not be determined, two internal amino acid sequences, V(L or S)SAEPN? MAIGL and LQLED(G or E)?T were determined after digesting the purified enzyme with V8 protease. When these two amino acid

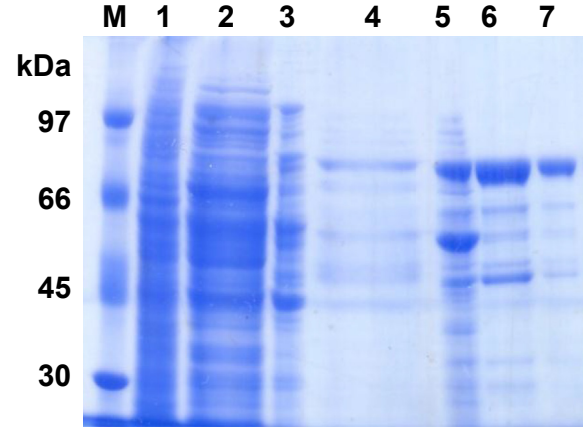

FIG. 1. SDS-PAGE of fucoidan deacetylase from *L. algae* H18. Lane M, marker proteins; lane 1, cell-free extracts (6.3 µg); lane 2, pooled fraction after DEAE-Sepharose chromatography (7.3 µg); lane 3, pooled fraction after Phenyl-Toyopearl chromatography (11 µg); lane 4, pooled fraction after Q-Sepharose chromatography (2.4 µg); lane 5, pooled fraction after Butyl-Toyopearl chromatography (7.6 µg); lane 6, pooled fraction after Mono-Q chromatography (2.9 µg); lane 7, pooled fraction after Superose chromatography (1.2 µg).