Abstract: Chitin oligosaccharide deacetylase (COD) is an enzyme that generates β-N-acetyl-D-glucosaminyl-(1,4)-D-glucosamine from N,N′-diacetylchitobiose. COD has been found only in species of *Vibrio* bacteria. However, a homology search in the sequence databases revealed that *Shewanella woodyi* ATCC51908 also encodes a protein with sequence similarity to COD. Analysis of the deduced amino acid sequence of the *S. woodyi* COD (Sw-COD) confirmed that the protein contains the same motifs as CODs from *Vibrio* bacteria. The COD-encoding gene, which includes a signal sequence, was cloned from the chromosomal DNA of *S. woodyi*, the expression plasmid containing this gene was constructed, and then the plasmid was introduced into *Escherichia coli* HMS174(DE3) cells. The recombinant Sw-COD (Sw-rCOD) was produced in culture medium with the aid of the signal peptide and purified from culture supernatant. The properties of Sw-rCOD (substrate specificity, optimal pH, etc.) were similar to those of the CODs from *Vibrio* bacteria.

Keywords: Chitin oligosaccharide deacetylase, *Shewanella woodyi*, recombinant protein, enzymological properties

Chitin oligosaccharide deacetylase (COD, EC 3.5.1.105) is an enzyme that hydrolyzes the acetamide bond of the second N-acetyl-D-glucosamine (GlcNAc) residue from the non-reducing end of chitin oligosaccharides. Historically, CODs have been identified and characterized from the following species of *Vibrio* bacteria: *Vibrio algolyticus* H-8, *Vibrio cholerae* El Tor N16961, *Vibrio parahaemolyticus* KN1699, *Vibrio sp.* SN184, and *Vibrio harveyi* ATCC BAA-1116. Each of these enzymes show highest activity against the N,N′-diacetylchitobiose [(GlcNAc)2] in chitin oligosaccharides. Previously, we reported that the β-N-acetyl-D-glucosaminyl-(1,4)-D-glucosamine (GlcNAc-GlcN), which is generated from (GlcNAc)2 by COD, functions in chitin deacetylase. Previously, we reported only in members of the genus *Vibrio* active site zinc ion are conserved in PDD region of CODs. Moreover, each of the two CBDs contain six aromat-
each of the bacterial COD homologs, including the *S. woodyi* protein (Fig. 1). Thus, the predicted protein from *S. woodyi* harbors all of the structural elements associated with COD activity in the *Vibrio* COD enzymes. These facts allow us to think that this protein of *S. woodyi* is obviously COD. This bacterium scarcely produced COD (data not shown). We therefore investigated the functional properties of *S. woodyi* COD in comparison to those of the *Vibrio* homologs using recombinant *S. woodyi* COD isolated following overproduction in *Escherichia coli* host cells.

*S. woodyi* ATCC51908(10) was obtained from the American Type Culture Collection (Manassas, USA). Bacteria belonging to the genus *Shewanella* were found in areas of seawater and freshwater.(11) The strain we used was isolated from deep sea.(10) Strain ATCC51908 harbors several chitinase genes, suggesting that this strain is bacterium that utilizes chitin as a nutrient source.

After the strain ATCC51908 cells were grown for 16 h at 28°C on an agar plate containing 1.87% (w/v) Marine broth medium supplemented with 50 µg/mL ampicillin. After harvesting the *E. coli* cells by centrifugation (3,300 × g for 3 min at 4°C), plasmid was isolated from the cells using a Miniprep kit (Qiagen). The target gene was excised from the plasmid using EcoRI (Toyobo Co., Ltd., Osaka, Japan) and ligated into pET-SwCOD and one resulting transformant was used for the production of Sw-rCOD. Nucleotide sequence analysis of the target gene in PET-SwCOD was performed using a Big Dye Terminator V3.1 Cycle Sequencing kit and an ABI 3130xl DNA sequencer (Applied Biosystems, Santa Clara, USA).

*E. coli* HMS174(DE3) cells were transformed with PET-SwCOD and one resulting transformant was used for the production of Sw-rCOD. The transformant cells were cultivated with shaking (160 rpm) at 30°C in 1 L of LB medium supplemented with 50 µg/mL ampicillin until the

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### Supporting Information

Fig. 1. Alignment of amino acid sequences of a putative COD from *Shewanella woodyi* ATCC51908 and CODs from several *Vibrio* strains.

These sequences correspond to the putative or known CODs from the following bacterial strains: 1. *Shewanella woodyi* ATCC51908; 2. *Vibrio alginolyticus* H-8 (GenBank Protein ID BAB21759); 3. *Vibrio cholerae* El Tor N16961 (GenBank Protein ID AAF94439); 4. *Vibrio paradueaeolyticus* KN1699 (GenBank Protein ID BAG70715); 5. *Fibrobacter sp.* SN184 (GenBank Protein ID BAG82921); and 6. *Fibrobacter harveyi* ATCC BAA-1116. Amino acids highlighted in black and gray are, respectively, identical and highly conserved (> 50%) among these proteins. Boxed N-terminal sequences represent signal peptides. Double-headed arrows indicate the extent of the carbohydrate-binding modules. Lower-case letters (a, b, c) with inverted triangles indicate structural elements as described in the text.
OD_{600} reached 0.45. Isopropyl-β-thiogalactopyranoside then was added into the culture broth at a final concentration of 0.5 mM and the culture was incubated for an additional 20 h under the same conditions. After removing the cells from 1 L of culture broth by centrifugation (3,000 × G for 10 min at 20ºC), (NH₄)₂SO₄ was added to the obtained culture supernatant to 80% saturation in order to precipitate the proteins. The resulting precipitate was collected by centrifugation (3,000 × G for 15 min at 4ºC), dissolved in a small amount of 20 mM sodium phosphate buffer (pH 7.0), and then dialyzed against the same buffer to yield the crude enzyme solution. This solution was loaded onto a DEAE-Sepharose Fast Flow resin (GE Healthcare, Buckinghamshire, England) column (size: ø2.5 × 15 cm) pre-equilibrated with the same buffer. Proteins were eluted from the column with a linear gradient of 0 to 0.8 M (NH₄)₂SO₄ in the same buffer (total volume: 400 mL). The eluate containing the protein that show COD activity was concentrated to 1 mL using an Amicon Ultra-15 Centrifugal Filter Device (Merck KGaA) and loaded onto a Bio-Gel P-100 Fine resin (Bio-Rad Laboratories) column (size: ø1.5 × 90 cm) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0). The eluate containing the protein that show COD activity was re-equilibrated to 20 mM sodium phosphate buffer (pH 7.0) containing 0.7 M (NH₄)₂SO₄ by ultrafiltration using a Stirred Cell Model 8400 equipped with Ultrafiltration Discs YM-10 (Merck KGaA). Target protein was further purified by column chromatography using a Phenyl Sepharose High Performance resin (GE Healthcare) column (size: ø1.5 × 8 cm) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 0.7 M (NH₄)₂SO₄. Proteins were eluted from the column with a linear gradient of 0.7 to 0 M (NH₄)₂SO₄ in 20 mM sodium phosphate buffer (pH 7.0) (total volume: 400 mL). The eluate containing the protein that show COD activity was dialyzed against 20 mM sodium phosphate buffer (pH 7.0) and stored at 4ºC. The purified protein ran as a single band with a molecular mass of approximately 48.6 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). Sw-rCOD was purified 7.2-fold with 17.2% recovery from crude enzyme solution. The specific activity of the purified enzyme against (GlcNAc)₂ was 7.8 unit (U)/mg of protein. Assays of COD activity were conducted according to the colorimetric method reported by Dishe and Borenfreund using (GlcNAc)₂ as a substrate. This enzyme showed the highest activity around pH 7 when assayed at 37ºC, and at 37ºC when assayed at pH 7.0 (Fig. 3). These data indicated that the optimal reaction pH and temperature of Sw-rCOD are similar to those of the Vibrio CODs. Next, we investigated the substrate specificity of Sw-rCOD under the standard assay conditions using chitin oligosaccharides of various polymerization degrees. As shown in Table 1, Sw-rCOD showed activity not only for (GlcNAc)₂ but also some still lower activity for N,N',N''-triacetyltchitotriose (GlcNAc₃). Although very low, this enzyme exhibited activity against N,N',N'',N'''-tetraacetyltchitotetraose (GlcNAc₄) too. In this assay condition, the activity of Sw-rCOD against GlcNAc and N,N',N'',N''''-pentaacetyltchitoctaose (GlcNAc₆) was not detected. Such a specificity has also been observed in the CODs from Vibrio parahaemolyticus KN1699, Vibrio sp. SN184, and V. harveyi ATCC BAA-1116. Treatment of the compound produced from (GlcNAc)₂ by Sw-rCOD with β-N-acetyhexosaminidase gave GlcNAc and GlcN (Supplemental Fig. 1; See J. Appl. Glycosci. Web site), indicating that this COD hydrolyzes acetamide bond of reducing end GlcNAc residue of (GlcNAc)₆. These facts indicate that there are no remarkable differences in the properties of Sw-rCOD compared to those of the Vibrio CODs.

To perform a kinetic study of the Sw-rCOD reaction, 8 mL of 20 mM sodium phosphate buffer (pH 7.0) containing 10.24 µg of Sw-rCOD and (GlcNAc)₂ (1.0, 2.0, 3.0, 4.0, or 5.0 mM) was incubated at 37ºC. Aliquots (1 mL/time point) were withdrawn from each reaction mixture every 5 min for 30 min, and were heated at 95ºC for 30 min in a hot dry bath to stop the enzymatic reaction. Heat-denatured enzyme was used in the control reaction mixture. Enzyme activity was determined according to the above-mentioned colorimetric method. The values of V_{max} and K_{m} were obtained from double-reciprocal plots of the reaction curves. The molecular mass of Sw-rCOD (44.0 kDa) was calculated from deduced amino acid sequence. Based on the results from three independent experiments, the parameter values (mean ± SEM) were determined as follows: V_{max}, 15.7 ±
reported the kinetic parameters of recombinant k and the Vp-rCOD is 2.6-fold lower than that of Sw-rCOD; the cat Sw-rCOD; the k cat-rCOD is 2.8-fold higher than that of Vp.

V. harveyi ATCC BAA-1116, respectively. n.d., no detectable activity. The reactions were performed under the standard assay conditions using GlcNAc and chitin oligosaccharides, which were purchased from Seikagaku Biobusiness (Tokyo, Japan), as the substrates. The values of U/mg of protein (mean ± SEM) were determined by three independent experiments. These data are derived from our previous papers.

Substrate specificity of CODs.

### Table 1.

| Substrate | Specific activity (U/mg of protein) |
|-----------|-------------------------------------|
|           | Sw-rCOD | Vp-rCOD | Vsp-rCOD | Vh-rCOD |
| GlcNAc    | n.d.     | n.d.     | n.d.     | n.d.     |
| (GlcNAc)  | 7.66 ± 0.55 | 25       | 7.8      | 9.47     |
| (GlcNAc)  | 1.72 ± 0.09 | 7.5      | 2.2      | 3.69     |
| (GlcNAc)  | 0.22 ± 0.06 | n.d.     | n.d.     | 0.23     |
| (GlcNAc)  | n.d.     | n.d.     | n.d.     | n.d.     |

Vp-rCOD, Vsp-rCOD, and Vh-rCOD correspond to the recombinant CODs of V. parahaemolyticus KN1699, Vibrio sp. SN184, and V. harveyi ATCC BAA-1116, respectively.

0.46 μmol·min⁻¹·mg⁻¹ of protein; Kₘ, 0.67 ± 0.07 mM; kcat, 11.5 s⁻¹; and kcat / Kₘ, 17.2 mM⁻¹·s⁻¹. Recently, we reported the kinetic parameters of recombinant V. parahaemolyticus KN1699 COD (Vp-rCOD). Comparison of the parameters indicate that the Vₘₐₓ value of Sw-rCOD is 2.5-fold lower than that of Vp-rCOD; the Kₘ value of Sw-rCOD is 2.8-fold higher than that of Vp-rCOD; the kcat value of Sw-rCOD is 2.6-fold lower than that of Vp-rCOD; and the kcat / Kₘ value of Sw-rCOD is 7.1-fold lower than that of Vp-rCOD. These data indicate that the catalytic function of Sw-rCOD is inferior to that of Vp-rCOD. Amino acid sequence identity between Sw-rCOD and Vp-rCOD is 66.9%. We believe that the difference in catalytic efficiency between these CODs reflects the differences in their primary structures.

In conclusion, although there were differences in catalytic efficiency, S. woodyi COD and Vibrio CODs shared similar properties. In future work, we plan to identify and characterize CODs from bacteria of various genera.

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