Note

A Chitin Oligosaccharide Deacetylase from *Vibrio* sp. SN184: Gene Cloning, Overexpression, Purification and Characterization

(Received June 22, 2009; Accepted August 4, 2009)

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Abstract: The chitinolytic bacterial strain SN184, which produces chitin oligosaccharide deacetylase (COD), was identified a species of the genus *Vibrio*. The COD gene was cloned from the chromosomal DNA of SN184 and its nucleotide sequence was analyzed. The gene encoded a 427 amino acid protein, including the 22 amino acid signal sequence. The deduced amino acid sequence of this protein showed high similarity to those of several bacterial CODs belonging to carbohydrate esterase family 4. An expression plasmid containing the COD gene of strain SN184 was constructed and inserted into *Escherichia coli* cells; the recombinant enzyme was secreted into the culture medium with the aid of the signal sequence. The recombinant enzyme was purified from the culture supernatant and its substrate specificity was investigated. These studies confirmed that the recombinant COD hydrolyzed *N*-acetyl groups of the second *N*-acetyl-D-glucosamine residue from the nonreducing-ends of di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose.

Key words: chitin oligosaccharide deacetylase, *Vibrio*, gene cloning, recombinant enzyme, enzyme characterization

Chitin, a water-insoluble β -(1,4)-linked polymer of *N*-acetyl-D-glucosamine (GlcNAc), is one of the most abundant renewable forms of biomass. In the natural environment, chitin is decomposed mainly by microorganisms that can utilize it as a nutrient source. Various hydrolases are involved in chitin degradation [*i.e.*, chitinase (EC 3.2.1.14), β -*N*-acetylhexosaminidase (EC 3.2.1.52), chitin deacetylase (EC 3.5.1.41) and chitin oligosaccharide deacetylase (COD, EC 3.1.1)]. Chitinivorous microorganisms produce water-soluble mono- or oligosaccharides from chitin using one or several of these chitinolytic enzymes, and then transport these soluble saccharides into the cell for further degradation. In a previous paper, we reported that *Vibrio parahaemolyticus* KN1699 produces the heterodisaccharide β -*N*-acetyl-D-glucosaminyl-(1,4)-D-glucosamine (GlcNAc-GlcN) as the primary chitin degradation product using following two types of extracellular enzymes: glycoside hydrolase family 18 chitinase, which produces *N,N'*-diacetylchitobiose, (GlcNAc)₂, from chitin, and carbohydrate esterase (CE) family 4 COD, which hydrolyzes the *N*-acetyl group at the reducing end GlcNAc residue of (GlcNAc)₂.^{1–3)} Recently, we clarified that GlcNAc-GlcN is not only a nutrient for strain KN1699, but also functions as an inducer of chitinase production by this bacterium.⁴⁾ Moreover, effect of this heterodisaccharide on the chitinase induction was confirmed also in other chitin-decomposing *Vibrio* strains harboring CE family 4 COD genes. These findings suggest that CODs involved in the synthesis of this signal compound are key enzymes for chitin catabolism in some species of *Vibrio*.

To date, aside from COD of strain KN1699, only two CODs, one from *Vibrio alginolyticus* H-8^{5,6)} and one from *Vibrio cholerae* EI Tor N16961,⁷⁾ have been purified and characterized. Although these three enzymes have high amino acid sequence homologies (80–99%), their specificities for chitin oligosaccharides are different, indicating the importance of characterizing other microbial CODs. We therefore screened natural sources for bacteria that secrete enzymes that can convert (GlcNAc)₂ to GlcNAc-GlcN. As a result, we isolated a target bacterium (strain SN184) from the bacteria that adhered on the surface of α -chitin flakes (wrapped with tea bag filter paper) sunk in the sea near Tsumekizaki (Shimoda City, Shizuoka Prefecture, Japan). Gram-stain results indicated that strain SN184 is Gram-negative. The genotype of strain SN184 was investigated by comparing the nucleotide sequence of its 16S rDNA (GenBank accession no. AB469367) to the sequence database BLASTN. The results confirmed that the isolate is most closely related to many strains of the genus *Vibrio* (about 97% identity). Although we tried to determine the species of this isolate by investigating its physiological characteristics, it was not possible to determine the species of this bacterium using the methodology outlined in Bergey's Manual of Systematic Bacteriology.⁸⁾ The bacterium was therefore named *Vibrio* sp. SN184.

To clone the COD gene of strain SN184, we constructed PCR primers, 5'-CAACCGGTTGCATTATTCGTG-3' (forward primer) and 5'-ACATGATGATAGTAGGG AACAG-3' (reverse primer), from highly conserved nucleotide sequences of upstream and downstream regions around the COD genes of *V. parahaemolyticus* KN1699 (1284 bp, GenBank accession no. AB275387) and *V. alginolyticus* H-8 (1284 bp, GenBank accession no. AB

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Fig. 1. Alignment of amino acid sequences of putative COD from the nucleotide sequences of the PCR product.

Amino acids highlighted in black and gray indicate identical and highly homologous amino acids, respectively. Double-headed arrows indicate the sequences that were confirmed to encode the secretion signal peptides on the products from COD genes of both *V. parahaemolyticus* KN1699 and *V. alginolyticus* H-8.^{3,6)}

055217) and performed PCR (Ex *Taq* DNA polymerase; 30 cycles: denaturation at 94°C for 1 min; annealing at 45°C for 1 min; elongation at 72°C for 1.5 min) using these synthetic primers (2 pmol) and chromosomal DNA (200 ng) extracted from strain SN184 cells. As a result, a PCR product consist of approximately 1500 base pairs was confirmed by agarose gel electrophoresis. From the results of nucleotide sequence analysis, genes (1284 bp) that were presumed to encode CE family 4 COD were found on this DNA fragment. Nucleotide sequences were determined using a ThermoSequenase Fluorescence-labeled primer cycle sequencing kit (GE Healthcare Bio-Science, Buckinghamshire, UK) and an automated DNA sequencer DSQ-2000L (Shimadzu Corp., Kyoto, Japan). The amino acid sequence deduced from the nucleotide sequence of this gene showed high similarity (79–86%) to those of CE family 4 CODs from *V. parahaemolyticus* KN1699,³⁾ *V. alginolyticus* H-8⁶⁾ and *V. cholerae* EI Tor N16961,⁷⁾ clearly demonstrating that strain SN184 harbors a CE family 4 COD gene (GenBank accession no. AB 469368) (Fig. 1).

The amount of enzyme produced by strain SN184 is very small, so we used recombinant strategies to overproduce the COD, allowing its characteristics to be investigated. Facile purification of the recombinant COD (rCOD) is achieved only in the absence of large amounts of other proteins from the transformed *Escherichia coli* cells. Therefore, we engineered a version of rCOD that would be secreted into the *E. coli* culture medium with the aid of a signal peptide. Amplification of the DNA fragment containing the ribosome-binding sequence and the COD gene that includes the putative signal sequence was performed by PCR (enzyme, cycles and conditions were as described for COD gene cloning) using strain SN184 chromosomal DNA (200 ng) and synthetic oligonucleotide primers (2 pmol), 5'-CGCGGATCCAGGAA TAGAAAATGAAATTAAATAAAC-3' (forward primer) and 5'-CCGCTCGAGCTATAGAGCTGTGAACAAGG-3' (reverse primer) (underlined letters designate the *Bam*HI site of the forward primer and the *Xho*I site of the reverse primer. Letters in italics designate added non-complimentary nucleotides). Resulting PCR products were digested with *Bam*HI (Toyobo Co., Ltd., Osaka, Japan)

and *Xho*I (Nippon Gene Co., Ltd., Tokyo, Japan), then the resulting DNA fragment was ligated into the *Bam*HI and *Xho*I sites of pET-21 (+) (Merck, Darmstadt, Germany) to yield the expression plasmid. *E. coli* BL21 (DE 3) was transformed with this plasmid, and the transformants were selected on LB medium-agar plates supplemented with 50 µg/mL ampicillin. *E. coli* cells harboring the expression plasmid were cultivated at 37°C in 200 mL of LB medium supplemented with 50 µg/mL ampicillin, and the production of rCOD was induced by the addition of isopropyl-β-thiogalactopyranoside (IPTG, final concentration; 1 mM). Addition of IPTG to the culture broth immediately promoted the production of rCOD in the *E. coli* cells, while its secretion into the culture medium required more time. After 8 h cultivation, total COD activity in the culture medium was at the same level as that in *E. coli* lysates. It was confirmed by SDS-PAGE that the protein (45 kDa) presumed to be rCOD was the main product in the *E. coli* culture medium, while other proteins secreted by the *E. coli* cells were present in very low concentrations (data not shown). These results indicate that the conditions used in the present investigation are favorable for the purification of rCOD. The rCOD was purified from the culture supernatant by (NH₄)₂SO₄ precipitation (80% saturation) and ion-exchange column chromatography using DEAE-Toyopearl 650M resin (Tosoh Corp., Tokyo, Japan). The rCOD was eluted from the column (φ1.6 × 30 cm) with a linear gradient of 0 to 0.4 M NaCl in 20 mM sodium phosphate buffer (pH 7.0). The resulting recombinant protein gave a single band of molecular mass 45 kDa on SDS-PAGE (data not shown). Using the method presented here, rCOD was purified 11.6-fold from the culture supernatant with a total recovery of 11.4%. The N-terminal amino acid sequence of the recombinant protein was determined to be QTAPKGTIYLTFF, showing that the signal peptide region (MKLNKLAMVTFGLTAL-SQFSFA) of the expression product was removed as the protein crossed the *E. coli* cell membrane. The N-terminal amino acid sequences of the protein were determined using a Model 492 Procise protein sequencer (Applied Biosystems Inc., Foster City, USA).

The rCOD was most active at pH 7.0–7.5 and 45°C, indicating that COD of strain SN184 is similar to that of

Table 1. Substrate specificity of rCOD.

Substrate	Specific activity (U/mg of protein)
GlcNAc	ND*
(GlcNAc) ₂	7.8
(GlcNAc) ₃	2.2
(GlcNAc) ₄	ND
(GlcNAc) ₅	ND
(GlcNAc) ₆	ND

GlcNAc and chitin oligosaccharides were purchased from Seikagaku Co. (Japan). The activity of rCOD was assayed by the method previously reported, and the units (U) of enzymatic activity were determined. * ND, no detectable activity.

V. cholerae EI Tor N16961 in terms of optimum reaction pH and temperature. In the chitin oligosaccharides used, the recombinant enzyme showed hydrolysis activity for (GlcNAc)₂, as well as lower activity for (GlcNAc)₃ at pH 7.0 and 37°C (Table 1). To confirm the structure of the reaction products, we purified the compounds produced from (GlcNAc)₂ and (GlcNAc)₃ by column chromatography using the cation exchange resin Dowex 50W-X8 (The Dow Chemical Company, Midland, USA) and analyzed their structure by positive ion fast atom bombardment mass spectrometry (FABMS) using a JMX SX-102A instrument (Jeol Ltd., Tokyo, Japan) and ¹H-NMR (400 MHz, D₂O, 20°C) using a Mercury 400 spectrometer (Varian Inc., Palo Alto, USA). The mass spectra of the compound produced from (GlcNAc)₂ corresponded to [M+H]⁺ species at *m/z* of 383, indicating that this compound is the disaccharide consisting of GlcNAc and GlcN. ¹H-NMR signals of this disaccharide corresponded well to those obtained previously with GlcNAc-GlcN,^{1,4,9)} indicating that this disaccharide is GlcNAc-GlcN. This result shows that the position specificity of rCOD for the deacetylation of (GlcNAc)₂ is the same as that of CODs from *V. parahaemolyticus* KN1699,¹⁾ *V. alginolyticus* H-8⁵⁾ and *V. cholerae* EI Tor N16961.⁷⁾ The mass spectra of the compound produced from (GlcNAc)₃ corresponded to [M+H]⁺ species at *m/z* 586, indicating that this compound is the trisaccharide consisting of two GlcNAc residues and one GlcN residue. This compound was digested with *Aspergillus oryzae* β-*N*-acetylhexosaminidase (Sigma-Aldrich Inc., USA), which releases a GlcNAc residue from the non-reducing end of chitin oligosaccharides.^{10,11)} Two reaction products (**a** and **b**) were confirmed by silica gel thin layer chromatography (TLC) analysis (Fig. 2). The products were subjected to silica gel preparative layer chromatography (PLC) and isolated from the PLC plate, and their structures were analyzed by FABMS and ¹H-NMR. TLC plates (Silica Gel 60, 0.25 mm) and PLC plates (Silica Gel 60, 2 mm) were obtained from Merck & Co., Inc. The mass spectra of compound **a** corresponded to [M+H]⁺ species at *m/z* of 222, indicating that this compound is GlcNAc. The mass spectra of compound **b** corresponded to [M+H]⁺ species at *m/z* of 383, indicating that this compound is the disaccharide consisting of GlcNAc and GlcN. In the ¹H-NMR signals of this disaccharide, chemical shifts (ppm) of H-2 of the reducing end sugar rings appeared at 3.935 (α-anomer) and 3.720 (β-anomer), and those of the non-reducing end sugar rings

Fig. 2. TLC analysis of compounds obtained by treating the oligosaccharide produced from (GlcNAc)₃ by rCOD with *A. oryzae* β-*N*-acetylhexosaminidase.

β-*N*-Acetylhexosaminidase (1.9 U) was added to 200 μL of 50 mM sodium phosphate buffer (pH 6.0) containing 3 mg of the trisaccharide, and then the mixture was incubated at 37°C. At designated time points, 1-μL aliquots of the reaction mixture were withdrawn and applied to the TLC plate. The reaction products were developed twice using 5:4:3 (v/v) *n*-butanol/methanol/16% aqueous ammonia as the mobile phase and visualized by spraying with an aqueous solution of 2.4% (w/v) phosphomolybdic acid, 5% (v/v) H₂SO₄ and 1.5% (v/v) H₃PO₄ followed by heating.

appeared at 2.773 (α-anomer) and 2.750 (β-anomer). These chemical shift data indicate that compound **b** is β-D-glucosaminyl-(1→4)-*N*-acetyl-D-glucosamine (GlcN-GlcNAc). These data indicate that the oligosaccharide produced from (GlcNAc)₃ by rCOD is *N*-acetyl-β-D-glucosaminyl-(1→4)-β-D-glucosaminyl-(1→4)-*N*-acetyl-D-glucosamine (GlcNAc-GlcN-GlcNAc) and show that the position specificity of rCOD for the deacetylation of (GlcNAc)₃ is the same as that of COD from *V. cholerae* EI Tor N16961.⁷⁾ We confirmed that COD from *V. parahaemolyticus* KN1699 shows high activity against (GlcNAc)₂, significantly less activity against (GlcNAc)₃, and almost no activity against (GlcNAc)₄₋₆.¹⁾ It was reported that COD from *V. alginolyticus* H-8 is specific for (GlcNAc)₂ and has no activity against larger chitin oligosaccharides.⁵⁾ Although (GlcNAc)₂ is the best substrate for *V. cholerae* EI Tor N16961 COD, this enzyme also has activity against (GlcNAc)₃₋₆.⁷⁾ These findings indicate that the substrate specificities of *Vibrio* CODs are diverse even if their primary structure is highly similar. In the present study, it became clear that the substrate specificity of COD we newly isolated from *Vibrio* sp. SN184 is most similar to that of the *V. parahaemolyticus* KN1699 enzyme. Recently, we found the CE family 4 COD genes also from *Vibrio campbellii* and *Vibrio proteolyticus*.⁴⁾ We have a plan to investigate the substrate specificities for CODs of these *Vibrios*.

This research was supported by grants from the High-Tech Research Center Project of the Ministry of Education, Culture, Sports, Science and Technology of Japan to promote advanced scientific research, and College of Bioresource Sciences, Nihon University. We thank Dr. Kenji Ueda for screening of the bacterium and Naoko Saito, Natsuko Sekino, Mika Hyodo and Nozomi Murayama for their technical assistance.

REFERENCES

- 1) K. Kadokura, A. Rokutani, M. Yamamoto, T. Ikegami, H. Sugita, S. Itoi, W. Hakamata, T. Oku and T. Nishio: Purification and characterization of *Vibrio parahaemolyticus* extracellular chitinase and chitin oligosaccharide deacetylase involved in the production of heterodisaccharide from chitin. *Appl. Microbiol. Biotechnol.*, **75**, 357–365 (2007).
- 2) K. Kadokura, Y. Sakamoto, K. Saito, T. Ikegami, T. Hirano, W. Hakamata, T. Oku and T. Nishio: Production and secretion of a recombinant *Vibrio parahaemolyticus* chitinase by *Escherichia coli* and its purification from the culture medium. *Biosci. Biotechnol. Biochem.*, **71**, 2848–2851 (2007).
- 3) K. Kadokura, Y. Sakamoto, K. Saito, T. Ikegami, T. Hirano, W. Hakamata, T. Oku and T. Nishio: Production of recombinant chitin oligosaccharide deacetylase from *Vibrio parahaemolyticus* in the culture medium of *Escherichia coli* cells. *Biotechnol. Lett.*, **29**, 1209–1215 (2007).
- 4) T. Hirano, K. Kadokura, T. Ikegami, Y. Shigeta, Y. Kumaki, W. Hakamata, T. Oku and T. Nishio: Heterodisaccharide 4-*O*-(*N*-acetyl- β -D-glucosaminyl)-D-glucosamine is a specific inducer of chitinolytic enzyme production in *Vibrios* harboring chitin oligosaccharide deacetylase genes. *Glycobiology*, **19**, 1046–1053 (2009).
- 5) K. Ohishi, M. Yamagishi, T. Ohta, M. Motosugi, H. Izumida, H. Sano, K. Adachi and T. Miwa: Purification and properties of two deacetylases produced by *Vibrio alginolyticus* H-8. *Biosci. Biotechnol. Biochem.*, **61**, 1113–1117 (1997).
- 6) K. Ohishi, K. Murase, T. Ohta and H. Etoh: Cloning and sequencing of the deacetylase gene from *Vibrio alginolyticus* H-8. *J. Biosci. Biotechnol.*, **90**, 561–563 (2000).
- 7) X. Li, L.-X. Wang, X. Wang and S. Roseman: The chitin catabolic cascade in the marine bacterium *Vibrio cholerae*: Characterization of a unique chitin oligosaccharide deacetylase. *Glycobiology*, **17**, 1377–1387 (2007).
- 8) J.J. Farmer, J.M. Janda, F.W. Brenner, D.N. Cameron and K. M. Birkhead: Genus I. *Vibrio* Pacini 1845, 411^{AL}. in *Bergey's Manual of Systematic Bacteriology*, vol. 2. *The Proteobacteria. Part B. The Gammaproteobacteria*, 2nd Ed., G.M. Garrity, D.J. Brenner, N.R. Krieg and J.T. Staley, eds., Springer, Berlin Heidelberg/New York, pp. 494–546 (2001).
- 9) K. Tokuyasu, H. Ono, K. Hayashi and Y. Mori: Reverse hydrolysis reaction of chitin deacetylase and enzymatic synthesis of β -D-GlcNAc-(1 $\rightarrow$ 4)-GlcN from chitobiose. *Carbohydr. Res.*, **322**, 26–31 (1999).
- 10) T. Mega, T. Ikenaka and Y. Matsushima: Studies on *N*-acetyl- β -D-glucosaminidase of *Aspergillus oryzae*. I. Purification and characterization of *N*-acetyl- β -D-glucosaminidase obtained from Takadiastase. *J. Biochem.*, **68**, 109–117 (1970).
- 11) T. Mega and Y. Matsushima: Comparative studies of three exo- β -glycosidases of *Aspergillus oryzae*. *J. Biochem.*, **85**, 335–341 (1979).

Vibrio sp. SN184 由来のキチンオリゴ糖デアセチラーゼの遺伝子クローニング, リコンビナント酵素生産, およびその精製と諸性質調査

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キチンオリゴ糖デアセチラーゼ (COD, EC 3.1.1) は, *N*-アセチルグルコサミン (GlcNAc) が β -(1,4)-グリコシド結合で連結したオリゴ糖 (キチンオリゴ糖) の部分脱アセチル化を触媒する酵素である. 以前, われわれは, (GlcNAc)₂ から COD によって生産されたヘテロ2糖 (GlcNAc-GlcN) が, 本酵素の遺伝子を有する数種の *Vibrio* 属細菌において, 栄養となるばかりでなくキチナーゼ生産の誘導物質として機能していることを見出した. このように, COD はある種の細菌のキチン分解代謝において重要な役割を担っていることがわかった. しかし, COD に関しては, 現在のところ3種の *Vibrio* 属細菌が生産するものについての報告しかなく, 本酵素の生物機能, 特異性および生物界における分布などについては不明な点が多いため, われわれは, COD に関するより多くの知見を得るため, 自然界より COD 生産菌のスクリーニングを行ったところ, 静岡県下田市の爪木崎沖海底に沈めたキチン片に付着していた細菌から carbohydrate esterase family 4 に属する分泌型 COD を生産する細菌 (*Vibrio* sp. SN184) を単離することができた. 本菌による COD 生産量はきわめて少なかったため, 分泌シグナル配列を含む COD 遺伝子をクローニングし, 大腸菌によりリコンビナント酵素 (rCOD) を培養液中に過剰生産させた. 得られた rCOD を精製し, GlcNAc とキチンオリゴ糖 (重合度; 2–6) に対する特異性を調べたところ, (GlcNAc)₂ と (GlcNAc)₃ に対して活性を示した. また, その活性は (GlcNAc)₃ よりも (GlcNAc)₂ に対して高かった (約3倍). このような特異性は, 3種の *Vibrio* 属細菌由来の COD の中でも *V. parahaemolyticus* KN1699 のものと類似していた. また, 本酵素が (GlcNAc)₂ から GlcNAc-GlcN を, (GlcNAc)₃ から GlcNAc-GlcN-GlcNAc を生産したことから, キチンオリゴ糖の脱アセチル化は, 非還元性末端 GlcNAc 残基の隣りの GlcNAc 残基で起こることがわかった. この結果は, これまでに報告された3種の *Vibrio* 属細菌の COD の反応位置特異性と同一であった.