Synthesis of Long-Chain Chitooligosaccharides by a Hypertransglycosylating Processive Endochitinase of *Serratia proteamaculans* 568

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We describe the heterologous expression and characterization of a 407-residue single-domain glycosyl hydrolase family 18 chitinase (*SpChiD*) from Gram-negative *Serratia proteamaculans* 568 that has unprecedented catalytic properties. *SpChiD* was optimally active at pH 6.0 and 40°C, where it showed a K_m of 83 mg ml⁻¹, a k_{cat} of 3.9×10^2 h⁻¹, and a k_{cat}/K_m of 4.7 h mg⁻¹ ml⁻¹ on colloidal chitin. On chitobiose, the K_m , k_{cat} , and k_{cat}/K_m were 203 μM, 1.3×10^2 h⁻¹, and 0.62 h⁻¹ μM⁻¹, respectively. Hydrolytic activity on chitooligosaccharides (CHOS) and colloidal chitin indicated that *SpChiD* was an endo-acting processive enzyme, with the unique ability to convert released chitobiose to *N*-acetylglucosamine, the major end product. *SpChiD* showed hypertransglycosylation (TG) with trimer-hexamer CHOS substrates, generating considerable amounts of long-chain CHOS. The TG activity of *SpChiD* was dependent on both the length and concentration of the oligomeric substrate and also on the enzyme concentration. The length and amount of accumulated TG products increased with increases in the length of the substrate and its concentration and decreased with increases in the enzyme concentration. The *SpChiD* bound to insoluble and soluble chitin substrates despite the absence of accessory domains. Sequence alignments and structural modeling indicated that *SpChiD* would have a deep substrate-binding groove lined with aromatic residues, which is characteristic of processive enzymes. *SpChiD* shows a combination of properties that seems rare among family 18 chitinases and that may resemble the properties of human chitotriosidase.

Chitin is a β-1,4-linked, insoluble linear polymer of *N*-acetylglucosamine (NAG) and is the second most abundant polysaccharide, after structural biopolymers of plants such as cellulose and hemicelluloses. Chitin provides structural and chemical resistance in the exoskeletons of crustaceans and arthropods, as well as in the cell walls of fungi. In nature, chitin is an important source of energy for variety of organisms. Degradation of cellulosic or chitinous biomass is achieved by mixtures of hydrolytic exo- and endo-acting enzymes that act in a synergistic manner. There is a renewed interest in these enzymatic machineries because they have the potential to convert biomass to fermentable sugars, although the costs for such enzymes are a major limiting factor for commercial processes.

Chitinases (EC 3.2.1.14) hydrolyze chitin to NAG, chitobiose, or smaller chitooligosaccharides (CHOS). Chitinases are found in a wide range of organisms, including bacteria, fungi, plants, and animals and are closely associated with the physiological roles of their substrates (4). Chitinases can be divided into two major categories: exochitinases acting from termini and randomly acting endochitinases (6, 22). Based on amino acid sequence similarity, chitinases are classified into families 18 and 19 of the glycoside hydrolases (GH), described in the Carbohydrate Active enZYme (CAZy) database (<http://www.cazy.org/>) (14). Family 18 chitinases have catalytic domains of triosephosphate isomerase [TIM barrel (β/α)₈ fold] with a conserved DxDxE motif on the β4 strand and catalyze the hydrolytic reaction by a substrate-assisted mechanism (42). Family 18 chitinases are represented in most living organisms, whereas family 19 chitinases are mostly found in plants and contribute to defense against chitinous pathogens.

The CHOS are of interest to the food, agriculture, and medicine-related industries in light of the diverse applications for these molecules. The use of oligochitosan as a broad-spectrum vaccine

against plant diseases (48) highlights the need to produce specific CHOS for crop protection and production. The capacity to produce CHOS on a large scale would allow the use of synthetic CHOS of desired length and sequence to induce immunity in plants. CHOS elicit a variety of defense reactions in plants and also in mammals. Despite their biological interest and potential agro-nomical usefulness, CHOS with well-defined structures remain poorly accessible. CHOS with 2 to 6 degrees of polymerization (DP) were obtained from chitin by chemical or enzymatic depolymerization or metabolic engineering (2, 17, 30). Most biological activities require CHOS DP of ≥4 (12), but the synthesis of oligomers with ≥6 DP has been a daunting task. The transglycosylation (TG) activity of chitinases has been actively investigated for synthesis of long-chain CHOS.

A few of the GHs carry out TG and establish new glycosidic bonds between donor and acceptor saccharides, besides hydrolysis of glycosidic bonds. Efficient TG requires an enzyme with an active-site architecture that disfavors correct positioning of the hydrolytic water molecule and/or favors binding of incoming carbohydrate molecules, through strong interactions in the aglycon subsites (46). TG by family GH 18 chitinases from viruses to humans is known. Chitinase from *Autographa californica* multiple nucleopolyhedrovirus produced TG products DP6 and DP8 from

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4.6 to 5.0 mM DP4 to DP6 substrates (10). Chitotriosidase from human macrophages synthesized the TG products up to DP6 to DP9 from 5 mM DP5 substrate (1).

Enzymes acting on crystalline polysaccharides need to associate with the insoluble substrate, disrupt the polymer packing, and, importantly, guide a single polymer chain into the catalytic center, a mechanism called “processivity.” Processive enzymes often have long and deep substrate-binding clefts or even tunnels, as illustrated by the early structures of processive cellulases (29, 7). The substrate-binding sites in processive chitinases and cellulases are lined with aromatic residues, such as tryptophan. These residues are thought to facilitate processivity by functioning as a flexible and hydrophobic sheath along which the polymer chain can slide during the processive mode of action (8, 44). Processivity improves the catalytic efficiency of chitinases because the enzyme remains closely associated with the detached single polymer chain between hydrolytic steps (15). Furthermore, the detached chain is prevented from reassociating with the crystalline material, which would make it less accessible.

Native chitinases produced by several bacteria and their variants were generated and characterized (23, 24). *Serratia marcescens* produces three chitinases and one chitin-binding protein (CBP) (41). *Bacillus circulans* secretes three chitinases (5) in the presence of chitin. Most of the bacterial chitinases have a multi-domain organization, but a few have only a catalytic domain, for example, ChiCH from *B. cereus* 28-9 (16) and ChiB from *Stenotrophomonas maltophilia* (K. Suma and A. R. Podile, unpublished data). The genome sequence of *Serratia proteamaculans* 568, isolated as a root endophyte from a *Populus trichocarpa* plant (36), revealed the presence of at least 11 open reading frames (ORFs) potentially involved in chitin turnover, coding for four family 18 chitinases (28), three family 33 CBPs (27), one family 20 *N*-acetylhexosaminidase, two proteases (chitin-binding protease and metalloprotease), and one nonspecific polysaccharide deacetylase (PDA), showing that *S. proteamaculans* 568 is a rich source of enzymes associated with chitin degradation. Here, we describe the cloning of an ORF encoding a single GH18 domain of *S. proteamaculans* 568, its heterologous expression in *Escherichia coli*, and its complete characterization. The unique single-domain *S. proteamaculans* ChiD (*SpChiD*) showed copious TG activity on DP3 to DP6 CHOS substrates and was identified as a processive endochitinase that has a potential to synthesize CHOS with DP up to 13. Further, *SpChiD* retained the TG products for a prolonged duration.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions, biochemicals, and enzymes. *Serratia proteamaculans* 568 (a kind gift from Daniel van der Lelie) was used as the source of genomic DNA for *S. proteamaculans* chitinase D (*SpchiD*) gene cloning. The plasmid pET-22b(+) and *E. coli* Rosetta-gami 2(DE3) (Novagen, Madison, WI) were used for heterologous expression. *S. proteamaculans* 568 was grown with shaking at 28°C in LB medium (Difco, Detroit, MI) for 16 h. *E. coli* was grown in LB broth (1% peptone, 0.5% yeast extract, 1% NaCl) at 37°C. Ampicillin (100 µg ml⁻¹) and chloramphenicol (25 µg ml⁻¹) were added to the LB broth as required. Oligonucleotide primers were purchased from Eurofins India (Bangalore, India). Restriction enzymes, T4 DNA ligase, and *Pfu* DNA polymerase were from MBI Fermentas (Ontario, Canada). Isopropyl-β-D-thiogalactoside (IPTG), ampicillin and all other chemicals were purchased from Calbiochem or Merck (Darmstadt, Germany) or from Hi-Media Labs (Mumbai, India). The polymeric substrates α-chitin (extracted from

shrimp shells, 60-mesh powder), β-chitin (extracted from squid pen, 200 µM), and chitosan substrates (fraction of acetylated units [F_A] = 0.10, extracted by homogeneous *N*-deacetylation of milled shrimp shell chitin, 40-mesh powder) were kindly provided by Dominique Gillete (Mahtani Chitosan, Veraval, India). Colloidal chitin and glycol chitin were prepared as described by Berger and Reynolds (3) and Yamada and Imoto (47), respectively. CHOS with different degrees of polymerization (DP) were purchased from Seikagaku Corporation (Tokyo, Japan). Avicel was procured from Sigma-Aldrich (St. Louis, MO).

DNA manipulations and cloning of *SpchiD*. *S. proteamaculans* 568 genomic DNA (gDNA) was isolated using a DNeasy kit (Qiagen, Dusseldorf, Germany). Plasmid DNA preparation, restriction digestion, PCR, and recombinant DNA construction were done according to standard methods as described by Sambrook and Russell (31). The SignalP server (<http://www.cbs.dtu.dk/services/SignalP/>) predicted the gene *SpchiD* (GenBank accession no. ABV41826.1) to contain an N-terminal signal peptide-encoding sequence (57 bp). A 1.22-kb segment of *SpchiD* without the signal peptide was PCR amplified from *S. proteamaculans* 568 gDNA using gene-specific forward (5'-TAA TAC CAT GGG TGC CGG CAT GGC TCA TG-3') and reverse (5'-AAT AAC TCG AGC TGT TTC CCG TTA ATC C-3') primers with *Pfu* DNA polymerase and cloned into the *Nco*I and *Xho*I (underlined) sites of the pET-22b(+) vector. The resulting plasmid, pET 22b(+)-*SpchiD*, was transformed into *E. coli* Rosetta-gami 2(DE3). Transformants were selected on ampicillin- and chloramphenicol-containing LB plates. The insert sequence was confirmed by automated DNA sequencing (Eurofins, Bangalore, India).

Expression and purification of *SpChiD*. Expression and purification of *SpChiD* were done as described previously by Neeraja et al. (23). Nitrilotriacetic acid (Ni-NTA) affinity chromatography was used to purify the C-terminal His tag-carrying *SpChiD*. The *SpChiD*-containing fractions were pooled, concentrated (30,000-*M_r* cutoff; Macrosep Centrifugal Devices, Pall Corporation), buffer exchanged with 50 mM sodium acetate buffer (pH 6.0), and stored at 4°C until use.

Protein measurement. Purified protein was quantified with a bicinchoninic acid (BCA) protein assay kit (Novagen) using a standard calibration curve constructed from bovine serum albumin (BSA). For the chitin binding assay, the protein was measured from the absorbance at 280 nm using the molar extinction coefficients (ϵ) calculated from the amino acid composition of the protein as described by Pace et al. (26).

Zymogram analysis for *SpChiD*. A dot blot assay was performed to detect the activity of purified *SpChiD*. A composite gel impregnated with 0.1% glycol chitin was prepared. Five microgram of *SpChiD* was spotted on to the gel and placed in humid chamber at 37°C overnight. After incubation, the gel was stained with 0.01% calcofluor white M2R in 0.5 M Tris-HCl (pH 8.9) for 10 min at 4°C. Finally, the brightener solution was removed. The gel was washed with distilled water for 10 min at 4°C. The lytic zone was visualized on a UV transilluminator.

Chitinase assay. The reducing-end assay was carried out as described by Neeraja et al. (23) with slight modifications. Reaction mixtures contained 3 µM *SpChiD*, 25 mg ml⁻¹ colloidal chitin, and 50 mM sodium acetate buffer (pH 6.0) and were incubated at 40°C for 1 h with shaking at 190 rpm.

Steady-state kinetics and pH and temperature optima of *SpChiD*. Kinetic parameters of the *SpChiD* were determined using colloidal chitin or chitobiose as the substrate. The reaction mixtures (200 µl), containing 0 to 100 mg ml⁻¹ colloidal chitin or 0 to 1,500 µM chitobiose as the substrate and 3 µM *SpChiD* in 50 mM sodium acetate buffer (pH 6.0), were incubated at 40°C for 1 h and subjected to reducing-end assay as described above. The kinetic values were obtained using the nonlinear regression function available in GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). The *SpChiD* activity was also measured at pH 2.0 to 12.0 and at 20 to 100°C under the standard assay conditions as mentioned above.

Substrate specificity. The substrate specificity of purified *SpChiD* on different chitin and nonchitin substrates was tested. The substrates (2.5%,

FIG 1 Substrate specificity of *SpChiD*. The reaction mixture contained 3 μM *SpChiD* and 2.5% (wt/vol) of one of the polymeric substrates; incubation was at pH 6.0 and 40°C for 1 h and monitoring by reducing-end assay. One unit of chitinase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar per min at 40°C. Error bars represent standard deviations from triplicate experiments.

wt/vol) α -chitin, β -chitin, colloidal chitin, glycol chitin (water-soluble chitin), chitosan ($F_A = 0.10$), chitosan chlorhydrate (water soluble chitosan with $F_A = 0.10$), Avicel (microcrystalline cellulose), and carboxymethyl cellulose (CM-cellulose) (water-soluble cellulose) were used. Activity was assayed under standard assay conditions. One unit was defined as the amount of chitinase that liberated 1 μmol of reducing sugar per minute.

Insoluble-substrate binding. The standard 1-ml binding assay mixture contained 3 μM *SpChiD* and 1 mg ml^{-1} substrate (α -chitin, β -chitin, colloidal chitin, or Avicel) in 50 mM sodium acetate buffer, pH 6.0. As a control for nonspecific adsorption, *SpChiD* was replaced by BSA in the reaction mixture. To minimize enzyme-mediated hydrolysis of the bound substrate, the binding assay mixture was incubated for 1 h at 4°C with rotary shaking at 1,300 rpm in a Thermomixer (Thermomixer Comfort; Eppendorf, Hamburg, Germany). The supernatant containing unadsorbed protein was separated from the insoluble polysaccharides at $16,100 \times g$ for 15 min. The protein concentration in the supernatant was measured by recording the absorbance at 280 nm. The difference between the amount of protein added to the mixture and that in the supernatant was considered adsorbed protein. All assays were performed in triplicate and with blanks (buffer plus 1.0 mg ml^{-1} chitin) and controls to correct for specific binding of the protein (buffer plus *SpChiD*) and nonspecific adsorption (buffer plus BSA).

Soluble-substrate binding. The binding of *SpChiD* to different soluble polysaccharides was evaluated by affinity electrophoresis as described by Hardt and Laine (13) with slight modifications. Different soluble polymeric substrates, *viz.*, glycol chitin, laminarin, and CM-cellulose, were used. Proteins (3 μM *SpChiD* or noninteracting BSA) were electrophoresed in 8.0% polyacrylamide under nondenaturing conditions at 4°C and visualized by staining with Coomassie blue G-250.

Hydrolytic activity of *SpChiD* on CHOS and colloidal chitin. Hydrolysis or TG of chitoooligosaccharides (CHOS) (DP2 to DP6) was assessed in a 150 μl of a reaction mixture containing 2 mM substrate and 560 nM purified *SpChiD* in 50 mM sodium acetate buffer, pH 6.0. Reaction mixtures were incubated at 40°C for 0 (immediately after addition of enzyme) to 720 min. To stop the reaction, 10 μl of the reaction mixture was transferred to an Eppendorf tube containing 10 μl of 0.1 N NaOH and stored at -20°C until analysis by thin-layer chromatography (TLC). Aliquots (20 μl) of the reaction mixtures were chromatographed on a silica gel plate (TLC Silica Gel 60; Merck Co., Germany) with *n*-butanol, methanol, 25% ammonia solution, and water (5:4:2:1 [vol/vol/vol/vol]). The products were detected by spraying the plate with aniline-diphenylamine reagent (400 μl of aniline, 400 mg of diphenylamine, 20 ml of acetone, and 3 ml of

85% phosphoric acid) and baking it at 180°C using a hot air gun (Black & Decker, Idstein, Germany) for 3 min.

For high-pressure liquid chromatography (HPLC), the sample preparation and analysis of reaction products formed by *SpChiD* from DP3 to DP6 substrate were same as described for TLC analysis. However, various concentrations of *SpChiD* (560 nM unless stated otherwise) and CHOS (3.5 mM unless stated otherwise) were used for HPLC analysis. A 75- μl portion of the reaction mixture was transferred to an Eppendorf tube containing an equal amount of 70% acetonitrile to stop the reaction. Twenty microliters of the reaction mixture was injected into an HPLC (Shimadzu, Tokyo, Japan) equipped with a Shodex Asahipack NH2P-50 4E column (4.6 mm [inner diameter] by 250 mm; Showa Denko K.K.) using a Hamilton syringe (Hamilton Bonaduz, Switzerland). Reaction mixtures were analyzed at 25°C. The mobile phase consisted of 67% acetonitrile and 33% MilliQ H_2O , the flow rate was set to 0.7 ml min^{-1} , and eluted CHOS were monitored at 210 nm. A CHOS HPLC mixture containing equal weights of oligomers ranging from DP1 to DP6 was used for standard graph preparation. Standard calibration curves of CHOS moieties were constructed separately for each oligosaccharide. These data points yielded a linear curve for each standard sugar with r^2 values of 0.997 to 1.0, allowing molar concentrations of CHOS to be determined with confidence.

The HPLC analysis of hydrolysis products from colloidal chitin (25 mg ml^{-1}) was done as described above. However, the reaction mixtures were pelleted at $16,100 \times g$ for 10 min at 4°C prior to the HPLC analysis. The supernatant was further concentrated (Eppendorf [Hamburg, Germany] concentrator) under reduced pressure at 25°C until the complete evaporation of solvent and finally dissolved in 20 μl of 35% acetonitrile for HPLC analysis.

Product analysis by MALDI-TOF MS. The products from DP3 to DP6 CHOS substrates were analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) using 45-min (DP4, DP5 and DP6) and 15-min (DP3) reaction mixtures. A portion of the reaction mixture (40 μl) was concentrated under reduced pressure at 25°C until complete evaporation of the solvent and dissolved in 4 μl of HPLC-grade MilliQ H_2O (Merck, Mumbai, India). Two microliters of a 9- mg ml^{-1} mixture of 2,5-dihydroxybenzoic acid (DHB) in 30% acetonitrile was applied to a ground-steel target plate (MTP 384 ground-steel target plate TF; Bruker Daltonics, Bremen, Germany), and 2 μl sample was mixed into the DHB droplet and dried under a stream of air and analyzed using Ultraflex MALDI-TOF/TOF (Bruker Daltonics GmbH, Bremen, Germany) with an autoflex 123 smart beam. The instrument was operated in positive acquisition mode and controlled by the FlexControl

FIG 2 Insoluble-substrate binding of *SpChiD*. The reaction mixture, containing 3 μM *SpChiD* and 1 mg ml^{-1} of one of the polymeric substrates, was incubated at pH 6.0 and 4°C for 1 h. The mixture was centrifuged, and the amount of unbound protein in the supernatant was estimated. The amount of protein bound was recorded as the difference between initial and unbound protein. Error bars represent standard deviations from triplicate experiments.

3.0 software package. All spectra were obtained using the reflectron mode with an acceleration voltage of 25 kV, a reflector voltage of 26, and pulsed ion extraction of 40 ns in the positive ion mode. The acquisition range was from m/z 50 to 3000. The data were collected from averaging 500 laser shots, with the lowest laser energy necessary to obtain sufficient signal-to-noise ratios. Peak lists were generated from the MS spectra using Bruker Flex Analysis software (version 3.0).

Sequence alignment and homology modeling of *SpChiD*. The catalytic domains of well-studied *S. marcescens* chitinases (*SmChiA*, *SmChiB*, and *SmChiC*) and Heva (hevine from *Hevea brasiliensis*) were aligned along with that of *SpChiD* using Clustalw2 (www.ebi.ac.uk/Tools/msa/clustalw2/) as described by van Aalten et al. (43). The model of *SpChiD* was generated with Modeler9v8 (<http://www.salilab.org/modeler/>) using chitinase II (Protein Data Bank [PDB] ID 3QOK) from *Klebsiella pneumoniae* as a structural template. Forty individual models for *SpChiD* and corresponding Ramachandran plots also were generated for checking the protein structure quality using PROCHECK. The model shown in Fig. 9 was generated using PyMOL (<http://www.pymol.org/>).

RESULTS

Of the 11 ORFs that could be important in chitin degradation by *Serratia proteamaculans* 568, the amino acid sequence for the ORF encoding *SpChiD* displayed 78% identity to chitinase II from *K. pneumoniae* (ACZ01996), 40% to chitinase from *Bacillus subtilis* (ACU33923), 35% to ChiC from *B. cereus* m1293 (EEK46733), 33% to ChiA from *S. maltophilia* (AAB70917) and Chi80 from *Bacillus ehimensis* (BAC76694), and 26% to ChiB from *S. marcescens* (ACX42072). The ORF codes for a chitinase with an N-terminal signal peptidase site (between Ala-19 and Gly-20) and a single family 18 catalytic domain (Ser-29 through Asp-406) with no accessory domains.

Heterologous expression, purification, and dot blot assay of *SpChiD*. *SpchiD* was cloned using PCR and heterologously expressed in *E. coli*. Periplasmic proteins were isolated from the induced culture pellet and subjected to Ni-NTA affinity chromatography for purification. The molecular mass (44.4 kDa) calculated from the amino acid sequence without the signal peptide was in reasonable agreement with that assessed by SDS-PAGE (see Fig. S1A in the supplemental material). Recombinant *SpChiD* was active on a substrate-containing gel (see Fig. S1B in the supplement-

FIG 3 Soluble-substrate binding of *SpChiD*. Affinity nondenaturing gel electrophoresis was performed at 4°C with continuous polyacrylamide gels consisting of 8.0% (wt/vol) polyacrylamide. Proteins (3 μM *SpChiD* or noninteracting BSA) were electrophoresed without substrate (A) or with 0.1% (wt/vol) substrate glycol chitin (B), laminarin (C), or CM-cellulose (D) incorporated. Proteins were visualized by Coomassie blue G-250 staining after electrophoresis.

tal material). Purified *SpChiD* was used for further characterization.

Kinetic analysis and pH and temperature optima of *SpChiD*.

The kinetic parameters of the hydrolytic activity of *SpChiD* were determined separately with colloidal chitin and chitobiose substrates. Velocity measurements with increasing substrate concentrations fitted to Michaelis-Menten kinetics revealed that the K_m , k_{cat} , and k_{cat}/K_m values were 83 mg ml^{-1} , $3.9 \times 10^2 \text{ h}^{-1}$, and $4.7 \text{ h mg}^{-1} \text{ ml}^{-1}$, respectively, for colloidal chitin and 203 μM , $1.3 \times 10^2 \text{ h}^{-1}$, and $0.6 \text{ h}^{-1} \mu\text{M}^{-1}$, respectively, for chitobiose. The hydrolytic activity of *SpChiD*, examined over a broad pH range from pH 2.0 to 12.0, indicated optimum activity in 50 mM sodium acetate buffer at pH 6.0, with a relative activity of >60% at pH 5.0 and 9.0, while no activity was detected at pH 2.0 and 12.0 (see Fig. S2A in the supplemental material). *SpChiD* was optimally active at 40°C, retained up to 80% and 50% activity at 20 and 60°C, respectively (see Fig. S2B in the supplemental material), and had no detectable activity at 100°C.

Hydrolyzing activity against various polymeric substrates.

SpChiD showed highest activity on chitosan chlorhydrate ($F_A = 0.10$), followed by glycol chitin, colloidal chitin, β -chitin, chitosan ($F_A = 0.10$), and α -chitin (Fig. 1). The relative activity of *SpChiD* was 50% on glycol chitin, 30% on colloidal chitin and β -chitin, and 23% on chitosan ($F_A = 0.10$) and α -chitin. Avicel and CM-cellulose were not suitable substrates for *SpChiD*.

Binding of *SpChiD* to polysaccharides.

Insoluble-substrate binding of *SpChiD* was assayed using α -chitin, β -chitin, colloidal chitin, and Avicel. *SpChiD* showed affinity toward both chitinous and cellulosic substrates. *SpChiD* bound preferably to β -chitin among the insoluble chitinous substrates, followed by colloidal/ α -chitin, and bound significantly less to Avicel (cellulosic substrate) (Fig. 2). Binding of the *SpChiD* to soluble polysaccharides was assessed by native PAGE with and without polysaccharides. The mobility of *SpChiD* was lower in the presence of glycol chitin than in the presence of CM-cellulose, laminarin, or no substrate, indicating that the *SpChiD* was bound to soluble glycol chitin (Fig. 3).

Hydrolytic activities of *SpChiD* on CHOS and colloidal chitin.

To assess the activity of the *SpChiD* on oligomeric and polymeric substrates, the hydrolytic products were analyzed using TLC. The relative size and intensity of color of the spots visible on the gel were

FIG 4 Time course of chitoooligosaccharide hydrolysis of *SpChiD* as analyzed by TLC. Reaction mixtures were incubated at 40°C for 0, 1, 3, 5, 15, 30, 45, 60, 120, 180, and 720 min as indicated below the chromatograms. The products of the reaction mixtures were subjected to TLC and detected with aniline-diphenylamine reagent. Panels A to D show hydrolysis of CHOS of DP3, DP4, DP5, and DP6, respectively. Lanes S, standard mix ranging from DP1 to DP6; lanes C, substrate control without *SpChiD*. Arrows indicate transglycosylation products.

considered a measure of concentration. With DP2 to DP6 CHOS substrates, *SpChiD* released NAG (as the major product) and chitobiose as end products. *SpChiD* rapidly hydrolyzed DP3 and DP4 and less rapidly hydrolyzed the DP2, DP5, and DP6 substrates (Fig. 4; see

Fig. S3 in the supplemental material). TG products were produced by *SpChiD* with DP3 to DP6 CHOS substrates.

The HPLC profiles showed that *SpChiD* initially released DP1 to DP4 products (predominantly chitobiose) from colloidal chi-

FIG 5 Time course of hydrolysis of colloidal chitin by *SpChiD*. The reaction mixture (1 ml) was incubated with 3 μ M *SpChiD* for different time periods from 0 to 720 min at 40°C. The reaction products were analyzed by isocratic HPLC. (A) The topmost profile shows a standard mixture of CHOS ranging from DP 1 to DP6. The remaining four profiles indicate the reactions at different incubation times. The inset shows the magnified low-peak-area products at a specific retention time. Control represents the substrate without *SpChiD*, while standard represents CHOS ranging from DP1 to DP6. (B) Overview of concentrations of CHOS products generated during hydrolysis of colloidal chitin. Products were quantified from respective peak areas of products by using standard calibration curves of CHOS ranging from DP1 to DP6.

FIG 6 Time course of DP5/DP6 hydrolysis and TG catalyzed by *SpChiD*. The reaction mixture was incubated with 560 nM *SpChiD* and 3.5 mM DP5/DP6 for different time periods from 0 to 720 min at 40°C. Products were analyzed by isocratic HPLC. (A and B) HPLC profiles of reaction products from DP5 (A) and DP6 (B) substrates. The topmost profile shows a standard mixture of CHOS ranging from DP1 to DP6. The other profiles show the reaction products from DP5 and DP6 substrates at the indicated incubation times. The inset shows a magnified view of the low-peak-area products. Control represents the substrate without *SpChiD*. Peak identities of CHOS of $\geq$ DP6 were determined using MALDI-TOF MS. (C and D) Overview of concentrations of CHOS products generated during reaction time courses with DP5 (C) and DP6 (D) substrates. Products were quantified from respective peak areas by using standard calibration curves of CHOS ranging from DP1 to DP6.

tin, and at the end of the reaction, NAG was the major product (Fig. 5). The reaction of *SpChiD* with DP3 substrate yielded DP1 to DP6 products. NAG and chitobiose were hydrolytic products, while DP4 to DP6 products formed due to TG (see Fig. S4A and C in the supplemental material). DP4 and DP5 products were produced instantaneously (within the sample processing time, referred as 0 min), while DP6 product appeared from 3 min. TG products (DP4, DP5, and DP6) were a small portion (7.1%) of the products at the beginning, increased to 13.0% by 15 min, and decreased subsequently. *SpChiD* produced DP1 to DP9 products from DP4 substrate, among which DP1 to DP3 and DP5 to DP9 were hydrolysis and TG products, respectively (see Fig. S4B and D in the supplemental material). DP5 to DP8 products were produced from the earliest time point and detectable until 120 min, while DP9 product was detectable up to 90 min, along with DP1 to DP6 products. Hydrolysis of DP4 substrate resulted mainly in production of chitobiose at up to 120 min. DP5 and DP6 CHOS were the quantifiable TG products from the standard CHOS (DP1

to DP6). The TG products (DP5 and DP6) were a minor fraction (5.7%) at 0 min and steadily increased to 10.84% by 45 min.

SpChiD generated DP1 to DP10 products from DP5 substrate, among which DP1 to DP4 and DP6 to DP9 were hydrolytic and TG products, respectively (Fig. 6A and C). DP6 to DP9 products were detected from 0 to 120 min, while DP10 product appeared during 1 to 90 min, along with DP1 to DP9 products. Hydrolysis of DP5 substrate resulted in DP3 oligomer as a major product during 0 to 45 min, in contrast to the case for DP3 and DP4 substrates. DP6 product was the only quantifiable TG product with the available standard CHOS mixture. At the start of the reaction, very little (0.02%) DP6 product was formed, which increased to 4.40% by 90 min.

SpChiD produced DP1 to DP10 products from DP6 substrate, among which DP1 to DP5 and DP7 to DP10 products were hydrolysis and TG products, respectively (Fig. 6B and D). TG products (DP7 to DP10) were detectable from 0 min onwards, up to 120 min. Hydrolysis of DP6 substrate yielded mainly DP2 product

during 0 to 120 min. At the end of 720 min, DP1 to DP5 products were present. The amount of DP1 product was larger than those of the DP2, DP3, DP4, and DP5 products.

Mass-based identification of products of *SpChiD* from DP3 to DP6 substrates was done using MALDI-TOF MS to identify CHOS products of $\geq$ DP6. Reaction products ranging from DP2 to DP7, DP2 to DP10, DP2 to DP12, and DP2 to DP13 were detected from DP3, DP4, DP5, and DP6 substrates, respectively (Fig. 7). The majority of the oligosaccharide species formed as Na adducts of the oligosaccharide Na salt.

Hydrolysis by and TG efficiency of *SpChiD* were assessed using DP6 substrate with various concentrations of enzyme and substrate. A 10-fold-lower concentration of enzyme (56 nM) resulted in production of a range of reaction products (DP1 to DP10) similar to that obtained under standard conditions (560 nM), but the substrate degradation was slow. Production of DP4 product was high up to 720 min compared to the predominant DP2 product detected under normal conditions (Fig. 8A and D). At 0 min of reaction time, the production of DP1, DP2, DP3, DP4, and DP5 products was 12.07%, 24.16%, 15.81%, 32.02%, and 15.92%, respectively. Along with DP1 to DP5 products, TG products (DP7 to DP9) were also detectable up to 720 min. At 720 min, the yields of DP1, DP2, DP3, DP4, and DP5 products were 18.02%, 27.58%, 19.71%, 20.09%, and 14.58%, respectively.

A 10-fold-higher concentration of *SpChiD* resulted in significantly increased accumulation of hydrolytic products DP1 to DP5 (Fig. 8B and E). Among the hydrolytic products, 99.1% were DP1 to DP3 and 0.90% was DP4 and DP5 products. The only TG product detectable at 30 min was DP7. DP2 product was predominant from the start of the reaction until 45 min (as observed under standard conditions). Later, DP1 product increased steeply. At the start of the reaction, the proportions of DP1, DP2, DP3, DP4, and DP5 products were 40.11%, 50.82%, 8.46%, 0.39%, and 0.02%, respectively. The substrate utilization rate was high, and complete hydrolysis of the substrate was observed within 60 min. By 720 min, all the products were hydrolyzed to NAG.

A 10-fold decrease in substrate concentration resulted primarily in accumulation of hydrolytic products (DP1 to DP5) and one TG product (DP7) at 90 min and yielded chitobiose as a major product at the initial time periods (Fig. 8C and F). At the beginning of the reaction, DP1, DP2, DP3, DP4, and DP5 products represented 15.65%, 29.92%, 15.05%, 27.50%, and 11.85%, respectively. By 720 min, only NAG and chitobiose were present. The amount of NAG was 2.9-fold higher than that of chitobiose. We could not assess the product profile with a 10-fold-higher concentration of substrate because of its poor solubility.

Sequence analysis and modeling of *S. proteamaculans* chitinases. Sequence analysis showed that the catalytic modules of *SmChiA*, *SmChiB*, and *SpChiD* have an α/β fold insertion between β -sheets 7 and 8 of the TIM-barrel fold, while it was absent in *SmChiC* like in hevamine (see Fig. S5 in the supplemental material). For modeling *SpChiD*, the sequences were submitted to

BLASTP at NCBI to search for templates using parameters such as protein BLAST against the protein data bank. *SpChiD* displayed high sequence identity (78%) to the chitinase II from *K. pneumoniae* (ACZ01996). A model of *SpChiD* was generated by Modeler9v8 using the structure of chitinase II from *K. pneumoniae* (PDB ID 3QOK) as the template (Fig. 9). The amino acid residues Y29, Y89, W120, Y149, W158, W166, W210, Y219, Y232, F260, and W296 were identified as surface-exposed aromatic amino acid residues in the substrate-binding groove. The superimposition of the final model C α atoms on the template structure gave a root mean square deviation (RMSD) of 0.202 Å.

DISCUSSION

The vast amount of chitin waste released from the aquatic food industry, where crustaceans (prawn, crab, shrimp, and lobster) constitute the main agricultural products, creates an environmental problem due to the recalcitrance of chitin. CHOS and related compounds are an important class of signaling molecules involved in cell-to-cell interactions (32). CHOS are useful as immunopotentiating, antitumor, antifungal, and antibacterial agents, in addition to being marketed as human dietary supplement to boost the immune system. The smaller CHOS with DP2 to DP6 are obtained by chemical or enzymatic depolymerization, but most of the biologically active CHOS are larger than chitohexaose (DP6). Enzymatic processes were developed to facilitate complete enzymatic synthesis of CHOS larger than DP6 using chitinases with TG activity (30). Production of chitin derivatives with suitable enzymes therefore is pertinent.

Two bacterial GH18 single-domain chitinases were biochemically characterized. *SpChiD* resembles, in terms of domain organization, exochitinase ChiCH (36 kDa) from *Bacillus cereus* 28-9 (16) and endochitinase ChiB (41 kDa) from *S. maltophilia* (Suma and Podile, unpublished data). However, no TG activity was reported for these two single-domain GH18 chitinases.

The K_m of *SpChiD* toward colloidal chitin was 5-fold-higher than that of ChiCH from *B. cereus* 28-9 (K_m , 17.0 mg ml⁻¹) (16). *SpChiD* was useful for the hydrolysis of soluble chitin and chitosan (Fig. 1), similar to the case for the ChiC of both *S. marcescens* (34) and *Alteromonas* sp. strain O-7 (25). *SpChiD* was optimally active at pH 6.0 and 40°C (see Fig. S2A and B in the supplemental material), like the chitinase II from *B. amyloliquefaciens* V656 (45). *SpChiD*, though was able to bind to polymeric substrates and showed less hydrolytic activity as it contained only the catalytic GH18 domain without accessory domains. The ChiCH from *B. cereus* 28-9, with only a catalytic GH18 domain, displayed $\sim$ 30% binding to colloidal chitin and Avicel (16). The electrophoretic mobility of *SpChiD* was affected in the presence of glycol chitin (Fig. 3). In contrast to ChiCH from *B. cereus* 28-9 (16), *SpChiD* showed affinity toward soluble chitin substrate. The difference in the hydrolyzing/binding activity of *SpChiD* may not be solely due to the difference in the binding activity or absence of accessory binding domains. The interactions from several aromatic residues

FIG 7 MALDI-TOF MS analysis of products from DP3 to DP6 substrate hydrolysis and TG catalyzed by *SpChiD*. After HPLC analysis of reaction products from DP3 to DP6 substrates (15-min sample for DP3 and 45-min sample for DP4, DP5, and DP6), 40 μ l of reaction sample was concentrated under reduced pressure at 25°C and finally dissolved in 4 μ l of MilliQ H₂O. The reaction mixture (2 μ l) was mixed with an equal volume of 2, 5-dihydroxybenzoic acid (2,5-DHB), and the resultant solution was employed for mass measurements using an Ultraflex MALDI-TOF/TOF instrument. Peaks in all MALDI-TOF MS spectra are labeled according to their observed atomic mass and the DP of the oligosaccharide. (A to D) MALDI-TOF MS spectrum of the enzymatic products from DP3 to DP6 substrates, respectively. (E to H) Magnifications for low-peak-area products from these substrates.

FIG 9 Three-dimensional model of *SpChiD*. The model of *SpChiD* was generated by Modeler9v8 (<http://www.salilab.org/modeler/>) using chitinase II (PDB ID 3QOK) from *K. pneumoniae* as the structural template. The core domain is shown in a blue cartoon representation. The region representing the α/β domain that constitutes one of the “walls” of the substrate-binding cleft is colored pink. Solvent-exposed aromatic amino acids in the substrate-binding cleft are shown in a stick representation with carbon, oxygen, and nitrogen atoms colored light green, red, and dark blue, respectively. The catalytic glutamate is shown in a stick representation with carbon, oxygen, and nitrogen atoms colored yellow, red, and dark blue, respectively. The figure was prepared using PyMOL (<http://www.pymol.org/>).

located at the catalytic domains of chitinases probably are essential for substrate binding by *SpChiD* (38).

TLC analysis revealed that chitobiose could also be the substrate for *SpChiD*, suggesting chitobiase activity (see Fig. S3 in the supplemental material). *SpChiD* liberated products 1 unit shorter than the starting substrate (i.e., it produced DP5, DP4, DP3, DP2, and DP1 from DP6 substrate) and also formed higher-DP CHOS products from the DP3 to DP6 substrates. We could not clearly detect >DP6 products on TLC plates due to resolution constraints. The data suggested an endo mode of hydrolysis by *SpChiD* coupled with TG activity (Fig. 4). *SpChiD* showed a more complete degradation of short-chain substrates, with the following order of rate of degradation: DP3 > DP4 > DP5 $\approx$ DP6 $\approx$ DP2.

Formation of NAG from CHOS or polymeric chitinous substrates (Fig. 5) by *SpChiD* was a unique property, since chitobiose is the major end product of several bacterial chitinases. Formation of chitobiose by chitinases could be correlated with the structure of polymers such as cellulose and chitin, in which consecutive sugars are rotated by 180°. As a consequence of that, only every second sugar will have a productive configuration when the polymer is threaded through the active site of an enzyme (49).

The TG reaction occurred swiftly, yielding picomole quantities of the elongated oligomers. MALDI-TOF MS spectra revealed the formation of a few higher-DP TG products, such as DP7, DP10, DP11 to DP12, and DP11 to DP13 from DP3, DP4, DP5, and DP6

substrates, respectively (Fig. 7), compared to those seen in HPLC analysis. *SpChiD* is the only chitinase (especially of bacterial origin) having extensive TG activity, and it synthesizes longer CHOS from a lower concentration of substrate and retains TG products for a prolonged duration.

Dose-responsive effects of the enzyme and substrate on *SpChiD* hydrolysis and TG were studied by altering the enzyme and substrate concentrations by 10-fold (Fig. 8). A 10-fold-lower concentration of enzyme yielded products similar to those obtained under standardized conditions (DP1 to DP10), but the substrate degradation was slow. The formation of DP8 product, among the TG products, was high until 30 min. *SpChiD* synthesized DP8 product by combining the major hydrolytic product chitobiose with DP6 substrate. As the DP2 and DP4 products were in unequal proportion, in the initial stages of the reaction, *SpChiD* mostly preferred to hydrolyze the second glycosidic bond. At a 10-fold-lower concentration of *SpChiD*, the formation of DP4 was more than that of DP2 due to utilization of DP2 for TG. A 10-fold-higher concentration of *SpChiD* resulted in a single TG product. This could be due to the increased hydrolytic activity suppressing the TG activity. Similarly, a 10-fold-lower concentration of substrate also resulted in formation of only one TG product. We therefore propose that the TG efficiency, in terms of number and concentration of TG products of *SpChiD*, depends mainly on the ratio of enzyme and substrate concentrations. Formation of TG products was inversely proportional to the enzyme concentration and directly proportional to the substrate concentration.

The TG reaction catalyzed by GHs takes place through two steps: an acceptor molecule binds to the catalytic cleft, and then the glycosidic linkage is newly generated (21, 20, 18). Thus, the amino acid residues responsible for acceptor binding appear to be important for TG activity. Mutation of the middle Asp in the diagnostic DxDxE motif, which interacts with the catalytic Glu during the catalytic cycle, yielded strong TG mutants (50). Correlation between a lower K_m and increased TG was observed for the β -mannanase Man5A from the blue mussel *Mytilus edulis* (19), endo- β -N-acetylglucosaminidase from *Mucor hiemalis* (39), and chitinase A from *Vibrio carchariae* (33).

A deep substrate-binding groove is a characteristic feature of chitinases that act with an exo mode of action or in a processive manner (15, 40). The α/β fold insertion between β -sheets 7 and 8 of the TIM-barrel fold, which is responsible for deepening the substrate-binding groove, was present in *SmChiA*, *SmChiB*, and *SpChiD*, while it was absent in Heva and *SmChiC* (see Fig. S5 in the supplemental material). Shallow catalytic clefts are typically seen among endo-acting, nonprocessive carbohydrate-degrading enzymes. Detailed studies using chitosan as the substrate have shown that *SmChiC* is indeed a nonprocessive enzyme, while *SmChiA* and *SmChiB* are processive enzymes (15, 37). Because of the presence of the α/β fold insertion, *SpChiD* could be considered a processive enzyme similar to *SmChiA* and *SmChiB*. All the residues known to be important for catalysis in family 18 chitinases (serine in the diagnostic SXGG motif and glutamate in DDXE motif) are present in *SpChiD*. Serine was thought to help

FIG 8 Dose-responsive effects of substrate (DP6) and enzyme on hydrolysis/TG catalyzed by *SpChiD*. Reactions mixture containing 3.5 mM and 0.35 mM substrate and different concentrations of enzyme (56 and 5,600 nM) were incubated for different time periods from 0 to 720 min at 40°C. The reaction products were analyzed by isocratic HPLC. (A) *SpChiD* (56 nM) and DP6 substrate (3.5 mM); (B) *SpChiD* (5,600 nM) and DP6 substrate (3.5 mM); (C) *SpChiD* (560 nM) and DP6 substrate (0.35 mM); (D, E, and F) estimated concentrations of CHOS products shown in panels A to C, respectively.

in the stabilization of a temporary surplus of negative charge that develops on the first aspartate of the catalytic sequence motif during catalysis, while glutamate in the active site acts as the catalytic acid (42, 35).

SpChiD has very low sequence identity (<25%) with other well-characterized chitinases, except with chitinase II from *K. pneumoniae*, for which the enzyme characterization was not available. The overall structure of SpChiD resembles that of the human chitotriosidase, although the alignment shows that these two enzymes have only 23% identity. The human chitotriosidase is also a transglycosylating chitinase (1, 9). The product profiles of the two enzymes are similar except that the human chitotriosidase produces DP2 as major product from polymer and CHOS substrates (1). Like in the human chitotriosidase (11), the substrate-binding cleft of SpChiD is lined with solvent-exposed aromatic residues. Some chitinases with such deep clefts have long loops that form a “roof” over the substrate-binding cleft (43, 42); such a roof is absent in both of these chitinases. Both SmChiA and SmChiB are exochitinases and have a deep active-site cleft, which is partially closed by overhanging loops. SpChiD and the human chitotriosidase show more open clefts, suggesting an endo-acting property of these two enzymes. Based on hydrolytic activity and the structure model, SpChiD could be categorized as a processive endochitinase similar to the human chitotriosidase (9).

SpChiD is the first characterized single-catalytic-domain-containing GH18 family bacterial endochitinase. Among the three characterized single-domain chitinases, SpChiD showed significant TG activity on DP3 to DP6 substrates and produced products of up to DP13. Due to the low hydrolytic activity of SpChiD, the TG products were retained for a longer duration than for other reported chitinases with TG activity. The unique features of SpChiD, coupled with other enzymatic processes that generate CHOS of $\geq$ DP6, would be useful for the industrial production of long-chain CHOS for important biological applications. Importantly, the feature of very high TG activity is accompanied by a hitherto rather unprecedented activity among family 18 chitinases, namely, the ability to convert chitobiose, the primary product of processive action, to monomeric sugars.

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