A Structurally Novel Chitinase from the Chitin-Degrading Hyperthermophilic Archaeon *Thermococcus chitonophagus*

Ayumi Horiuchi,[a] Mehwish Aslam,[a] Tamotsu Kanai,[a,b] Haruyuki Atomi[a,b]

Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto, Japan[a]; JST, CREST, Chiyoda-ku, Tokyo, Japan[b]

ABSTRACT

A structurally novel chitinase, *Tc*-ChiD, was identified from the hyperthermophilic archaeon *Thermococcus chitonophagus*, which can grow on chitin as the sole organic carbon source. The gene encoding *Tc*-ChiD contains regions corresponding to a signal sequence, two chitin-binding domains, and a putative catalytic domain. This catalytic domain shows no similarity with previously characterized chitinases but resembles an uncharacterized protein found in the mesophilic anaerobic bacterium *Clostridium botulinum*. Two recombinant *Tc*-ChiD proteins were produced in *Escherichia coli*, one without the signal sequence [*Tc*-ChiD(ΔS)] and the other corresponding only to the putative catalytic domain [*Tc*-ChiD(ΔBD)]. Enzyme assays using *N*-acetylglucosamine (GlcNAc) oligomers indicated that both proteins hydrolyze GlcNAc oligomers longer than $(GlcNAc)_4$. Chitinase assays using colloidal chitin suggested that *Tc*-ChiD is an exo-type chitinase that releases $(GlcNAc)_2$ or $(GlcNAc)_3$. Analysis with GlcNAc oligomers modified with *p*-nitrophenol suggested that *Tc*-ChiD recognizes the reducing end of chitin chains. While *Tc*-ChiD(ΔBD) displayed a higher initial velocity than that of *Tc*-ChiD(ΔS), we found that the presence of the two chitin-binding domains significantly enhanced the thermostability of the catalytic domain. In *T. chitonophagus*, another chitinase ortholog that is similar to the *Thermococcus kodakarensis* chitinase ChiA is present and can degrade chitin from the nonreducing ends. Therefore, the presence of multiple chitinases in *T. chitonophagus* with different modes of cleavage may contribute to its unique ability to efficiently degrade chitin.

IMPORTANCE

A structurally novel chitinase, *Tc*-ChiD, was identified from *Thermococcus chitonophagus*, a hyperthermophilic archaeon. The protein contains a signal peptide for secretion, two chitin-binding domains, and a catalytic domain that shows no similarity with previously characterized chitinases. *Tc*-ChiD thus represents a new family of chitinases. *Tc*-ChiD is an exo-type chitinase that recognizes the reducing end of chitin chains and releases $(GlcNAc)_2$ or $(GlcNAc)_3$. As a thermostable chitinase that recognizes the reducing end of chitin chains was not previously known, *Tc*-ChiD may be useful in a wide range of enzyme-based technologies to degrade and utilize chitin.

Chitin is a β-1,4-linked insoluble linear polymer of *N*-acetylglucosamine (GlcNAc) and is the main component of the exoskeleton of crustaceans and insects, as well as the cell walls of fungi. Chitin is the second most abundant natural polysaccharide after cellulose, and its annual formation rate is in the order of 10^{10} to 10^{11} tons (1). At present, the majority of chitin remains unused, and thus the development of effective methods to convert chitin into useful biomaterials and/or bioenergy is important to maintain our supplies of edible polysaccharides, such as starch.

Chitinases are enzymes responsible for the hydrolysis of chitin polymer, and they produce GlcNAc and/or its oligomers as products. Chitinases are found in a variety of organisms, including bacteria, archaea, fungi, viruses, animals, and plants (1, 2). Chitinases from a number of bacteria have been studied in detail. Members of the *Actinomycetes* usually contain several chitinase genes, for example, *Streptomyces coelicolor* A3(2) contains 11 GH18 family chitinase genes and two GH19 chitinase genes (3, 4). These chitinases include enzymes with different substrate specificities that are active toward various states of chitin (4). The GH19 chitinases from *Actinomycetes* exhibit antifungal activity and have been suggested to play a role in the restriction of fungal growth (4–6). The chitinolytic machinery of *Serratia marcescens*, a member of the order *Enterobacteriales*, has also been extensively studied, lead-ing to the identification of multiple chitinases with different catalytic properties (7).

In general, the GH18 family chitinases are the largest in number (>8,000 in the CAZy database), and >400 enzymes have been characterized. GH19 family chitinases also form a large group and are found almost exclusively in *Bacteria* and *Eucarya* (mainly in plants) (8–12). Although the numbers are small, chitinases from other families have also been characterized. A GH23 protein encoded by a goose-type (G-type) lysozyme gene from a moderately thermophilic bacterium, *Ralstonia* sp. strain A-471, displays chitinolytic activity but not lysozyme activity (13, 14). A GH48 protein (APAP I) from the leaf beetle, *Gastrophysa atrocyanea*, also

exhibits chitinase activity (15), while most other members in this family display hydrolytic activity for cellulose or its derivatives. There is also a report on a structurally novel type chitinase from the hyperthermophilic acidophilic archaeon *Sulfolobus tokodaii* (16), which has not yet been classified to any of the glycosyl hydrolase (GH) families.

The presence of more than a dozen chitinases from thermophiles has been reported, but only a limited number are found in hyperthermophiles. The chitinase first characterized from a hyperthermophile is the chitinase ChiA from the hyperthermophilic archaeon *Thermococcus kodakarensis* (*Tk*-ChiA) (17). *Tk*-ChiA contains dual catalytic domains, both belonging to the GH18 family, and enzymatic analyses on full-length and truncated proteins indicated that the amino-terminal domain catalyzes an exo-type cleavage of the chitin chain, while the carboxy-terminal domain catalyzes an endo-type cleavage. The presence of two catalytic domains in *Tk*-ChiA with different specificities results in a synergistic effect on chitin degradation (17, 18). *Tk*-ChiA also contains three chitin-binding domains (ChBDs). The ChBD located at the amino terminus belongs to family 5 of the carbohydrate-binding module (CBM), while the other two domains of the CBM family 2 are located consecutively between the two catalytic domains. In the hyperthermophilic archaeon *Pyrococcus furiosus*, two putative chitinase genes located next to each other on the genome were expressed in *Escherichia coli*, and their products (ChiA and ChiB from *P. furiosus* [*Pf*-ChiA and *Pf*-ChiB, respectively]) were found to display chitinase activity (19, 20). The catalytic domains of *Pf*-ChiA and *Pf*-ChiB are structurally and enzymatically orthologous to the amino- and carboxy-terminal catalytic domains of *Tk*-ChiA, respectively. It has been pointed out that the *Pf*-ChiA and *Pf*-ChiB genes are separated due to a single-base insertion in the locus, and that its deletion would lead to a gene structurally similar to that of *Tk*-*chiA*. Deletion of the base from the *P. furiosus* genome resulted in a strain that grew well on chitin (21).

Thermococcus chitonophagus is a unique hyperthermophilic archaeon isolated from an enriched culture of a sample from a hydrothermal vent site using medium containing chitin as the sole carbon and energy source (22). In *T. chitonophagus*, the presence of three proteins showing chitinolytic activity has been suggested. A protein with an apparent molecular mass of 70 kDa (Chi70) was purified from the cell membrane fraction. Chi70 exhibits an endo-chitinase activity, with *N,N*′-diacetylchitobiose [(GlcNAc)$_2$] being the major hydrolysis product (23). Another enzyme with an apparent molecular mass of 50 kDa, Chi50, was partially purified from the culture supernatant and exhibits an exo-chitinase activity that exclusively produces (GlcNAc)$_2$ from colloidal chitin (23). In addition, activity staining on a gel after electrophoresis of whole-cell extracts of *T. chitonophagus* indicated the presence of a 90-kDa protein, designated Chi90, that can hydrolyze 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (chitobiase activity) (23). A portion of the Chi70 gene has been identified (24), but the complete primary structures of these enzymes are still unknown.

In this study, we have identified and characterized a structurally novel chitinase from *T. chitonophagus*. The enzyme, *Tc*-ChiD, does not exhibit sequence similarity to any protein that has been demonstrated to exhibit chitinase activity. The enzymatic properties of the protein suggest that *Tc*-ChiD recognizes the reducing end of the chitin chain and releases (GlcNAc)$_2$ as the major product.

MATERIALS AND METHODS

Strains and culture conditions. *T. chitonophagus* DSM 10152 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Cultivation of *T. chitonophagus* was performed basically with the same method applied for *T. kodakarensis* (25). *T. chitonophagus* was grown under anaerobic conditions at 85°C in a nutrient-rich medium (ASW-YT) supplemented with elemental sulfur (S^0). ASW-YT medium was composed of 0.8× artificial seawater (0.8× ASW), 5.0 g liter^{-1} yeast extract, 5.0 g liter^{-1} tryptone, and 0.8 mg liter^{-1} of resazurin (as a redox indicator). After autoclaving, Na$_2$S solution was added to the medium until it became colorless, and S^0 (2.0 g liter^{-1}) was added prior to inoculation.

Escherichia coli DH5α and *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene) were used for plasmid construction and heterologous gene expression, respectively. *E. coli* cells were cultivated at 37°C in Luria-Bertani (LB) medium containing ampicillin (100 μg liter^{-1}). Unless mentioned otherwise, all chemicals were purchased from Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

Genomic DNA extraction and draft genome analysis of *T. chitonophagus*. *T. chitonophagus* was cultivated in ASW-YT-S^0 medium at 85°C for 12 h. The genomic DNA was extracted using the NucleoBond AXG kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Draft genome analysis was performed at TaKaRa Bio, Inc. (Otsu, Japan) using the HiSeq system (Illumina, San Diego, CA, USA).

Construction of expression plasmids of *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD). Two recombinant *Tc*-ChiD proteins, *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD), were heterologously produced in *E. coli*. *Tc*-ChiD(ΔS) (residues 26 to 805) contains the entire *Tc*-ChiD protein except for the putative signal sequence. *Tc*-ChiD(ΔBD) (residues 321 to 805) contains only the predicted catalytic region. The expression plasmids were constructed as follows. Using the genomic DNA of *T. chitonophagus* as the template, two primer sets (Tc-nChi-f/Tc-nChi-r and Tc-nChi-dBD-f/Tc-nChi-r) were used to amplify the regions corresponding to *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD), respectively. The amplified fragments were digested with NdeI and SalI and inserted into the respective sites of pET21a (Stratagene). The resulting plasmids were designated pET-*Tc*-ChiD(ΔS) and pET-*Tc*-ChiD(ΔBD), respectively.

Preparation of recombinant *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD). *E. coli* cells harboring pET-*Tc*-ChiD(ΔS) or pET-*Tc*-ChiD(ΔBD) were cultivated at 37°C in LB medium until the optical density at 660 nm reached ~0.3. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM to induce overexpression. After cultivation of cells for a further 4 h, cells were harvested by centrifugation (5,000 × *g* for 20 min at 4°C). The cell pellets were resuspended with 50 mM Tris-HCl (pH 7.5). These cell suspensions were disrupted by sonication, and crude cell extracts were obtained by centrifugation (5,000 × *g* for 20 min at 4°C). The cell extracts were further incubated at 80°C for 15 min and centrifuged (20,400 × *g* for 10 min at 4°C). Supernatants after the centrifugation were applied to an anion-exchange column, Resource Q (GE Healthcare Biosciences), equilibrated with 50 mM Tris-HCl (pH 7.5). Both *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD) were eluted by a linear gradient of NaCl (0 to 1 M) using the protein purification system ÄKTA avant 25 (GE Healthcare Biosciences). For the purification of *Tc*-ChiD(ΔS), the peak fractions after anion-exchange column were collected, and (NH$_4$)$_2$SO$_4$ was added to a final concentration of 1.5 M. The mixture was centrifuged (5,000 × *g* for 20 min at 4°C), and the resulting precipitate was dissolved in 50 mM Tris-HCl (pH 7.5) plus 150 mM NaCl. For the purification of *Tc*-ChiD(ΔBD), (NH$_4$)$_2$SO$_4$ was added to the peak fractions after anion-exchange chromatography at a final concentration of 1.0 M, and the mixture was applied to a hydrophobic interaction column, Resource ISO (GE Healthcare Biosciences), and eluted with a linear gradient of (NH$_4$)$_2$SO$_4$ (1 to 0 M). *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD) proteins after each step were collected and further applied to a gel filtration column, Superdex 200 (GE Healthcare Biosciences) equilibrated with 50 mM Tris-HCl (pH 7.5) plus 150 mM NaCl. Fractions containing *Tc*-ChiD(ΔS) or *Tc*-ChiD(ΔBD)

were concentrated using Vivaspin 2 (Sartorius, Göttingen, Germany). The protein concentrations were determined by the Bio-Rad protein assay system (Bio-Rad, Hercules, CA), using bovine serum albumin as a standard.

Preparation of colloidal chitin. Chitin from crab shells (2.5 g; Nacalai Tesque) was mixed with 125 ml of phosphoric acid (85% [wt/vol] in H_2O) and stirred at 4°C for 24 h. The suspension was poured into 1.25 liter of deionized H_2O and centrifuged (15,300 × g for 10 min at 4°C). The resulting precipitate was washed with deionized H_2O until the pH of the suspension became neutral, and it was suspended with 100 ml of deionized H_2O. The concentration of chitin in the suspension was determined by measuring dry chitin weight.

Chitinase assay by thin-layer chromatography. Chitin degradation activity of *Tc*-ChiD(ΔS) or *Tc*-ChiD(ΔBD) was measured using the GlcNAc oligomers G2 (N,N'-diacetylchitobiose), G3 (N,N',N''-triacetylchitotriose), G4 (N,N',N'',N'''-tetraacetylchitotetraose), G5 (N,N',N'',N''',N''''-pentaacetyl-chitopentaose), and G6 ($N,N',N'',N''',N'''',N'''''$-hexaacetylchitohexaose) (Seikagaku Corporation, Tokyo, Japan) (Megazyme, Co. Wicklow, Ireland) or their *p*-nitrophenyl (*p*NP) derivatives (*p*NP-G2 [*p*-nitrophenyl N,N'-di-acetylchitobioside], *p*NP-G3 [*p*-nitrophenyl N,N',N''-triacetylchitotrioside], *p*NP-G4 [*p*-nitrophenyl N,N',N'',N'''-tetraacetylchitotetraoside], and *p*NP-G5 [*p*-nitrophenyl N,N',N'',N''',N''''-pentaacetylchitopentaoside]) (Seikagaku Corporation). In a typical reaction, GlcNAc oligomers (1.4 mg ml^{-1}) or *p*-nitrophenyl GlcNAc oligomers (1 mM) were added with enzyme (0.67 μM) in 50 mM sodium phosphate (pH 5.0), and reactions were performed at 85°C for a defined time, like 5, 15, 30, 60, or 120 min. Reaction mixtures were spotted onto a silica gel plate (DC Kieselgel 60; Merck Co., Berlin, Germany) and developed with a solvent composed of 1-butanol–methanol–ammonia solution (25% in water)–water (5:4:2:1 [vol/vol/vol/vol]). The products were determined by spraying the plate with aniline-diphenylamine reagent (4 ml of aniline, 4 g of diphenylamine, 200 ml of acetone, and 30 ml of 85% phosphoric acid) and baking at 180°C for 3 min.

Effects of pH and temperature on chitinase activity. Chitinase activity was measured under various pH and temperature conditions with the Schales procedure, with some modification (26). Colloidal chitin was used as the substrate (final concentration, 0.2% [wt/vol]), and the standard assay was performed at 85°C in 50 mM sodium phosphate (pH 5.0) for 10 min. Reactions were terminated by cooling samples on ice, and the amount of reducing sugar generated was measured using GlcNAc as the standard. The pH dependence of chitinase activity was determined at 85°C with the following buffers: 50 mM sodium citrate (pH 3.0 to 5.0), 50 mM sodium phosphate (pH 4.5 to 6.0), and 50 mM 2-morpholinoeth-anesulfonic acid monohydrate (MES) (pH 6.0 to 7.0). The optimal temperature was examined between 50 and 95°C in 50 mM sodium phosphate buffer (pH 5.0) using colloidal chitin as the substrate.

Thermostability of the recombinant *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD) proteins. The purified *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD) proteins in 50 mM Tris-HCl (pH 7.5) supplemented with 150 mM NaCl were incubated for various periods of time at 80, 90, or 100°C. After cooling the protein solutions on ice, the remaining chitinase activity was measured by the modified Schales procedure described above.

Nucleotide sequence accession numbers. The gene sequences of *T. chitonophagus chiA*, *chiC*, and *chiD* have been deposited in the DDBJ/EMBL/GenBank databases under the accession numbers LC134313, LC134314, and LC121823, respectively.

RESULTS

Draft genome analysis of *T. chitonophagus* and identification of *Tc*-ChiD. As *T. chitonophagus* is able to grow using chitin as the sole carbon source, the organism is expected to possess an efficient system for chitin degradation and assimilation. In order to elucidate the chitin degradation process, we performed a genomic analysis of *T. chitonophagus*. The genomic DNA of *T. chitonophagus* was isolated and analyzed by HiSeq 2000 (Illumina) using a paired-end sequencing strategy. From the total nucleotide reads of

>2,073 Mbp, 20 contigs were assembled that constituted 1,964,779 nucleotides with a G+C content of 44.91%. Open reading frame (ORF) prediction resulted in the identification of 2,168 ORFs.

In order to identify genes involved in chitin degradation, we searched for protein sequences that included regions displaying sequence similarity to the following elements in *Tk*-ChiA: (i) the exo-chitinase domain, (ii) the endo-chitinase domain, (iii) the ChBD of CBM family 2, and (iv) the ChBD of CBM family 5. In all four cases, an orthologous gene for *Tk*-*chiA* was identified. The gene consists of 3,870 bp and encodes a protein (*Tc*-ChiA) of 1,289 amino acid residues (approximately 142 kDa) (see Fig. S1 and S2 in the supplemental material). *Tc*-ChiA displays a domain structure identical to that of *Tk*-ChiA: a signal sequence, a ChBD of the CBM family 5, a GH18 family catalytic domain (74% identical to the exo-chitinase domain of *Tk*-ChiA), two consecutive ChBDs of the CBM family 2, and a second GH18 family catalytic domain (73% identical to the endo-chitinase domain of *Tk*-ChiA). Using the exo-chitinase domain of *Tk*-ChiA, we identified another gene encoding a putative chitinase adjacent to *Tc*-*chiA*. The gene consists of 1,806 bp and encodes a protein (*Tc*-ChiC) of 601 amino acid residues (approximately 68.1 kDa) containing a signal sequence, a ChBD of the CBM family 5, and a GH18 family catalytic domain (66% identical to the exo-chitinase domain of *Tk*-ChiA) (see Fig. S1 and S3 in the supplemental material). *Tc*-ChiC contains a putative transmembrane region at the carboxy terminus.

A third gene was identified with a search using the *Tk*-ChiA ChBD of CBM family 2. The gene consists of 2,418 bp and encodes a protein (designated here *Tc*-ChiD) of 805 amino acid residues (approximately 90 kDa) (see Fig. S1 and S4 in the supplemental material). The amino-terminal sequence of *Tc*-ChiD includes two basic residues (KR), followed by a hydrophobic stretch of 11 residues, suggesting that it corresponds to a signal sequence for secretion. Following the signal sequence, two putative ChBDs (ChBD1, residues 33 to 133; ChBD2, residues 181 to 280) are present. Both domains belong to CBM family 2, and they are 41.2% identical to each other. These ChBDs show high sequence similarities with the second (39.6% and 59.2% identical, respectively) and third (39.0% and 56.6%, respectively) ChBDs of *Tk*-ChiA (17) and the ChBD (38.0% and 56.7%, respectively) of *Pf*-ChiB (20) (see Fig. S5 in the supplemental material). According to three-dimensional (3D) structural analyses of ChBDs from *Tk*-ChiA and *Pf*-ChiB, the chitin-binding surface is composed of three solvent-exposed tryptophan residues (Trp^{636}, Trp^{669}, and Trp^{687} for ChBD2 of *Tk*-ChiA; Trp^{779}, Trp^{812}, and Trp^{830} for ChBD3 of *Tk*-ChiA; and Trp^{274}, Trp^{308}, and Trp^{326} for *Pf*-ChiB) (27–29). These tryptophan residues are also conserved in both ChBDs of *Tc*-ChiD. In contrast to the amino-terminal region, the function of the carboxy-terminal region of *Tc*-ChiD could not be clearly predicted based on primary structure alone. A homology search using the region spanning residues 321 to 805 of *Tc*-ChiD identified a homologous group of proteins found in various strains of *Clostridium botulinum*, a mesophilic anaerobic bacterium (see Table S1 in the supplemental material). The proteins in this group are mostly annotated as hypothetical proteins, but some are predicted to be related to chitinase, such as H04402_02911 in *C. botulinum* strain H04402 065 (annotated as chitinase Jessie 3) and CLL_A1377 in *C. botulinum* strain B Eklund 17B (NRP) (annotated as secreted chitodextrinase). Although the annotation of CBO2831 from *C. botulinum* strain Hall
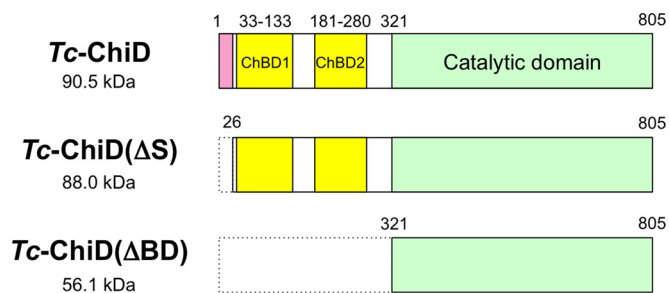
FIG 1 Structural features of the ChiD from *T. chitonophagus* (*Tc*-ChiD) and truncated *Tc*-ChiD proteins [*Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD)]. A putative signal sequence (pink, residues 1 to 25), two chitin-binding domains (ChBDs) (yellow, residues 33 to 133 and 181 to 280), and a putative catalytic domain (green, residues 321 to 805) are indicated.

FIG 2 SDS-polyacrylamide gel electrophoresis of *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD) purified from recombinant *E. coli* strains. Purified *Tc*-ChiD(ΔS) (87.8 kDa) and *Tc*-ChiD(ΔBD) (56.1 kDa) were applied (1 μg each).

A is a hypothetical protein, Sebaihia et al. (30) described that it encodes a predicted secreted protein that is weakly similar to chitinases but has no apparent catalytic or chitin-binding domains. *Tc*-ChiD and its homologs in *C. botulinum* strains do not contain the conserved motifs frequently observed in chitinases, such as the DXDXE catalytic motif of GH18 family chitinases or the Y-[FHY]-G-R-G-[AP]-x-Q-[IL]-[ST]-[FHYW]-[HN]-[FY]-NY motif found in GH19 family chitinases (31). To our knowledge, there is no report on the biochemical analysis of these proteins from *Clostridium*. The carboxy-terminal domain of *Tc*-ChiD also exhibits weak homology to eucaryal proteins found in the genus *Entamoeba* belonging to the phylum *Amoebozoa*, for example, Jessie 3 lectin of *Entamoeba histolytica* (EHI_152170) (32). *E. histolytica* Jessie 3 lectin harbors a unique ChBM of CBM family 55, followed by a domain showing weak similarity to the carboxy-terminal domain of *Tc*-ChiD (see Table S1). The Jessie 3 lectin of *E. histolytica*, produced as recombinant proteins fused with a maltose-binding domain, did not exhibit chitinase activity (33).

Overexpression and purification of the recombinant *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD). As there was a possibility that *Tc*-ChiD represented a structurally novel chitinase, we set out to elucidate the biochemical function of the protein. Two types of recombinant *Tc*-ChiD proteins were prepared. One includes the entire protein without the predicted signal sequence [*Tc*-ChiD(ΔS)], and the other contains only the region downstream of the two ChBDs [*Tc*-ChiD(ΔBD)] (Fig. 1). The two proteins were produced in *E. coli* using the T7 promoter/pET21a system and purified to homogeneity (Fig. 2), as described in Materials and Methods.

Chitinase activity of *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD). The chitinase activities of the recombinant *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD) proteins were examined by thin-layer chromatography (TLC) using (GlcNAc)$_n$ oligomers (n = 2 to 6) as the substrates (Fig. 3A and B). For both enzymes, degradation products were observed for (GlcNAc)$_n$ substrates longer than n = 4 [i.e., (GlcNAc)$_4$, (GlcNAc)$_5$, and (GlcNAc)$_6$], whereas (GlcNAc)$_2$ and (GlcNAc)$_3$ were not hydrolyzed. When using (GlcNAc)$_4$ as the substrate, the product was (GlcNAc)$_2$. (GlcNAc)$_5$ was converted into (GlcNAc)$_3$ and (GlcNAc)$_2$. When using (GlcNAc)$_6$ as the substrate, (GlcNAc)$_3$ and (GlcNAc)$_2$ were generated.

In order to determine whether the *Tc*-ChiD proteins were endo-type enzymes or whether they were exo-type enzymes that recog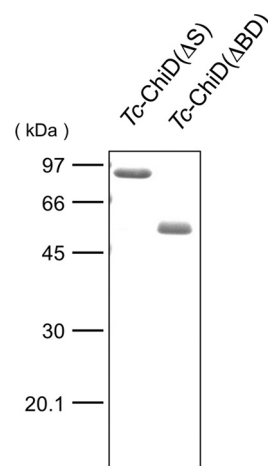nized the ends of (GlcNAc)$_n$ chains, we next examined the products generated from the colloidal chitin. As shown in Fig. 4, the main products were (GlcNAc)$_2$ and (GlcNAc)$_3$, indicating that both proteins recognized the ends of chitin oligomers and are exo-type enzymes. We also observed that reactions with *Tc*-ChiD(ΔS) resulted in a greater accumulation of product than those with *Tc*-ChiD(ΔBD).

In order to identify the end (reducing and/or nonreducing) recognized by *Tc*-ChiD, *p*-nitrophenyl GlcNAc oligomers, in which the reducing end is modified with *p*-nitrophenol (*p*NP), were used as the substrates. *p*NP-(GlcNAc)$_n$ (n = 2 to 5) substrates were individually incubated with *Tc*-ChiD(ΔS) or *Tc*-ChiD(ΔBD), and the free *p*NP released from the substrates was monitored by measuring absorption at 405 nm. Absorbance was not detected in any of the reactions (data not shown), indicating that both enzymes at least do not cleave the first glycosidic bond that links the reducing end of GlcNAc oligomer and *p*NP. In order to identify the degradation products, the reaction mixtures were analyzed by TLC (Fig. 5). As a result, *p*NP-(GlcNAc)$_2$ and *p*NP-(GlcNAc)$_3$ were not hydrolyzed by either enzyme. When *p*NP-(GlcNAc)$_4$ was used as the substrate, spots corresponding to *p*NP-(GlcNAc)$_2$ and (GlcNAc)$_2$ were detected. In the case of *p*NP-(GlcNAc)$_5$, spots corresponding to *p*NP-(GlcNAc)$_2$ and (GlcNAc)$_3$ were detected, and *p*NP-(GlcNAc)$_3$ could not be detected. These results suggest that *Tc*-ChiD recognizes the reducing end of chitin and cleaves the second glycosidic linkage.

In order to confirm that *Tc*-ChiD is specific toward chitin, the enzyme was incubated with other oligosaccharides, such as chitosan (β-1,4-linked glucosamine oligomers), cellulose (β-1,4-linked glucose oligomers), and amylose (α-1,4-linked glucose oligomers). Degradation products were not observed, confirming that *Tc*-ChiD is a chitinase (data not shown).

Enzymatic properties of *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD). Chitinase activities of *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD) were analyzed under near-optimal growth conditions of *T. chitonophagus*. Chitinase activities of *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD) were quantified by a modification of the Schales procedure using 0.2% (wt/vol) colloidal chitin as the substrate. When we examined the effects of temperature on activity, *Tc*-ChiD(ΔBD) displayed the
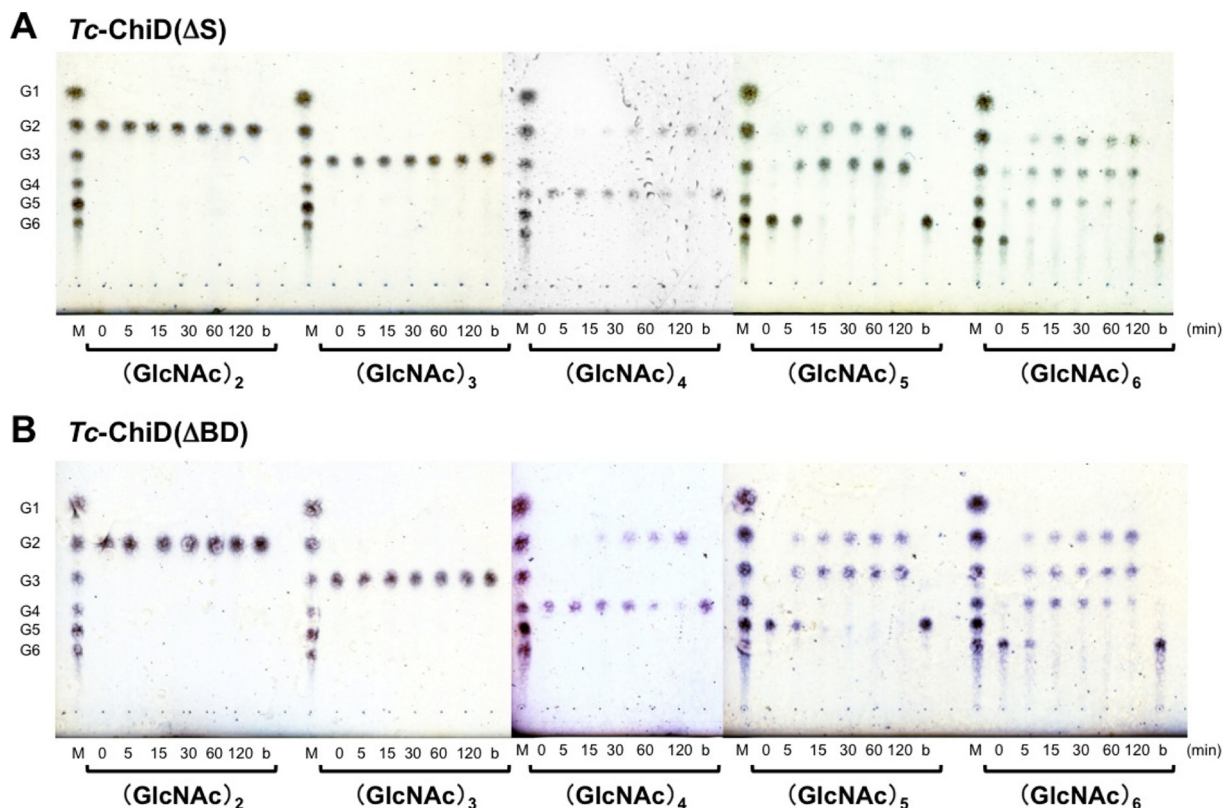
FIG 3 TLC of hydrolysis products of various GlcNAc oligomers incubated with *Tc*-ChiD(ΔS) (A) or *Tc*-ChiD(ΔBD) (B). Reaction mixtures containing 1.4 g liter^{-1} substrate in 50 mM sodium phosphate buffer (pH 5.0) were incubated with either *Tc*-ChiD(ΔS) or *Tc*-ChiD(ΔBD) (6.8 μM each) at 85°C for the indicated time periods (0 to 120 min). Lanes M, standard GlcNAc oligomers ranging from GlcNAc to (GlcNAc)$_6$ (G1 to G6); lanes b, blank control incubated without enzyme for 120 min at 85°C.

highest activity at 80°C (1.02×10^2 μmol min^{-1} μmol protein^{-1}), with a significant decrease observed at temperatures of >90°C, whereas *Tc*-ChiD(ΔS) exhibited the highest activity at 90°C (3.12×10^1 μmol min^{-1} μmol protein^{-1}) (Fig. 6A). Arrhenius plots of the data indicate that the activation energies of the reactions were 80.5 kJ mol^{-1} for *Tc*-ChiD(ΔS) and 24.3 kJ mol^{-1} for *Tc*-ChiD(ΔBD). The effects of pH on chitinase activity were also examined at 85°C under various pH conditions (pH 3.0 to 7.0). As a result, both enzymes showed high activity at a pH range from 4.0 to 4.5 (Fig. 6B). Interestingly, *Tc*-ChiD(ΔS) showed an increase in activity at around pH 6.0. In terms of thermostability, the two enzymes were incubated at 80, 90, or 100°C for various periods of time, and their residual activities were examined (Fig. 6C). As a result, the half-life of *Tc*-ChiD(ΔBD) was 40 min at both 80 and 90°C, and the residual activity dramatically decreased to approximately 10% within 5 min at 100°C. In the case of *Tc*-ChiD(ΔS), the half-lives at 80°C, 90°C, and 100°C were 10 h, 1.3 h, and 48 min, respectively. The results indicate that the catalytic domain alone [*Tc*-ChiD(ΔBD)] displays higher initial velocity, but the presence of the ChBDs of *Tc*-ChiD [*Tc*-ChiD(ΔS)] greatly contributes to increasing the thermostability of the protein. This is reflected in the results of Fig. 4, in which product formation was examined after relatively long reaction times at 85°C (4 to 36 h).

DISCUSSION

In this study, we have identified a structurally novel chitinase, *Tc*-ChiD, from the hyperthermophilic archaeon *T. chitonophagus*.
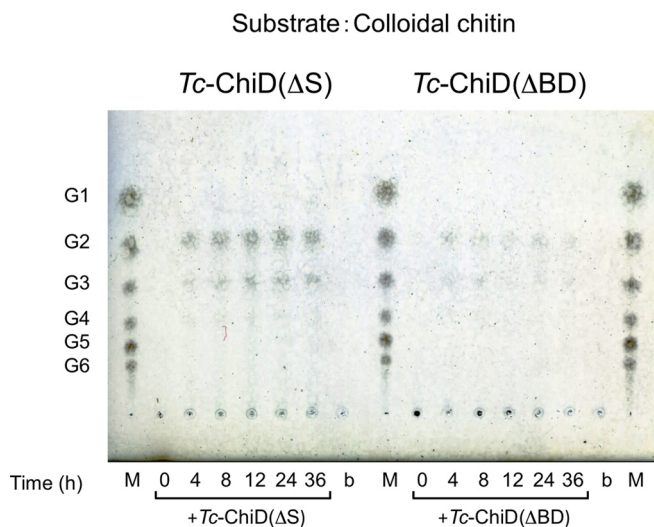

FIG 4 TLC analysis of reaction products of colloidal chitin incubated with *Tc*-ChiD(ΔS) or *Tc*-ChiD(ΔBD). Reaction mixtures containing 0.2% (wt/vol) colloidal chitin as a substrate in 50 mM sodium phosphate buffer (pH 7.0) were incubated with either *Tc*-ChiD(ΔS) or *Tc*-ChiD(ΔBD) (0.67 μM) at 85°C for the indicated time periods (0 to 36 h). The reaction products were analyzed after centrifugation. Lanes M, standard GlcNAc oligomers ranging from GlcNAc to (GlcNAc)$_6$ (G1 to G6); lanes b, blank control incubated without enzyme for 36 h at 85°C.
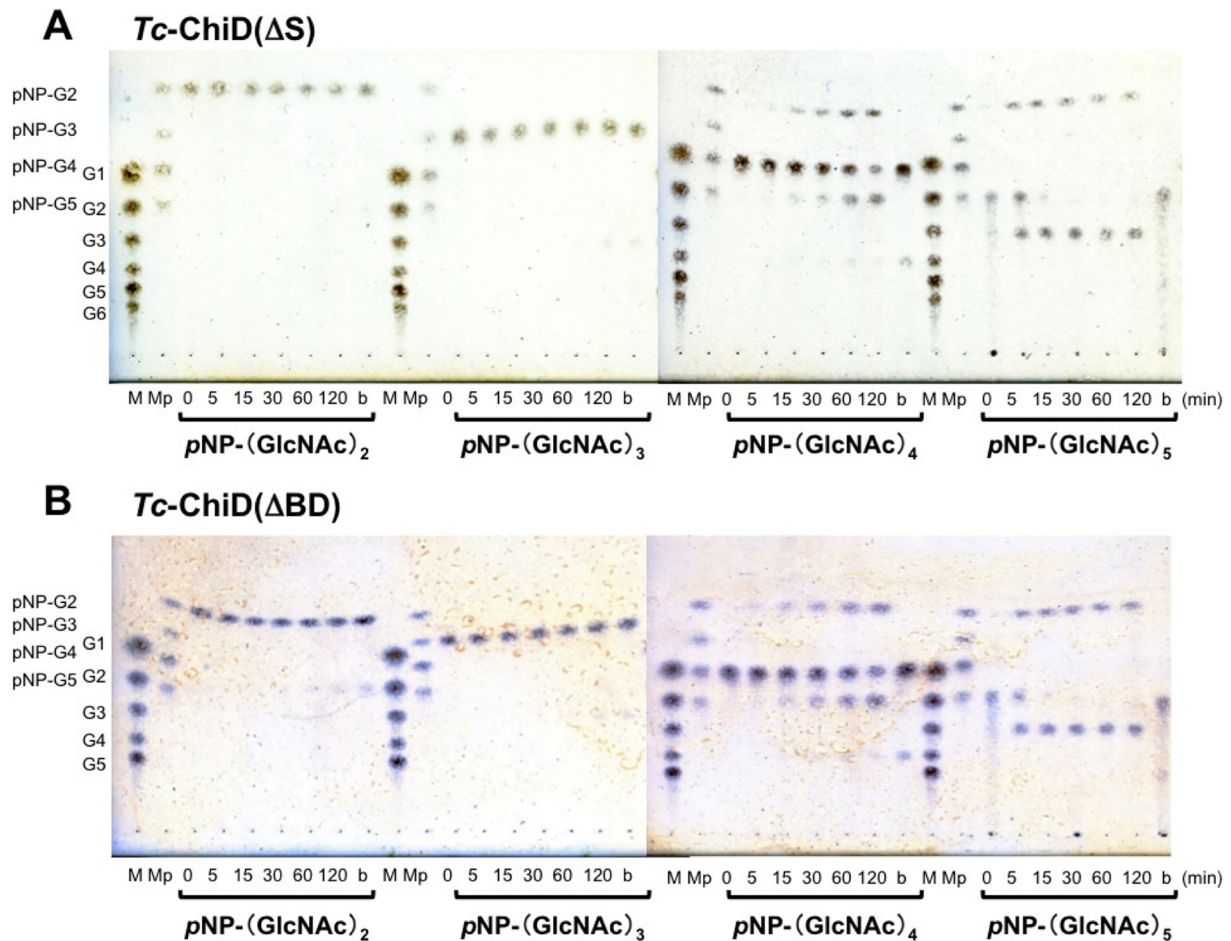
FIG 5 TLC of hydrolysis products of various *p*-nitrophenyl GlcNAc oligomers incubated with *Tc*-ChiD(ΔS) (A) or *Tc*-ChiD(ΔBD) (B). Reaction mixtures containing 1 mM substrate in 50 mM sodium phosphate buffer (pH 5.0) were incubated with either *Tc*-ChiD(ΔS) or *Tc*-ChiD(ΔBD) (6.8 μM) at 85°C for the indicated time periods (0 to 120 min). Lanes M, standard GlcNAc oligomers ranging from GlcNAc to (GlcNAc)$_6$ (G1 to G6); lanes Mp, standard *p*-nitrophenyl GlcNAc oligomers ranging from *p*NP-(GlcNAc)$_2$ to *p*NP-(GlcNAc)$_5$ (*p*NP-G2 to *p*NP-G5); lanes b, blank control incubated without enzyme for 120 min at 85°C.

Although we have tried to identify chitinases with similarity to *Tc*-ChiD, no chitinase sequences of the GH18 and GH19 families were found at least up to an 8.6 E value in a BLAST search (see Table S1 in the supplemental material). *Tc*-ChiD is specific for chitin and does not cleave chitosan, cellulose, or amylose. The enzyme is an exo-chitinase and mainly releases (GlcNAc)$_2$ units from the reducing ends of chitin chains (Fig. 7).

As indicated above, homologs of the *Tc*-ChiD protein are not widely distributed in nature. Most intriguingly, homologs of *Tc*-ChiD are not found in any of the closely related members of the *Thermococcales* and in any other archaeal genome. The most closely related homologs are confined to members of the bacterial genus *Clostridium*. As *Tc*-ChiD does not display structural similarity to any of the previously established chitinase proteins, the protein can be considered to represent a novel GH family.

Compared to chitinases, cellulases have been studied to a much greater extent. Exo-type cellulases (cellobiohydrolases) are divided into two groups: enzymes that recognize the reducing end (mainly belonging to families GH7 and GH48), and enzymes that recognize the nonreducing end (mainly belonging to GH6 and GH48) (34–37). It has been reported that organisms that harbor both types of exo-type cellulases along with an endo-type enzyme, such as *Thermomonospora fusca* and *Trichoderma reesei* (34), display relatively high efficiency in cellulose degradation. As for chitinases, a synergistic effect toward chitin degradation has been reported for *S. marcescens*, which harbors two exo-type chitinases, *Sm*-ChiA and *Sm*-ChiB, as well as an endo-type enzyme (38, 39). *Sm*-ChiA and *Sm*-ChiB are both members of the GH18 family, but *Sm*-ChiA hydrolyzes chitin from the reducing end, whereas *Sm*-ChiB recognizes the nonreducing end (38–40), enabling *S. marcescens* to degrade chitin chains from both ends. However, reports on chitinases that recognize the reducing end of chitin chains are still limited. *Tc*-ChiD is the first enzyme from an archaeon with such a mode of substrate recognition. As *Tk*-ChiA harbored two chitinase domains that exhibited endo-type activity and an exo-type activity that recognized the nonreducing end of chitin (17, 18), the corresponding protein in *T. chitonophagus* (*Tc*-ChiA) can also be expected to display similar properties. This would result in *T. chitonophagus* harboring both kinds of exo-type chitinases along with an endo-type enzyme, and it agrees well with
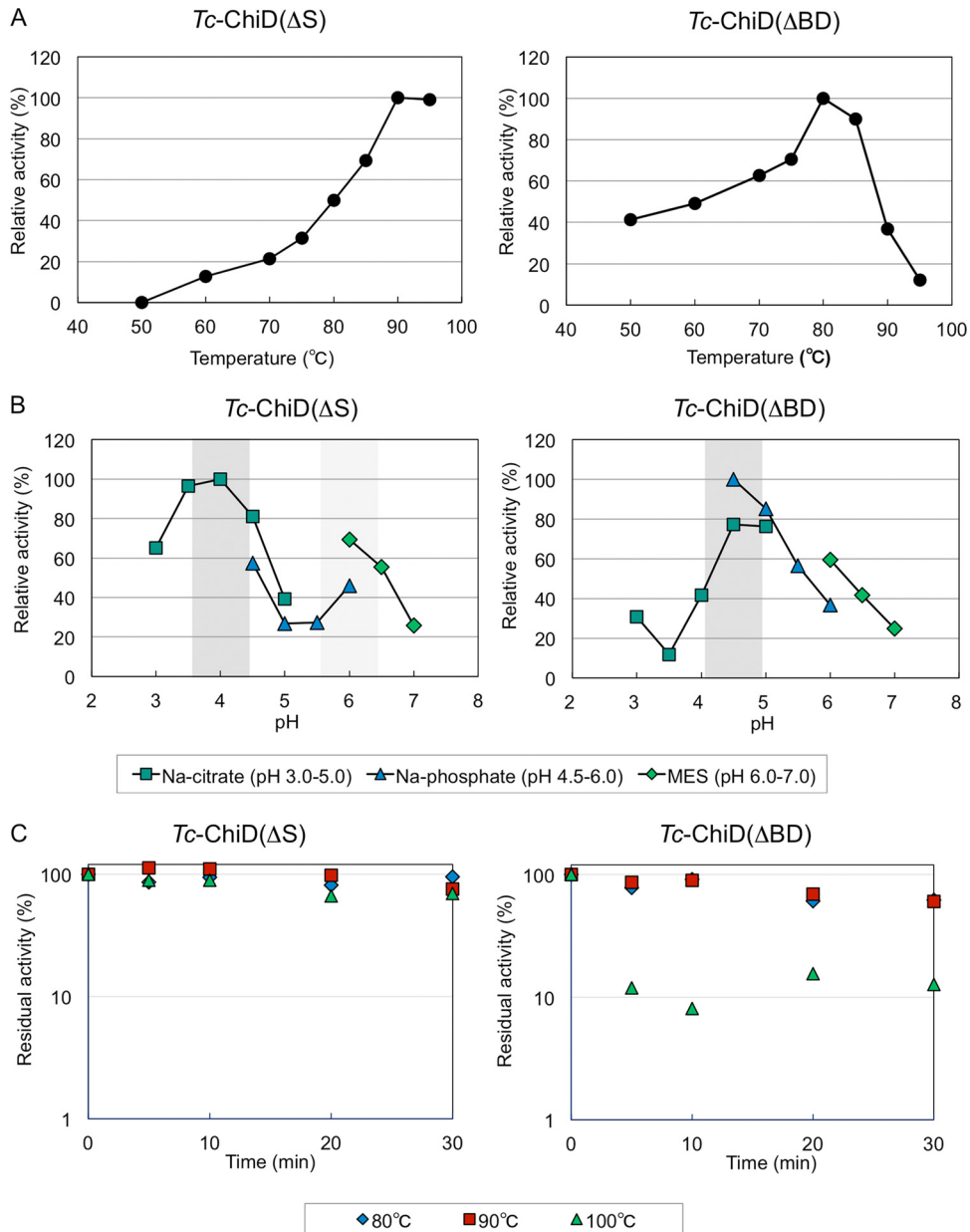
FIG 6 (A) Effects of temperature on the activities of *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD). The chitinase activities were determined at different temperatures (50 to 95°C) by using the Schales procedure, as described in Materials and Methods. (B) Effects of pH on the activities of *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD). The chitinase activities were determined at different pH conditions, as described in Materials and Methods. Symbols: dark green squares, sodium citrate (pH 3.0 to 5.0); blue triangles, sodium phosphate (pH 4.5 to 6.0); light green diamonds, MES (pH 6.0 to 7.0). (C) Thermostabilities of *Tc*-ChiD(ΔS) and *Tc*-ChiD(ΔBD). Purified enzymes were incubated in 50 mM Tris-HCl (pH 7.5) supplemented with 150 mM NaCl at 80°C (blue diamonds), 90°C (red squares), and 100°C (green triangles) for various time intervals. The residual activities were determined at 85°C.

the fact that this archaeon displays high levels of chitin degradation among the *Archaea*.

The putative chitinase genes on the *T. chitonophagus* genome allow us to estimate the sizes of the proteins they encode. As described in the introduction, *T. chitonophagus* has been reported to harbor at least three chitinases with molecular masses of approximately 90, 70, and 50 kDa (Chi90, Chi70, and Chi50, respectively) (23, 41). The *T. chitonophagus* genome sequence determined here suggests the presence of three chitinases: *Tc*-ChiA (142 kDa), *Tc*-ChiC (68 kDa), and *Tc*-ChiD (90 kDa). According to size alone,

Tc-ChiD examined in this study may correspond to Chi90. However, in contrast to the fact that Chi90 was reported as a chitobiase, or β-*N*-acetylglucosaminidase, *Tc*-ChiD displayed exo-chitinase activity only toward chitin chains with a length of ≥4 GlcNAc units. On the other hand, *Tc*-ChiC may correspond to the 70-kDa chitinase, Chi70. *Tc*-ChiA is too large to correspond to Chi90, Chi70, and Chi50. Further studies will be necessary to accurately predict a correspondence between the proteins identified biochemically and the genes identified by genome analysis.
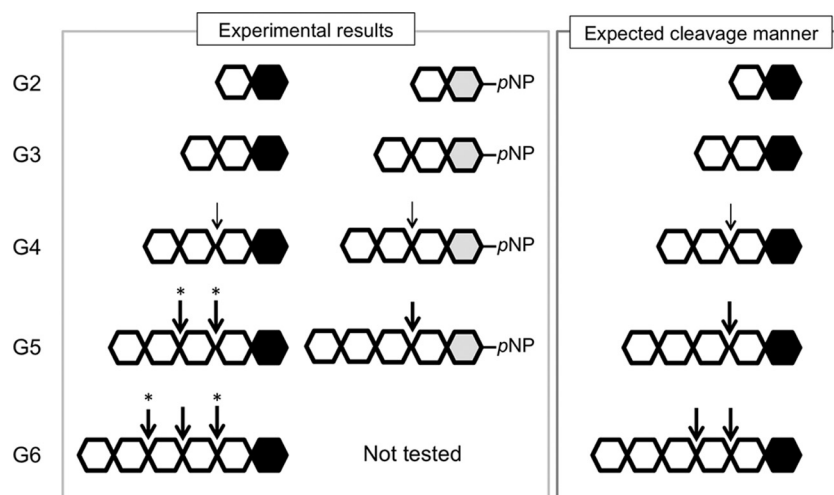
FIG 7 Schematic illustration of the reaction catalyzed by *Tc*-ChiD. Individual GlcNAc monomers are represented by hexagons. The black hexagons indicate the reducing ends of the GlcNAc oligomers, and those in the *p*NP-GlcNAc oligomers are in gray. Thin arrows indicate positions with lower activities. Asterisks indicate cleavage sites that cannot be distinguished in the experiments.

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