



Property and Function of a Novel Chitinase Containing Dual Catalytic Domains Capable of Converting Chitin Into *N*-Acetyl-D-Glucosamine

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A novel multifunctional chitinase (*CmChi3*)-encoding gene was cloned from *Chitinolyticbacter meiyuanensis* and actively expressed in *Escherichia coli*. Sequence analysis showed that *CmChi3* contains two glycoside hydrolase family 18 (GH18) catalytic domains and exhibited low identity with well-characterized chitinases. The optimum pH and temperature of purified recombinant *CmChi3* were 6.0 and 50°C, respectively. *CmChi3* exhibited strict substrate specificity of 4.1 U/mg toward colloidal chitin (CC) and hydrolyzed it to yield *N*-acetyl-D-glucosamine (GlcNAc) as the sole end product. An analysis of the hydrolysis products toward *N*-acetyl chitooligosaccharides (*N*-acetyl COSs) and CC substrates revealed that *CmChi3* exhibits endochitinase, *N*-acetyl- β -D-glucosaminidase (NAGase), and transglycosylase (TGase) activities. Further studies revealed that the N-terminal catalytic domain of *CmChi3* exhibited endo-acting and NAGase activities, while the C-terminal catalytic domain showed exo-acting and TGase activities. The hydrolytic properties and favorable environmental adaptations indicate that *CmChi3* holds potential for commercial GlcNAc production from chitin.

Keywords: chitin, *CmChi3*, dual functional domains, *N*-acetyl-D-glucosamine, hydrolysis pattern, chitinase

INTRODUCTION

Chitin, a linear β -1,4-linked biopolymer of *N*-acetyl-D-glucosamine (GlcNAc), is the second most abundant renewable biomass in nature after cellulose (Kaur and Dhillon, 2015). Chitin is present as a structural constituent in the exoskeletons of arthropods, crustacean shells, and fungal cell walls (Wan and Tai, 2013). About 6–8 million tons of waste crab, shrimp, and lobster shells are produced and discarded annually worldwide, which results in wastage of resources and environmental problems (Yan and Chen, 2015). GlcNAc, which is the monomeric unit of chitin, exhibits many bioactivities and used widely in several fields, such as the food, pharmaceutical, agriculture, and fine chemical industries (Chen et al., 2010). Furthermore, GlcNAc contains nitrogen and is an ideal

feedstock for manufacturing diverse intermediates of high value, such as 3-acetamido-5-acetylfuran (3A5AF) (Zang et al., 2021) and 5-hydroxymethylfurfural (5-HMF) (Zhou et al., 2020). Therefore, it would be economically and environmentally beneficial to produce GlcNAc from the abundant chitin resources, such as waste shrimp and crab shells.

Methods involving the conversion of chitin to GlcNAc have been widely explored to date (Husson et al., 2017; Zhang A. et al., 2021). In the commercial production of GlcNAc from chitin, acid hydrolysis at high temperatures is often used. However, with the increased awareness on environmental protection, chemical methods are not preferred as they are associated with pollution (Wei et al., 2017). Therefore, researchers have recently paid more attention to the enzymatic production of GlcNAc by the hydrolysis of chitin using chitin-degrading enzymes. This enzymatic process involves mild production conditions, provides high yields, and results in a product with high bioactivity (Chen et al., 2010).

Chitin-degrading enzymes, which are essential for chitin degradation, can be divided into endochitinase [randomly cleaves chitin at internal sites to release *N*-acetyl chitooligosaccharides (*N*-acetyl COSs)], exochitinase (hydrolyzes chitin oligosaccharides to liberate GlcNAc dimer), and *N*-acetyl- β -glucosaminidase (converts *N*-acetyl COSs to GlcNAc) (Dahiya et al., 2006). To efficiently convert chitin to GlcNAc, a multienzyme system containing at least one chitinase and one *N*-acetyl- β -glucosaminidase (NAGase) is often required. However, using multiple enzymes increases the costs, which limits their industrial application (Zhu et al., 2016). Therefore, identifying a multiple-function chitinase that can degrade chitin to GlcNAc and constructing a single-enzyme catalytic system are likely to be advantageous in reducing the cost and simplifying the conversion process. Several reports have shown that some chitinases have multiple catalytic functions and possess NAGase and chitinase activities (Fu et al., 2014; Zhang et al., 2018). However, these multifunctional enzymes always produce and accumulate GlcNAc dimer in the process of hydrolyzing chitin to prepare GlcNAc.

In our previous study, the chitinolytic bacterium *Chitinolyticbacter meiyuanensis* was isolated, and its extracellular chitin-degrading enzymes were found to efficiently hydrolyze chitin and produce GlcNAc as the sole product (Hao et al., 2011). Furthermore, several key chitinases and their coding genes were identified by combining whole-genome and peptide fingerprint analysis. Of these, a chitinase (ORF3769) containing two GH18 catalytic domains was observed to degrade chitin to GlcNAc without accumulating GlcNAc dimer. To the best of our knowledge, this is the first report on a chitinase with dual GH18 catalytic domains from *Chitinolyticbacter* species. However, its catalytic properties and specific hydrolysis pattern remain unclear.

In this study, the gene encoding ORF3769 (named *CmChi3*) was cloned from strain SYBC-H1 and heterologously expressed in *Escherichia coli* BL21(DE3). Sequence analysis, enzymatic properties, and hydrolysis pattern of *CmChi3* were investigated. Furthermore, the individual GH18 catalytic domains of *CmChi3* were studied to determine the hydrolysis

mechanism. The findings were expected to provide a detailed understanding of *CmChi3* and its usefulness in directly generating GlcNAc from chitin.

MATERIALS AND METHODS

Chemicals

Chitin was purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). The standards of *N*-acetyl COSs (purity: $\geq 95\%$) with a degree of polymerization between 2 and 6 were acquired from Qingdao Bozhi Biotechnology Co., Ltd. (Qingdao, China). Peptone and yeast extract were purchased from Oxoid Co., Ltd. (Beijing, China). All molecular reagents were purchased from TaKaRa Co., Ltd. (Dalian, China). Colloidal chitin (CC) was prepared as described by the method of Gao et al. (2015). Other chemicals and solvents used in this study were purchased from local suppliers and were of analytical grade.

Strains, Culture Conditions, and Plasmids

Chitinolyticbacter meiyuanensis SYBC-H1 (ATCC BAA-2140) used in this study was isolated and cultivated according to our previous study (Zhang et al., 2018). The *E. coli* strains, plasmids, and primers used in this study are listed in **Supplementary Table 1**. The *E. coli* strains were routinely cultivated aerobically at 37°C in Luria–Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, and 5 g/l NaCl). The transformants were selected on LB plates containing 50 mg/l of ampicillin.

Identification of Wild-Type *CmChi3* From *Chitinolyticbacter meiyuanensis* SYBC-H1

Wild-type *CmChi3* with chitinase activity was purified using chitinase–glycogen complex precipitation followed by autodigestion of the complex according to the method described in our previous study (Zhang et al., 2018). The corresponding protein (*CmChi3*) in the native polyacrylamide gel was sliced for peptide fingerprint analysis using the electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF MS/MS) technique (PROTECH, Inc., Suzhou, China). These masses were then compared with the theoretical values in Mascot website databases¹ to discern the amino acid sequences of the peptide fragments. The sequences were then aligned with the genome of strain SYBC-H1 to find *CmChi3* and its coding gene.

Gene Cloning

The genomic DNA of strain SYBC-H1 was used as the template for polymerase chain reaction (PCR) amplification. The plasmid templates with suitable combinations of primers listed in **Supplementary Table 1** were used to generate *CmChi3*, N-terminal GH18 catalytic domain (*CmChi3nGH18*), and C-terminal catalytic domain (*CmChi3cGH18*). The PCR conditions were as follows: 2 min at 95°C, followed by 35 cycles

¹<http://www.matrixscience.com>

of 95°C for 20 s, 55–62°C for 20 s, and 72°C for 30–90 s, with a final extension at 72°C for 5 min. After purifying by gel electrophoresis, the PCR products and the vectors were subjected to double digestion using restriction enzymes (*Nde*I and *Eco*RI; *Eco*RI and *Hind*III, respectively), followed by ligation using T4 DNA ligase (TaKaRa, Dalian, China). The recombinant plasmids were transformed into *E. coli* DH5 α and sequenced by GenScript Biotech (Nanjing, China).

Sequence Analysis of CmChi3

Nucleotide and amino acid sequences were analyzed using the Snap Gene v.1.1.3 software and the ExpASy ProtParam tool.² The conserved domains and glycoside hydrolase (GH) family classification were identified using the SMART website.³ DNA and protein sequence alignments were performed using the National Center for Biotechnology Information (NCBI) server with programs BLASTN and BLASTP,⁴ respectively. Phylogenetic trees were constructed using the neighbor-joining algorithm in MEGA v.7.0 software and assessed using 1,000 bootstrap replications. The presence of a signal peptide and the enzyme location were analyzed using the SignalP v.5.0 server⁵ and the Gneg-mPLOC server v.2.0,⁶ respectively. Protein homologous sequence alignment was performed using ClustalX v.2.1 software and ESPript v.3.0.⁷ The three-dimensional (3D) structure of CmChi3 was predicted using I-TASSER.⁸

Gene Expression and Protein Purification

The positive clones were directly screened by colony PCR, transformed into *E. coli* BL21(DE3), inoculated in 10 ml LB medium containing 50 mg/l of ampicillin, and cultured at 37°C in a shaker (200 rpm). Once the optical density (OD₆₀₀) of the culture broth reached 0.6–0.8, isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for induction, and the culture was further incubated at 18°C for 20 h. The cells were harvested by centrifugation (6,000 rpm for 10 min at 4°C); after which, they were resuspended in His-tag binding buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 8.0) and lysed by ultrasonication (JY92-IIN, Ningbo Xinzhi Biotechnology, Ltd., Ningbo, China). The cell debris was removed by centrifugation at 8,000 rpm for 30 min at 4°C, and the supernatant was retained as the crude enzyme. The recombinant proteins (chitinases) were purified using a fast protein liquid chromatography (FPLC) system (AKTA Pure 150; GE healthcare Co., Fairfield, CA, United States) with a Ni-nitrilotriacetic acid affinity chromatography (Ni-NTA) column (His Trap FF 5 ml). The target proteins were eluted with elution buffer (20 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, pH 8.0). The eluted fractions were passed through ultrafiltration tubes of 50 and 10 kDa (Millipore, Burlington, MA,

United States) to remove imidazole with 50 mM phosphate buffer saline (PBS, pH 7.0) and concentrate the enzyme solution. All steps in the enzyme purification were performed at 4°C.

Determination of Enzymatic Activity

Activity assay toward the various substrates was performed using the 3,5-dinitrosalicylic acid (DNS) method (Breuil and Saddler, 1985). Unless otherwise indicated, the reaction mixture containing the suitably diluted enzyme and different polysaccharide substrates at a final concentration of 10 g/l in 100 mM sodium phosphate buffer (pH 6.0) was incubated for 30 min at 50°C. The absorbance was measured at 540 nm, and a standard curve was constructed to determine the amount of reducing sugars produced. One unit of chitinase activity was defined as the amount of enzyme required to produce 1 μ mol of reducing sugar at 50°C in 1 min. All chitinase activities were assayed in triplicate, and the average enzyme activity with standard deviation was calculated.

Determination of Protein Concentration and Molecular Weight

Protein content was determined according to the method of Bradford (1976) using bovine serum albumin as the standard. The specific activity was expressed as units per milligram protein.

The molecular weights and purities of protein samples were analyzed by reductive sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 20 mM β -mercaptoethanol incubation. A premixed protein marker (TaKaRa Biotechnology Co., Ltd., Nanjing, China) containing 180–, 130–, 95–, 70–, 53–, 40–, 33–, 25–, 17–, and 10-kDa proteins was used as the molecular mass standards.

Enzymatic Characterization of Recombinant CmChi3

The optimal temperature for chitinase activity was determined over the range of 25–60°C in 100 mM sodium citrate buffer (pH 6.0) by using 10 g/l CC as the substrate. The thermostability of CmChi3 was determined by measuring the residual activity after pre-incubation of the purified enzyme in 100 mM sodium citrate buffer (pH 6.0) at 25–60°C in the absence of the substrate for 2 h. The optimal pH for the chitinase activity was assessed in several buffers at 50°C. The following buffers were used: 100 mM sodium citrate buffer, pH 3.0–6.0; 100 mM phosphate buffer, pH 6.0–8.0; Tris-HCl buffer, pH 8.0–9.0; and 0.4 mol/l glycine sodium hydroxide buffer, pH 9.0–10.0. For pH stability, the CmChi3 was pre-incubated in buffers with different pH at 4°C for 2 h, and the residual activities were determined using 10 g/l of CC as the substrate.

The effects of metal ions on the activity were also determined in this study. Purified CmChi3 was treated with 10 mM ethylenediaminetetraacetic acid (EDTA) for 5 h at 4°C and then dialyzed with 50 mM PBS (pH 7.0) to remove the EDTA. The activities were assayed as described previously and compared with that of an untreated enzyme solution incubated under similar conditions. For reactivation, the metal-free enzyme was incubated with metal ions (Ca²⁺, Cu²⁺, Co²⁺, K⁺, Na⁺, Al³⁺,

²<http://web.expasy.org/protparam/>

³<http://smart.embl-heidelberg.de/>

⁴<http://blast.ncbi.nlm.nih.gov/Blast.cgi>

⁵<http://www.cbs.dtu.dk/services/SignalP/>

⁶<http://www.csbio.sjtu.edu.cn/bioinf/Gneg-multi/>

⁷<http://esprpt.ibcp.fr/ESPript/cgi-bin/ESPript.cgi>

⁸<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>

Ba²⁺, Ni²⁺, Zn²⁺, Mg²⁺, Mn²⁺, Fe³⁺, and Fe²⁺) at a final concentration of 10 mM for 30 min, and the residual activity was determined. The activity prior to EDTA treatment was used as the control (100%).

Substrate Specificity and Kinetic Parameters of Recombinant *CmChi3*

The substrate specificity was determined using 1% (*w/v*) solutions of various polysaccharides [CC, α -chitin powder, β -chitin powder, carboxymethyl cellulose (CMC), hemicellulose powder, and chitosan powder] under the optimum conditions. The amount of reducing sugars released from these polysaccharide substrates was estimated using the DNS method as described previously.

The kinetic parameters against CC under optimum conditions for recombinant *CmChi3* were measured. The initial velocities were determined by incubating 20 μ g of purified *CmChi3* with CC of concentrations ranging from 1 to 20 g/l at 50°C in 1-ml reaction system (100 mM sodium citrate buffer, pH 6.0) for 30 min. The amount of reducing sugars released from these substrates was estimated using the DNS method as described previously. K_m and V_{max} values were obtained by Lineweaver-Burk plots (Price, 1985).

Hydrolysis Pattern of the Recombinant *CmChi3*, *CmChi3nGH18*, and *CmChi3cGH18* Toward Colloidal Chitin

The reaction system (1 ml of 100 mM sodium citrate buffer, pH 6.0) contained CC (10 g/l) and 20 μ g each of purified *CmChi3*, *CmChi3nGH18*, and *CmChi3cGH18*. The reaction was performed at 50°C for various time intervals. The samples were boiled for 5 min to stop the reaction.

Hydrolysis Pattern of the Recombinant *CmChi3*, *CmChi3nGH18*, and *CmChi3cGH18* Toward *N*-Acetyl Chitooligosaccharides

Reaction mixtures containing purified enzymes (0.1 μ g) and *N*-acetyl COSs (DP 2–6) at a final concentration of 10 g/l were incubated in a 20- μ l volume of 100 mM sodium citrate buffer (pH 6.0) at 50°C for various time intervals. The reactions were stopped by boiling for 5 min.

High-Performance Liquid Chromatography Analysis of the Products

The products were detected using an Agilent 1260 series liquid chromatography system (Agilent Technologies, Santa Clara, CA, United States), according to our previous study (Zhang et al., 2018).

Nucleotide Sequence Accession Number

The sequence of the chitinase gene *CmChi3* was deposited in the GenBank database under the accession number MZ559373.

RESULTS AND DISCUSSION

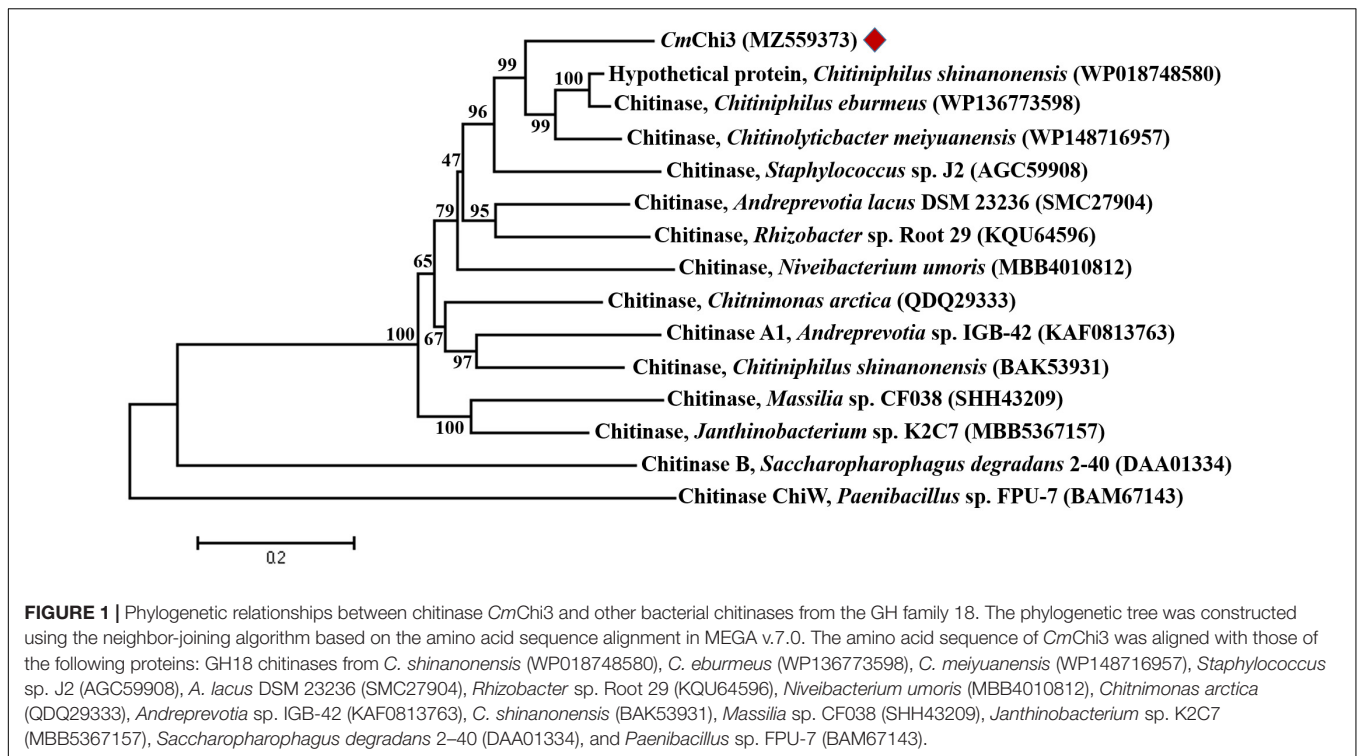
Sequence Analysis of *CmChi3*

The *CmChi3* gene (2,943 bp) encodes a protein of 980 amino acids with a calculated molecular mass and the predicted isoelectric (pI) point of 103 kDa and 6.64, respectively. The signal peptide prediction indicated the presence of a putative N-terminal signal peptide (residue 1–33) in the sequence of *CmChi3*, which signified that *CmChi3* should be a secretory protein and explained the identification of *CmChi3* in the fermentation broth of strain SYBC-H1.

According to the Carbohydrate-Active enZYmes (CAZy) database,⁹ chitinolytic enzymes are mainly divided into GH families 18, 19, and 20 based on amino acid sequence similarity. BLASTP analysis showed that *CmChi3* belonged to GH family 18 (GH18) and shared the highest identity (74.52%) with the GH18 chitinase from *Chitiniphilus eburneus* (WP_136773598), followed by a hypothetical protein (73.57%) from *Chitiniphilus shinanonensis* (WP_018748580). However, these coding genes have not been expressed and studied. Among the characterized GH18 chitinases, *CmChi3* showed the highest identity (54.42%) with the GH18 chitinase from *Chromobacterium* sp. C-61 (AAP88583) (Park et al., 2007), followed by the GH18 chitinase (39.48%) from *Bacillus cereus* (AAM48520) (Huang and Chen, 2005) and GH18 chitinase (27.46%) from *Clostridium paraputrificum* (BAA34922) (Morimoto et al., 1999). For chitinases containing two GH18 catalytic domains, ChiW (27.13%) from *Paenibacillus* sp. (BAM67143) (Itoh et al., 2013), and ChiB (25.86%) from *Saccharophagus degradans* 2–40 (DAA01334) (Howard et al., 2004), the putative GH18 chitinase with the highest similarity to *CmChi3* and the verified GH18 chitinase were selected to construct the phylogenetic tree, which also showed that *CmChi3* exhibited a low sequence identity with most of the functionally characterized bacterial GH18 chitinases (Figure 1). Multiple alignments of the individual GH18 domains of *CmChi3* with other GH18 chitinases from different sources indicated sequence DxDxE in *CmChi3nGH18* and *CmChi3cGH18* is the highly conserved amino acid sequence among the GH18 family members. DxDxE is a commonly conserved catalytic motif in GH18 family members, in which glutamic acid (E) residue is considered as proton donors in the catalytic process (Supplementary Figure 1; Dai et al., 2021).

Domain structure prediction indicated that *CmChi3* possesses two GH18 catalytic domains (residues 37–329 and 564–967) (Figure 2A). This result is contrary to most GH18 chitinases, which usually possess one GH18 catalytic domain. To date, few microbial chitinases with dual GH18 catalytic domains have been characterized. Meanwhile, accessory binding modules and the catalytic domain can improve the substrate accessibility of chitinases. *CmChi3* also contains two family 5 carbohydrate-binding modules (residues 368–411 and 464–510), which showed that it might possess good binding abilities for the chitinous substrate. As shown in Figure 2, the 3D structure of *CmChi3* was predicted based on the structure model from I-TASSER, which revealed that the amino acid sites F71, S112, D147, D149,

⁹www.cazy.org/



E151, M217, D218, M265, W299, Y568, F600, D739, D741, E743, M813, Y815, D816, Y874, and W962 were the active sites of N- and C-terminal catalytic domains, respectively (**Supplementary Figure 1**). These amino acids possibly form active pockets.

Cloning, Expression, and Purification of the Recombinant *CmChi3*

The gene encoding *CmChi3* without the signal peptide sequence (2,844 bp) was successfully amplified and actively expressed in *E. coli* BL21(DE3). As shown in **Supplementary Figure 2**, the localization of *CmChi3* was in the supernatant (lane 1) of the cell-free extract of the recombinant *E. coli* BL21(DE3)-(pCold I-*CmChi3*), indicating that *CmChi3* was actively expressed. The recombinant *CmChi3* with N-terminal His-tag was purified by NTA affinity chromatography (lane 2). The SDS-PAGE analysis showed that purified recombinant *CmChi3* possesses a high purity with an approximate molecular weight of 110 kDa, which agrees with the 99.7 kDa calculated from the amino acid sequence (without signal peptide) containing the His-tag. The recombinant *CmChi3* eluted with 250 mM imidazole from a Ni-NTA resin with a recovery yield of 49.1% (**Supplementary Table 3**).

Effects of Temperature and pH on the Enzymatic Activity and Stability of Recombinant *CmChi3*

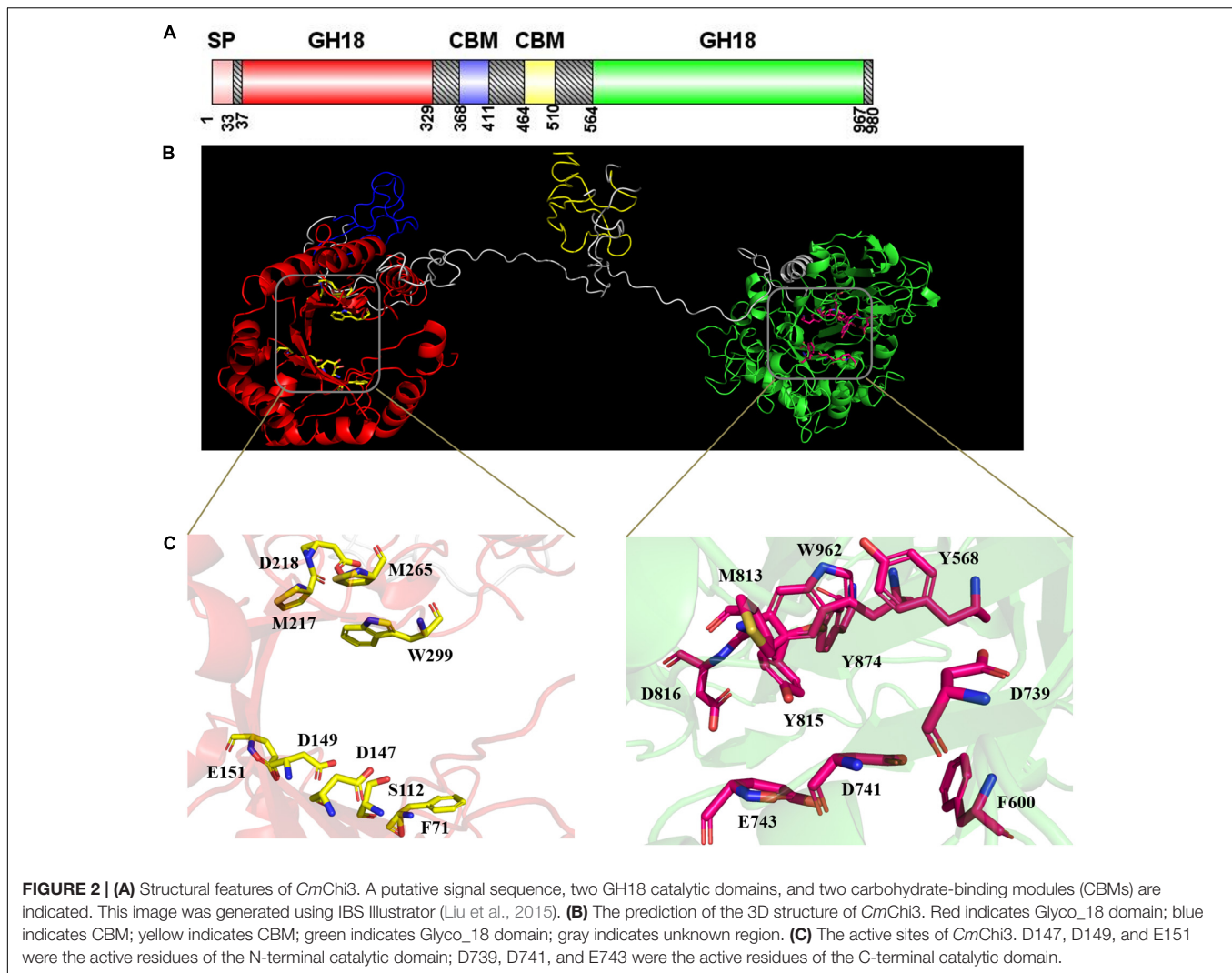
The effect of temperature on *CmChi3* activity was studied. The results showed that recombinant *CmChi3* exhibited maximum activity at a temperature of around 50°C (**Figure 3A**). This result is comparable to that of some bacterial chitinases, such as CsChiE from *C. shinanonensis* (50°C) (Rani et al., 2020) and PbChi70

from *Paenibacillus barengoltzii* (55°C) (Yang et al., 2016). Few chitinases with dual GH18 domains are ChiW from *Paenibacillus* sp. (50°C) (Itoh et al., 2014), Tk-ChiA from *Thermococcus kodakaraensis* KOD1 (85°C) (Tanaka et al., 1999), and chitinase P1724 from a wetland soil metagenome (40°C) (Dai et al., 2021). Additionally, *CmChi3* was stable and retained more than 90% activity for 2 h at temperatures below 40°C, but it was unstable at temperatures above 50°C.

The effects of pH on the recombinant *CmChi3* were determined in the range of pH 3.0–10.0. As shown in **Figure 3B**, *CmChi3* exhibited an optimum activity around pH 6.0. Meanwhile, the enzymatic activity of *CmChi3* dropped more than 50% at pH ≤ 4.0 and pH ≥ 8.5 (**Figure 3B**). This result is similar to that of other bacterial chitinases, such as CsChiE from *C. shinanonensis* (pH 6.0) (Rani et al., 2020) and spChiD from *Serratia proteamaculans* (pH 6.0) (Purushotham and Podile, 2012). However, the optimum pH of chitinases from *Microbispora* sp. V2 (pH 3.0) (Nawani et al., 2002), *Chitinibacter* sp. GC72 (pH 6.8) (Gao et al., 2015), and *Paenibacillus pasadenensis* NCIM 5434 (pH 10.0) (Loni et al., 2014) was different from *CmChi3*. With regard to pH stability, *CmChi3* retained more than 60% of its activity after storage at pH 5.0–8.5 for 2 h. This result is comparable to those of other chitinases from *C. meiyuanensis*, including *CmChi1* (pH 5.2–8.2) (Zhang et al., 2018) and *CmNAGase* (pH 4.0–8.5) (Zhang et al., 2020a).

Effects of Metal Ions on the Activity of Recombinant *CmChi3*

The effects of metal ions on *CmChi3* activity were investigated. All counter-ions of the used metal ions were Cl⁻. As shown



in **Table 1**, EDTA did not affect the enzymatic activity, which indicates that *CmChi3* is non-metal dependent. The activity was strongly inhibited by Cu^{2+} , Ni^{2+} , and Fe^{3+} . The presence of Fe^{2+} , Mg^{2+} , Ba^{2+} , and Na^{+} had a strengthening effect on *CmChi3* activity. Previous works also have shown that Cu^{2+} , Ni^{2+} , and Fe^{3+} inhibit the activity of chitinases. For example, the chitinase ChiW from *Paenibacillus* sp. was inhibited by Cu^{2+} and Fe^{3+} (Itoh et al., 2014). The chitinase ChiA-Ba43 from *Bacillus altitudinis* KA15 was inhibited by Ni^{2+} (Asmani et al., 2020).

Substrate Specificity and Kinetic Parameters of Recombinant *CmChi3*

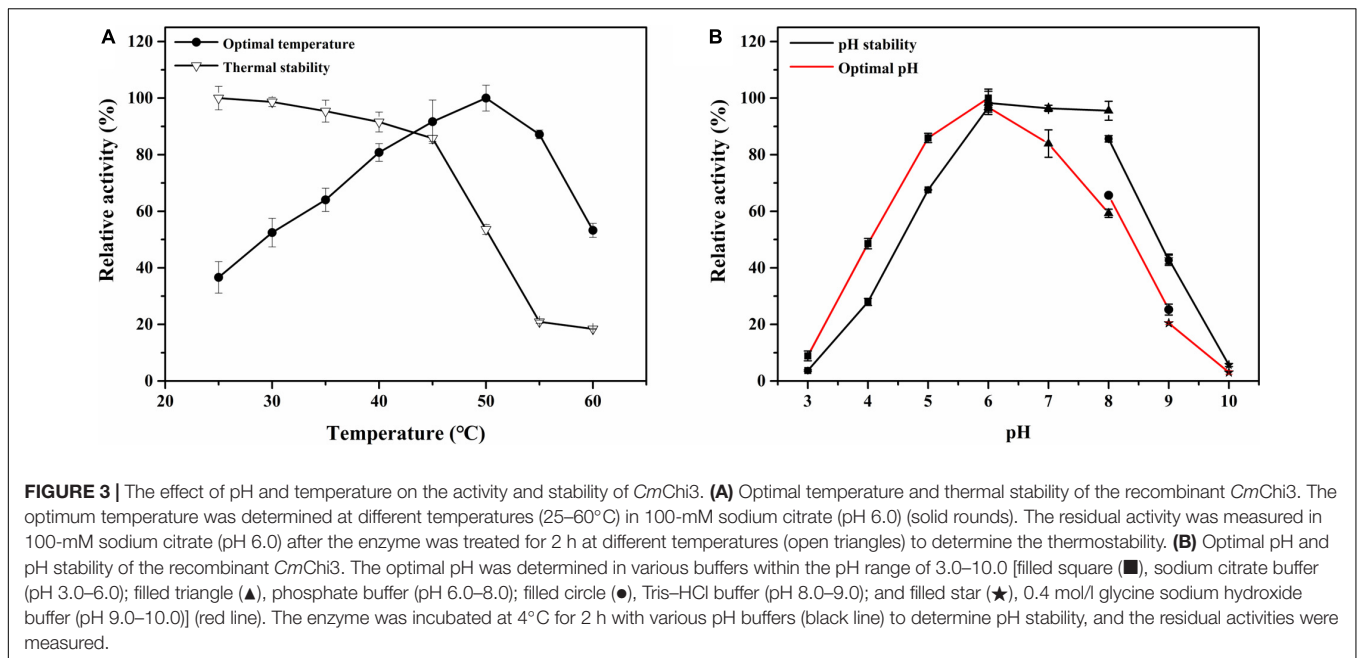
The hydrolysis ability of *CmChi3* toward various insoluble polysaccharide substrates was investigated. Of the substrates tested (**Table 2**), CC was most effectively hydrolyzed by the *CmChi3* with a specific activity of 4.1 U/mg, similar to the chitinase with dual catalytic domains, such as ChiW with a specific activity of 5.2 U/mg (Itoh et al., 2014). The activity was higher than that of most other reported chitinases, including chitinase from *Streptomyces speibonae* TKU048 (0.32 U/mg) (Thi Ngoc et al., 2019), chitinase CHI from *Paenibacillus*

chitinolyticus (0.75 U/mg) (Liu C. et al., 2020), and Tk-ChiA from *T. kodakaraensis* KOD1 (2.4 U/mg) (Tanaka et al., 2001), but lower than that of PxChi52 from *Paenibacillus xylanexedens* (16 U/mg) (Zhang W. et al., 2021) and *CmChi1* from *C. meiyuanensis* (15.3 U/mg) (Zhang et al., 2018). *CmChi3* displayed low activities toward α -chitin (0.02 U/mg) and β -chitin powder (0.3 U/mg). No activity was found toward CMC, hemicellulose, and chitosan (**Table 2**).

Furthermore, the kinetic parameters of recombinant *CmChi3* toward CC were investigated (**Supplementary Table 2**). The [s]-velocity plots of CC are shown in **Supplementary Figure 3**. The K_m , k_{cat} , and k_{cat}/K_m values were determined to be 7.53 ± 0.78 mg/ml, 9.08 ± 0.36 s⁻¹, and 1.2 ± 0.11 ml/s/mg for CC (**Supplementary Table 2**).

Hydrolysis Pattern of the Recombinant *CmChi3*

Colloidal chitin and *N*-acetyl chitooligosaccharides (DP 2–6) were used as hydrolytic substrates to evaluate the hydrolysis pattern of *CmChi3*. Our previous study confirmed that *CmChi3* can completely convert CC solely to GlcNAc when using a



high enzyme concentration (50 μg) (Zhang et al., 2020b). In this study, a low concentration of enzyme (20 μg) was used to study the specific intermediates of CC hydrolysis. As shown in **Figure 4A**, *CmChi3* hydrolyzed CC to mainly produce GlcNAc, with a small amount of (GlcNAc)₂ and (GlcNAc)₃ in 30 min. Then, it yielded GlcNAc as the sole end product after 120 min, suggesting that *CmChi3* possesses endochitinase and NAGase activities. Previously reported multifunctional chitinases *CmChi1* from *C. meiyuanensis* (Zhang et al., 2018) and *PbChi74* from *P. barengoltzii* (Fu et al., 2014) hydrolyzed CC to produce (GlcNAc)₂ in the initial hydrolysis stage. Also, (GlcNAc)₂ needed a longer reaction time to achieve full conversion to GlcNAc, which is different from our observation.

TABLE 1 | Effects of metal ions on the activity of recombinant *CmChi3*.

Metal ions	Chemicals	Concentration (mM)	Relative activity (%)
No addition	–	–	100
Ca ²⁺	CaCl ₂	10	98.8 ± 1.3
Co ²⁺	CoCl ₂	10	88.9 ± 0.2
Cu ²⁺	CuCl ₂ ·2H ₂ O	10	25.2 ± 4.0
K ⁺	KCl	10	106.2 ± 2.5
Na ⁺	NaCl	10	118.1 ± 4.0
Al ³⁺	AlCl ₃	10	71.7 ± 0.7
Ba ²⁺	BaCl ₂	10	158.5 ± 0.3
Zn ²⁺	ZnCl ₂	10	82.9 ± 2.9
Mg ²⁺	MgCl ₂	10	116.2 ± 0.3
Mn ²⁺	MnCl ₂	10	77.8 ± 0.9
Fe ²⁺	FeCl ₂	10	135.7 ± 4.6
Fe ³⁺	FeCl ₃	10	44.0 ± 5.0
Li ⁺	LiCl	10	98.6 ± 3.4
Ni ²⁺	NiCl ₂	10	63.0 ± 0.3
EDTA	EDTA	10	104.3 ± 0.9

For (GlcNAc)_{2–6}, *CmChi3* slowly hydrolyzed (GlcNAc)₂ into GlcNAc, showing that *CmChi3* possesses NAGase activity. This result is similar to that of other studies (Liu Y. et al., 2020). However, (GlcNAc)₂, (GlcNAc)_{2–3}, (GlcNAc)_{2–4}, and (GlcNAc)_{2–5} were each released from (GlcNAc)₃, (GlcNAc)₄, (GlcNAc)₅, and (GlcNAc)₆, respectively, which suggested that *CmChi3* has endocleavage activities. Additionally, small amounts of (GlcNAc)_{5–6} and (GlcNAc)₆ were observed when using (GlcNAc)₄ and (GlcNAc)₅ as substrates, respectively. This finding suggests that *CmChi3* has a weak TGase activity toward (GlcNAc)₄ and (GlcNAc)₅ (**Figure 4**). Conclusively, *CmChi3* is a multifunctional chitinase possessing endochitinase, NAGase, and TGase activities.

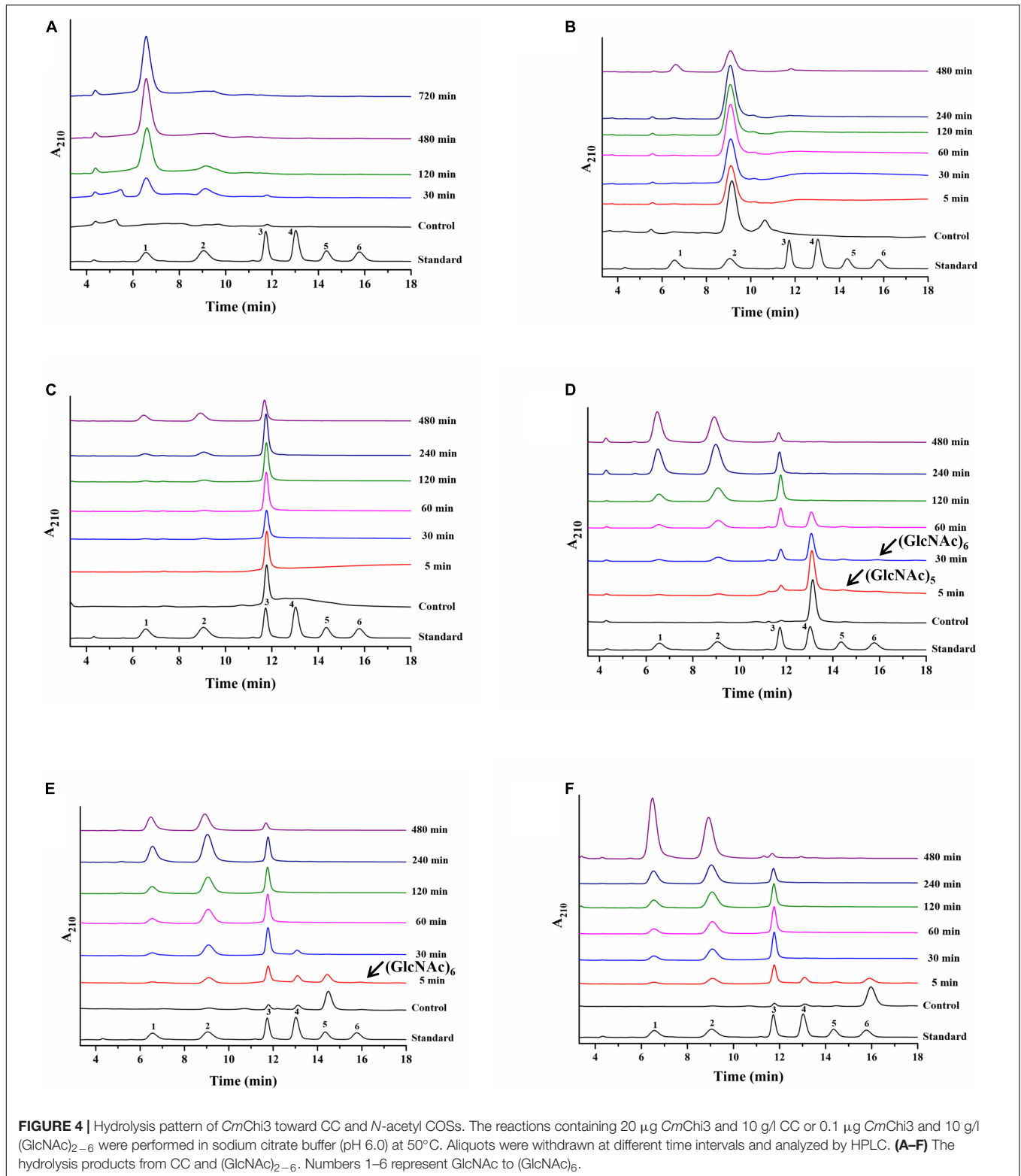
Hydrolysis Pattern of the Two GH18 Catalytic Domains, *CmChi3nGH18* and *CmChi3cGH18*

Recently, several reports have shown that a single enzyme containing more than one catalytic domain may be particularly powerful in degrading polysaccharides, such as a cellulase from the thermophilic bacterium *Caldicellulosiruptor bescii*, which

TABLE 2 | Substrate specificity of *CmChi3*.

Substrate	Specific activity (U/mg)
CC	4.1 ± 0.4
α -Chitin powder	0.02 ± 0.01
β -Chitin powder	0.3 ± 0.02
CMC	ND*
Hemicellulose	ND*
Chitosan	ND*

*Activity was not detected.



consists of GH9 and GH48 domains (Brunecky et al., 2013). Additionally, an enzyme from the actinobacterium *Jonesia denitrificans* contains AA10 lytic polysaccharide monoxygenase (LPMO) and GH18 catalytic domain (Mekasha et al., 2020).

According to previous reports, chitinases with dual catalytic domains often possess different catalytic activities. For example, in the chitinase Tk-ChiA from *T. kodakaraensis* KOD1 (Tanaka et al., 2001) and chitinase ChiB from *S. degradans* 2–40

(Itoh et al., 2013), the N-terminal and C-terminal GH18 domains possessed exo- and endo-activities, respectively. In another study, Larsbrink et al. (2016) showed that chitinase ChiA from *Flavobacterium johnsoniae* comprised two GH18 domains, one of which was proposed to have a predominantly endo-activity. In contrast, another one was predicted to have an exo-acting activity (Larsbrink et al., 2016).

To determine the hydrolysis pattern of individual GH18 domains of *CmChi3*, N-terminal (*CmChi3nGH18*) and C-terminal (*CmChi3cGH18*) GH18 catalytic domains were successfully expressed and purified (Supplementary Figure 4). Then, the hydrolysis pattern of *CmChi3nGH18* and *CmChi3cGH18* toward CC and (GlcNAc)_{2–6} was analyzed. As shown in Supplementary Figure 5, *CmChi3nGH18* hydrolyzed CC to produce (GlcNAc)_{1–3} (Supplementary Figure 5A) and hydrolyzed (GlcNAc)_{3–6} to (GlcNAc)_{1–2}, (GlcNAc)_{1–3}, (GlcNAc)_{1–4}, and (GlcNAc)_{1–5}, which showed that *CmChi3nGH18* possesses endochitinase activity. Additionally, *CmChi3nGH18* converted (GlcNAc)₂ into GlcNAc (Supplementary Figure 5B), thus exhibiting NAGase activity.

CmChi3cGH18 hydrolyzes CC solely into (GlcNAc)₂ (Supplementary Figure 5G). For (GlcNAc)_{2–6}, (GlcNAc)₂ could not be hydrolyzed. When using (GlcNAc)₃ as the substrate, the products were (GlcNAc)₂ and GlcNAc. (GlcNAc)₄ was mainly converted into (GlcNAc)₂. When using (GlcNAc)_{5–6} as the substrate, (GlcNAc)₂, (GlcNAc)₃, and (GlcNAc)₄ were generated (Supplementary Figure 5). These results showed that the enzyme has exochitinase activity. Also, *CmChi3cGH18* synthesized higher *N*-acetyl-COSs (GlcNAc)_{5–6} from (GlcNAc)₄ and (GlcNAc)₆ from (GlcNAc)₅, showing that it possesses TGase activity. This result also explains why little (GlcNAc)₃ was observed from (GlcNAc)₄ and (GlcNAc)₆, (GlcNAc)₄ was generated from (GlcNAc)₅, and (GlcNAc)₅ was obtained from (GlcNAc)₆.

The above results showed that *CmChi3nGH18* displayed endochitinase and NAGase activities and *CmChi3cGH18* showed exochitinase and TGase activities. The results agree with those of *CmChi3*.

CONCLUSION

In this study, a novel chitinase (*CmChi3*) containing dual GH18 catalytic domains and two carbohydrate-binding modules from

C. meiyuanensis was cloned, heterologously expressed in *E. coli*, and biochemically characterized. The analysis of the hydrolysis products revealed that *CmChi3* is a multifunctional chitinase, which possesses exochitinase, NAGase, and TGase activities. Further studies on the individual catalytic domains of *CmChi3* showed that N-terminal catalytic domain exhibited endo-acting and NAGase activities, while the C-terminal domain showed exo-acting and TGase activities. The multidomain structure of *CmChi3* is expected to produce GlcNAc from chitin. This work provides a reference to chitinase with multifunctional domains and GlcNAc enzymatic production from chitin.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

CW: writing-original draft preparation, formal analysis, and data curation. XC: investigation and methodology. NZ and YC: methodology. AZ: funding acquisition and writing-review and editing. KC and PO: conceptualization and supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.790301/full#supplementary-material>

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