Different Cleavage Specificities of the Dual Catalytic Domains in Chitinase from the Hyperthermophilic Archaeon Thermococcus kodakaraensis KOD1*

The chitinase from the hyperthermophilic archaeon Thermococcus kodakaraensis KOD1, Tk-ChiA, has an interesting multidomain structure containing dual catalytic domains and triple chitin-binding domains. To determine the biochemical properties of each domain, we constructed deletion mutant genes corresponding to the individual catalytic domains and purified the recombinant proteins. A synergistic effect was observed when chitin was degraded in the presence of both catalytic domains, suggesting different cleavage specificity of these domains. Analyses of degradation products from N-acetyl-chitooligosaccharides and their chromatogenic derivatives with thin layer chromatography indicated that the N-terminal catalytic domain mainly hydrolyzed the second glycosidic bond from the nonreducing end of the oligomers, whereas the C-terminal domain randomly hydrolyzed glycosidic bonds other than the first bond from the nonreducing end. Both catalytic domains formed diacetyl-chitobiose as a major end product and possessed transglycosylation activity. Further analysis of degradation products from colloidal chitin with high performance liquid chromatography showed that the N-terminal catalytic domain exclusively liberated diacetyl-chitobiose, whereas reactions with the C-terminal domain led to N-acetyl-chitooligosaccharides of various lengths. These results demonstrated that the N-terminal and C-terminal catalytic domains functioned as exo- and endochitinases, respectively. The biochemical results provide a physiological explanation for the presence of two catalytic domains with different specificity and suggest a cooperative function between the two on a single polypeptide in the degradation of chitin.

Chitin is a β-1,4-linked, insoluble linear polymer of N-acetyl-glucosamine (GlcNAc) and is the second most abundant organic compound on our planet following cellulose. Hence, the biological degradation of chitinous materials is an important process for the recycling of nutrients in most environments. Chitin-hydrolyzing enzymes are classified into three categories (endo-chitinases, exochitinases, and N-acetyl-β-glucosaminidases) according to the manner in which they cleave chitin chains. Endochitinases randomly cleave β-1,4-glycosidic bonds of chitin, whereas exochitinases cleave the chain from the nonreducing end to form diacetyl-chitobiose (GlcNAc2). N-Acetyl-β-glucosaminidases hydrolyze GlcNAc into GlcNAc or produce GlcNAc from the nonreducing end of N-acetyl-chitooligosaccharides. Many chitinases are composed of a catalytic domain joined to one or more chitin-binding domains (ChBDs), as in the case of various insoluble polysaccharide hydrolases including cellulases. This kind of substrate-binding domain is functional not only for accumulating catalytic sites on the surface of substrates but also for disrupting hydrogen bonds in the crystalline region of substrates and thereby facilitating subsequent hydrolysis by the catalytic domains.

Many chitinolytic bacteria, such as Bacillus circulans (3), Serratia marcescens (4), Streptomyces thermoviolaceus (5), Clostridium paraputrificum (6), Aeromonas sp. (7), and Pseudoalteromonas sp. (8), have been found to produce more than one kind of chitinase. The efficient chitin degradation is assumed to be performed by the combination of these multiple chitinases. Synergistic effects on degradation of chitin or cellulose have been observed in the simultaneous action of different types of hydrolases (9–11).

In contrast to bacterial, fungal, and plant chitinases, information concerning chitinase from the third kingdom, archaea, has been quite limited. A chitin-degrading hyperthermophilic archaeon, Thermococcus chitonophagus, was isolated from a deep sea hydrothermal vent environment (12). Putative chitinase genes were recently found in archaeal genomes of a hyperthermophile, Pyrococcus furiosus (13), and an extreme halophile, Halobacterium sp. NRC-1 (14). However, these archaeal chitinases have not yet been characterized. We have previously reported the first characterization of an archaeal chitinase from the hyperthermophilic archaeon Thermococcus kodakaraensis KOD1 (previously reported as Pyrococcus kodakaraensis KOD1) (15). The chitinase (Tk-ChiA) had a striking multidomain structure composed of two catalytic domains and three ChBDs (Fig. 1), in which both the catalytic domains were classified into family 18 of glycosyl hydrolases (16). Two internal ChBDs (ChBD2 and ChBD3) possessing almost identical sequences were classified into family 2 of carbohydrate-binding modules, and an N-terminal ChBD1 was classified into family 2 of carbohydrate-binding domains.

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1 The abbreviations used are: ChBD, chitin-binding domain; HPLC, high performance liquid chromatography.
2 P. M. Coutinho and B. Henrissat, afmb.cnrs-mrs.fr/~pedro/CAZY/cbm.html.
**Archaeal Endo- and Exochitinases on a Single Polypeptide**

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Media—** *Escherichia coli* TG-1 and BL21(DE3) were used as hosts for expression plasmids derived from *Chlorella* virus CVK2 (17) and PBCV-1 (18); however, catalytic properties of the individual domains have not been well characterized in both cases. In this study, we focused on the enzymatic properties of the individual catalytic domains of *Tk-ChiA* and found that the chitinase from *T. kodakarenseis* KOD1 possesses dual catalytic domains with different cleavage specificities on a single polypeptide.

**DNA Manipulations and Sequencing—** DNA manipulations were performed by standard methods, as described by Sambrook and Russell (19). Restriction enzymes and other modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) or Toyobo (Osaka, Japan). Small scale preparation of plasmid DNA from *E. coli* cells was performed with the Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany). DNA sequencing was performed with the ABI PRISM kit and Model 310 capillary DNA sequencer (Applied Biosystems, Foster City, CA). Nucleotide and amino acid sequence analyses were performed with GENETYX software (Software Development, Tokyo, Japan).

**Preparation of ChiAΔ5 and Other Mutants—** The expression plasmid for ChiAΔ5 was constructed by polymerase chain reaction as described below. Two oligonucleotides (sense, 5′-ACAACCCATATGATGAGGG-CTCCTCAGCCGC-3′; antisense, 5′-GCGATTCAGGCCAGGG-TGGTGGAGAACATTAC-3′ (underlining indicates an NdeI site and a BglII site in sense and antisense primers, respectively) and a phage DNA containing the *Tk-chiA* gene (20) were used as primers and template for DNA amplification, respectively. The amplified DNA (1,222 base pairs) was digested with NdeI and BglII and then ligated with the NdeI and BamHI sites of plasmid pET-25b(+) to create ChiAΔ5 expression plasmid. Expression and purification of ChiAΔ5 were performed with the same procedures as those described for *Tk-ChiA* (15). Preparations of *Tk-ChiA*, ChiAΔ2, ChiAΔ5, and ChiAΔ4 have been described previously (15). The protein concentration was determined by the Bio-Rad protein assay system (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The N-terminal amino acid sequences of purified proteins were determined by protein sequencer Model 491 cLC (Applied Biosystems).

**Enzyme Assays—** Chitinase was assayed by a modification of the

**FIG. 1.** Structural features of the chitinase from *T. kodakarenseis* (Tk-ChiA) and schematic drawings of the deletion mutants. A putative signal sequence, catalytic domains A and B, three ChBDs, and three linker-like regions are indicated.

**FIG. 2.** Synergistic effect on chitin degradation by deletion mutants of *Tk-ChiA*. The reaction mixture (1 ml) containing 1.7 mg of chitin in 50 mM sodium acetate buffer (pH 5.0) was incubated with enzyme at 80 °C. The amount of reducing sugar generated was measured by using the Schales procedure (21). Lane 1, *Tk-ChiA* (29 pmol); ○, ChiAΔ3 (58 pmol); ■, ChiAΔ5 (58 pmol); △, combination of ChiAΔ3 and ChiAΔ2 (29 pmol each); + (broken line), calculated activity for ChiAΔ3 and ChiAΔ2; ○, ChiAΔ5 (200 pmol); □, ChiAΔ4 (200 pmol); △, combination of ChiAΔ5 and ChiAΔ4 (100 pmol each); × (broken line), calculated activity for ChiAΔ5 and ChiAΔ4.

**FIG. 3.** A, SDS-polyacrylamide gel electrophoresis of ChiAΔ5 and ChiAΔ4 purified from recombinant *E. coli* strains. Lane 1, molecular mass marker; lane 2, purified ChiAΔ5 (45,512 Da); lane 3, purified ChiAΔ4 (33,832 Da). B, thermostabilities of ChiAΔ5 and ChiAΔ4 at 90 °C and 100 °C. ○, ChiAΔ5 at 90 °C; ■, ChiAΔ4 at 90 °C; ◊, ChiAΔ5 at 100 °C; □, ChiAΔ4 at 100 °C.

Schales procedure (21) with colloidal chitin as the substrate (final concentration, 0.17%). The preparation of colloidal chitin has been described previously (15). The standard assay was performed at 80 °C in 50 mM sodium acetate buffer (pH 5.0) for 10 min. The reaction was terminated by cooling the samples in an ice-cold bath, and the amount of reducing sugar generated was measured. To measure chitinase activity toward other substrates, colloidal chitin was replaced by chitin (chitin Ex), chitosan 7B, chitosan 8B, chitosan 9B, and chitosan 10B (Funakoshi, Tokyo, Japan). Ethylene glycol chitin (Seikagaku Corp., Tokyo, Japan) was used at a concentration of 0.15%. The optimal temperature and pH for chitinase activity were determined as described previously (15). The thermostability of the enzymes was measured by monitoring the remaining activity after heat treatment (90 °C or 100 °C) of 90 μg/ml enzyme in 47 mM Tris-HCl (pH 7.5) with or without 200 mM NaCl.

**Analyses of Degradation Products—** The analyses of degradation products from colloidal chitin, *N*-acetyl-chitoooligosaccharides (GlcNAc<sub><i>n</i></sub>g, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (GlcNAc<sub><i>n</i></sub>-PNP), and chitosan pentamer (Seikagaku Corp.) by silica gel thin layer chromatography (TLC) were performed as described previously (15). Degradation products from colloidal chitin at the early stage of the reaction were analyzed by high performance liquid chromatography (HPLC) equipped with a TSKgel Amide-80 column (4.6 × 25 mm; Tosoh, Tokyo, Japan). The produced *N*-acetyl-chitoooligosaccharides were eluted with 65% acetonitrile at a flow rate of 1 ml/min at 80 °C and then detected by absorbance at 205 nm. A chitoooligosaccharide mixture (Seikagaku Corp.) that contains equal weights of GlcNAc<sub><i>n</i></sub>-g was used as a standard.
RESULTS

Comparison of Primary Structures and Synergism of Two Catalytic Domains of Tk-ChiA—The chitinase from T. kodakaraensis KOD1 (Tk-ChiA) is composed of two catalytic domains and three ChBDs (Fig. 1) (15). Although both the N-terminal and C-terminal catalytic domains (catalytic domains A and B, respectively) are classified into family 18 of glycosyl hydrolases, the similarity between the catalytic domains is very low (17% identity within 420 amino acids). According to the classification of family 18 bacterial chitinases (22), catalytic domains A and B belong to different subfamilies, subfamilies A and C, respectively. These facts suggested that the two catalytic domains might possess some different features. We have already characterized two deletion mutants of Tk-ChiA, ChiAΔ3 and ChiAΔ2, which contain catalytic domain A with ChBD1 and catalytic domain B with the repeated ChBDs (ChBD2 and ChBD3), respectively (Fig. 1) (15). In the study, when colloidal chitin was used as a substrate, the sum of the specific activities of ChiAΔ3 and ChiAΔ2 was nearly equivalent to that of Tk-ChiA, indicating no synergistic effect. Here, we performed experiments using chitin as a substrate (Fig. 2). Activities of ChiAΔ3, ChiAΔ2, their combination, and Tk-ChiA were measured as μmol of reducing sugar released/nmol of catalytic domain. The results clearly indicated a synergistic effect between ChiAΔ3 and ChiAΔ2 because the activity of the combination of ChiAΔ3 and ChiAΔ2 was significantly higher than the specific activity calculated from individual activities during the reaction. The synergism coefficient, i.e. activity (ChiAΔ3 + ChiAΔ2) / activity ChiAΔ3 + activity ChiAΔ2), at 60 min was 1.49. The activity of Tk-ChiA, just joining ChiAΔ3 to ChiAΔ2, was also higher than the calculated activity of the deletion mutants. Such synergism raised the possibility that catalytic domains A and B of Tk-ChiA harbored distinct modes of cleavage on chitin.

Overexpression and Purification of Deletion Mutants—To examine the properties of each catalytic domain without the influence of ChBDs, a new deletion mutant (ChiAΔ5) consisting of only catalytic domain A was constructed (Fig. 1). In ChiAΔ5, the initial Met residue was flanked to Tyr-148 located between ChBD1 and catalytic domain A because the longer mutant protein was partially degraded during purification procedures, leading to a protein with Tyr-148 at the N terminus. E. coli cells harboring expression plasmid pET-ChiAΔ5 were induced by isopropyl-1-thio-β-D-galactopyranoside, and the protein was purified to apparent homogeneity by heat treatment and column chromatography, as described under “Experimental Procedures” (Fig. 3A, lane 2). The N-terminal amino acid sequence of purified ChiAΔ5 was MYGVPPVALD, which was identical to the predicted amino acid sequence. The other deletion mutant (ChiAΔ4) consisting of catalytic domain B was constructed and purified as described previously (Fig. 3A, lane 3) (15).

Characterizations of ChiAΔ5 and ChiAΔ4—We first investigated whether the catalytic domains ChiAΔ5 and ChiAΔ4, which lacked ChBD(s), showed synergism toward the degradation of chitin. When the activities of ChiAΔ5, ChiAΔ4, and their combination were measured with chitin as a substrate, we could still observe a significant synergistic effect by ChiAΔ5 and ChiAΔ4 (Fig. 2). The synergism coefficient was determined to be 1.51 at 60 min, which was equivalent to that of ChiAΔ3 and ChiAΔ2. The synergistic effect was also observed toward ethylene glycol chitin but was not observed toward colloidal chitin (data not shown).

The optimal temperature of ChiAΔ5 and ChiAΔ4 for colloidal chitin was determined to be 85 °C and 90 °C, respectively. The optimal pH of ChiAΔ4 was 4.5, whereas ChiAΔ5 exhibited high levels of activity over a wide pH range from 4.5 to 8.0. ChiAΔ5 and ChiAΔ4 were stable at 80 °C for 60 min in the pH range from 5 to 9 and from 4 to 9, respectively, and the activities were increased up to 110% by additional salt (0.2-1.0 M NaCl or KCl). The half-life of ChiAΔ5 at 90 °C was 5 min, and the enzyme was completely inactivated within 1 min at 100 °C (Fig. 3B). On the other hand, ChiAΔ4 was extremely thermo-
stable even at 100 °C, with a half-life of >7 h. Additions of 0.2 M NaCl did not affect the thermostabilities of ChiAΔ5 and ChiAΔ4 (data not shown).

Cleavage Specificities of ChiAΔ5 and ChiAΔ4—To determine the modes of cleavage by ChiAΔ5 and ChiAΔ4, we analyzed the reaction products formed from colloidal chitin and various N-acetyl-chitooligosaccharides (GlcNAc2–6) with TLC (Fig. 4).

When colloidal chitin was used as a substrate, only GlcNAc2 was detected in both cases. Hydrolysis of GlcNAc2 by ChiAΔ5 or ChiAΔ4 was not observed within 180 min; however, spots corresponding to GlcNAc could be detected after prolonged incubation (24 h), indicating that ChiAΔ5 and ChiAΔ4 possessed small degrees of activity toward GlcNAc (data not shown). GlcNAc3 was hydrolyzed to GlcNAc and GlcNAc2 by ChiAΔ5 and ChiAΔ4. Although both enzymes formed GlcNAc2 as a major end product from GlcNAc4–6, obvious differences between ChiAΔ5 and ChiAΔ4 were observed in their intermediate pathways.

**Fig. 5.** TLC of hydrolysis products by ChiAΔ5 (A) and ChiAΔ4 (B) from various p-nitrophenyl N-acetyl-β-chitooligosaccharides. The reaction mixture (100 μl) containing 0.16 mg of substrate in 10 mM sodium acetate buffer (pH 5.0) was incubated with enzyme (ChiAΔ5, 5 pmol; ChiAΔ4, 1 pmol) at 70 °C. The reaction mixtures (10 μl each) were concentrated under reduced pressure without heating and then analyzed. Lanes Std., standard N-acetyl-chitooligosaccharides ranging from GlcNAc (G1) to GlcNAc6 (G6); lanes PNP Std., standard p-nitrophenyl N-acetyl-chitooligosaccharides ranging from GlcNAc-PNP (G1PNP) to GlcNAc6-PNP (G6PNP). Schematic models of hydrolysis sites in GlcNAc or GlcNAc-PNP by ChiAΔ5 and ChiAΔ4 are represented at the bottoms of A and B, respectively. Arrow size indicates the relative degradation rate. White hexagons indicate GlcNAc residues, and black hexagons indicate GlcNAc residues of reducing ends or p-nitrophenyl groups.

**Fig. 6.** TLC of transglycosylation products by ChiAΔ5 and ChiAΔ4 from GlcNAc3. The reaction mixture (50 μl) containing 2.59 mg of GlcNAc3 in 50 mM sodium acetate buffer (pH 5.0) was incubated with enzyme (ChiAΔ5, 3 pmol; ChiAΔ4, 3 pmol) at 70 °C. The reaction mixtures were diluted and then analyzed. Lanes Std., standard N-acetyl-chitooligosaccharides ranging from GlcNAc (G1) to GlcNAc6 (G6).
products. ChiA\textsubscript{A5} produced oligosaccharides that were 1 unit shorter than the starting substrate, such as GlcNAc\textsubscript{5} from GlcNAc\textsubscript{5} (4 min) and GlcNAc\textsubscript{5} from the nonreducing end. ChiA\textsubscript{A4} hydrolyzed by both the enzymes. When GlcNAc\textsubscript{4} was added, the spots corresponding to GlcNAc\textsubscript{2}, GlcNAc\textsubscript{3}, GlcNAc\textsubscript{4}, and GlcNAc\textsubscript{5} were detected from colloidal chitin (Fig. 4); however, it was not clear whether these GlcNAc\textsubscript{4} products were produced directly from colloidal chitin or produced indirectly via longer oligosaccharide intermediates during hydrolysis. To clarify this, we analyzed the products from colloidal chitin at the early stage of the reaction (2, 4, 8, and 12 min) by HPLC. The weight of products was kept within 3% of that of the initial substrate in this experiment to avoid secondary hydrolysis. ChiA\textsubscript{A5} produced mainly GlcNAc\textsubscript{2}, together with a small amount of GlcNAc\textsubscript{4} (Fig. 7A). In contrast, ChiA\textsubscript{A4} produced GlcNAc\textsubscript{1,6}, and we also detected faint signals that were probably derived from GlcNAc\textsubscript{2} and GlcNAc\textsubscript{4} at the retention times of 10.7 and 12.6 min, respectively (Fig. 7B). The result of HPLC analysis supported the direct formation of GlcNAc\textsubscript{2} from colloidal chitin by ChiA\textsubscript{A5}, whereas ChiA\textsubscript{A4} liberated GlcNAc\textsubscript{1,6} from the high polymer substrate. It should be noted that GlcNAc\textsubscript{4} production rates from colloidal chitin with ChiA\textsubscript{A5} were much higher than those seen with ChiA\textsubscript{A4}, as shown in insets of Fig. 7.

**Specific Activities of ChiA\textsubscript{A5} and ChiA\textsubscript{A4} toward Various Substrates**—The specificities of ChiA\textsubscript{A5} and ChiA\textsubscript{A4} toward insoluble substrates (chitin and colloidal chitin) and soluble derivatives (ethylene glycol chitin and various degrees of deacetylated chitosans) were also determined as shown in Table I. The specific activities toward chitin were lowest among all substrates for both ChiA\textsubscript{A5} and ChiA\textsubscript{A4}. ChiA\textsubscript{A4} showed lower activity toward colloidal chitin than ChiA\textsubscript{A5}, as was demonstrated by the HPLC analysis (Fig. 7, insets). Whereas the activities of ChiA\textsubscript{A5} were similar toward colloidal chitin and chitosans with various degrees of deacetylation, ChiA\textsubscript{A4} showed remarkably high levels of activity toward soluble ethylene glycol chitin, chitosan 7B, chitosan 8B, and chitosan 9B. Both ChiA\textsubscript{A5} and ChiA\textsubscript{A4} showed hydrolytic activities against the highly deacetylated chitin (>98%) chitosan 10B. These activities are most likely to be dependent on residual GlcNAc units in the substrate because hydrolyses of chitosan pentamer by ChiA\textsubscript{A5} and ChiA\textsubscript{A4} could not be observed (data not shown).

**DISCUSSION**

The chitinase from the hyperthermophilic archaeon *T. kodakaraensis* KOD1, Tk-ChiA, possesses two catalytic domains on a single polypeptide (15). In this study, we examined the biochemical properties of the individual catalytic domains in detail. The results clearly indicated differences between the cleavage specificities of the two catalytic domains.

From the analyses of hydrolysates of N-acetyl-chitooligosaccharides with TLC (Figs. 4 and 5), we determined the cleavage sites in GlcNAc\textsubscript{3} or GlcNAc\textsubscript{2}-PNP by ChiA\textsubscript{A5} and ChiA\textsubscript{A4}, respectively (see illustrations in Fig. 5). ChiA\textsubscript{A5} showed exochitinolytic activity that mainly hydrolyzed the second glycosidic bond and slightly hydrolyzed the third one from the nonreducing end of chitin chains. On the other hand, ChiA\textsubscript{A4} showed endochitinolytic activity that randomly hydrolyzed gly-
cosidic bonds other than the terminal bond at the nonreducing end. Furthermore, we carried out HPLC analysis and successfully detected N-acetyl-chitooligosaccharides liberated from chitin at the early stage of hydrolysis, without the use of radiolabeled substrate. The results shown in Fig. 7 clearly indicate that ChiAΔ5 is an exochitinase, whereas ChiAΔ4 is an endochitinase, consistent with the conclusions from experiments using N-acetyl-chitooligosaccharides as substrates. The absence of hydrolytic activities of ChiAΔ5 and ChiAΔ4 against chitosan pentamer indicated that both catalytic domains did not possess chitosanase activity. This result agrees with previous findings that family 18 chitinases catalyze the hydrolysis of the glycosidic bond via substrate-assisted catalysis that required the N-acetyl group of the substrate (23, 24). Hence, although ChiAΔ5 and ChiAΔ4 showed activity even toward chitosan 10B (>98% deacetylated) as shown in Table I, the activities must be dependent on the residual GlcNAc units in the substrate.

Chitinase A1 from B. circulans WL-12 has been well studied and is one of the chitinases (besides the archaeal ones) most similar to catalytic domain A of Tk-ChiA (36% identity within 403 amino acids). Although it had been reported that chitinase A1 mainly hydrolyzed the second glycosidic bond from the nonreducing end of PNP-GlcNAc2, like ChiAΔ5 (25), recent three-dimensional structural analysis of chitinase A1 suggested that GlcNAc2 units were continuously split off from the reducing end of the chitin chain in this enzyme (26). Seven aromatic residues were proposed to guide a chitin chain into the catalytic site in chitinase A1, and six of them were also conserved in the catalytic domain A of Tk-ChiA (Trp-372, Trp-251, Trp-523, Trp-184, Tyr-187, and Tyr-223). From these facts, catalytic domain A may also hydrolyze mainly the second glycosidic bond from the reducing end when high polymer chitin is a substrate.

As described above, ChiAΔ4 (endochitinase type) could hydrolyze the bonds regardless of their positions in the high polymer chains, whereas the cleavage sites for ChiAΔ5 (exochitinase type) were limited at the ends of the chitin chains. The higher number of accessible cleavage sites for the endochitinase could explain the higher activities of ChiAΔ4 toward soluble ethylene glycol chitin, chitosan 7B, chitosan 8B, and chitosan 9B in comparison with those of ChiAΔ5 (Table I). The drastic decrease in activity toward chitosan 10B is likely to be due to the concentration of GlcNAc residues falling below the value needed for maximum velocity of the reaction. On the other hand, the activities of ChiAΔ5 were higher than those of ChiAΔ4 when colloidal chitin or chitosan 10B was used as a substrate. It is well known that the acid and alkaline treatments for preparations of the colloidal chitin and chitosan 10B are accomplished by low molecularization and a consequent increase of chitin ends (27, 28). Indeed, the viscosity of chitosan 10B was approximately one-third of that of the other chitosans used in our experiments. The larger number of chain ends in these substrates for recognition and cleavage by ChiAΔ5 probably contributes to the similar levels of activity toward colloidal chitin and chitosan 10B with less GlcNAc residues when compared with those toward other chitosans.

Due to its highly crystalline structure, chitin itself is hardly degradable. Indeed, the activities of ChiAΔ5 and ChiAΔ4 toward higher crystalline chitin were much lower when compared with other chitin derivatives. As shown in Fig. 2, ChiAΔ5 and ChiAΔ4 with different cleavage specificities exhibited a synergistic effect toward chitin. It can be assumed that the endochitinase (catalytic domain B) randomly produced ends of chitin chains accessible to the exochitinase (catalytic domain A), which then effectively released GlcNAc2 from the ends (Fig. 7, insets). Although many organisms produce more than one chitinase for efficient hydrolysis of chitin polymer (3–8), the single polypeptide Tk-ChiA alone could achieve synergistic hydrolysis of chitin. We have observed no synergistic effect between ChiAΔ5 and ChiAΔ4 toward colloidal chitin as a substrate. The reason for this phenomenon is probably the existence of a sufficient number of the ends in colloidal chitin formed by the low molecularization described above, which allowed ChiAΔ5 to efficiently hydrolyze the chains without the assistance of an endochitinase.

The deletion mutants, ChiAΔ5 and ChiAΔ4, are thermostable chitinases, and ChiAΔ4 in particular exhibited extreme thermostability (Fig. 3B). In addition, such thermostable enzymes generally possess stabilities not only toward heat but also toward detergents and organic solvents (29). These thermostable chitinases would be applicable as useful catalysts in the chitin industry. Moreover, ChiAΔ5 and ChiAΔ4 both possessed transglycosylation activity (Fig. 6). These catalytic properties may be advantageous in the efficient production of N-acetyl-chitooligosaccharides with biological activity (30, 31) using organic solvents as reaction media.

In the archaeal genomes of P. furiosus (the unfinished genome sequence is available at the Utah Genome Center website3) (13) and Halobacterium NRC-1 (14), two chitinase orthologues genes are present as a cluster (gene names: Pf_1168804 and Pf_1166613, and chi and VNG0818C, respectively). The deduced amino acid sequences of Pf_1168804 and Pf_1166613 are similar to the N-terminal region of Tk-ChiA divided at the middle of catalytic domain A and the C-terminal polypeptide without one of the two internal ChBDs, respectively. The two putative enzymes from Halobacterium NRC-1, both composed of N-terminal ChBD and C-terminal catalytic domain, are similar to the N-terminal half of Tk-ChiA. Among all chitinases for which primary structures have been determined, catalytic domains A and B of Tk-ChiA are the most closely related to the putative chitinases from P. furiosus (72–83% identities). The deduced amino acid sequences translated from chi and VNG0818C show comparatively higher similarities to catalytic domain A (43% and 33% identities, respectively) than to other chitinases from eucarya and bacteria. Further identification of archaeal chitinases would clarify whether archaeal chitinases comprise independent groups from eucaryal and bacterial enzymes in terms of primary structure.

The structure of Tk-ChiA, which is composed of dual catalytic domains and triple ChBDs on a single polypeptide, is very interesting. The two catalytic domains possess different cleavage specificities, and their simultaneous action showed a synergistic effect on the hydrolysis of high molecular chitin. The ChBDs enhanced the hydrolytic efficiency of these catalytic domains against the insoluble substrate (Fig. 2). Moreover, the multidomain structure of Tk-ChiA is expected to be effective to concentrate the different kinds of catalytic domains (endochitinase- and exochitinase-type enzymes) side by side on the surface of chitin crystals. The advantages are similar to those obtained by the "more evolved" cellulose in cellulose degradation. The biochemical evidence in this study provides a feasible physiological explanation for the unique structure of Tk-ChiA. This structure should contribute to efficient degradation of chitin, especially at the low enzyme and substrate concentrations found under natural conditions.

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