Characterization of an Exo-β-d-Glucosaminidase Involved in a Novel Chitinolytic Pathway from the Hyperthermophilic Archaeon Thermococcus kodakaraensis KOD1

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Received 17 March 2003/Accepted 16 June 2003

We previously clarified that the chitinase from the hyperthermophilic archaeon Thermococcus kodakaraensis KOD1 produces diacytelchitobiose (GlcNAc2) as an end product from chitin. Here we sought to identify enzymes in T. kodakaraensis that were involved in the further degradation of GlcNAc2. Through a search of the T. kodakaraensis genome, one candidate gene identified as a putative β-glycosyl hydrolase was found in the near vicinity of the chitinase gene. The primary structure of the candidate protein was homologous to the β-galactosidases in family 35 of glycosyl hydrolases at the N-terminal region, whereas the central region was homologous to β-galactosidases in family 42. The purified protein from recombinant Escherichia coli clearly showed an exo-β-d-gluacosaminidase (GlcNase) activity but not β-galactosidase activity. This GlcNase (GlmA\(T_k\)), a homodimer of 90-kDa subunits, exhibited highest activity toward reduced chitobiose at pH 6.0 and 80°C and specifically cleaved the nonreducing terminal glycosidic bond of chitooligosaccharides. The GlcNase activity was also detected in T. kodakaraensis cells, and the expression of GlmA\(T_k\) was induced by GlcNAc2 and chitin, strongly suggesting that GlmA\(T_k\) is involved in chitin catabolism in T. kodakaraensis. These results suggest that T. kodakaraensis, unlike other organisms, possesses a novel chitinolytic pathway where GlcNAc2 from chitin is first deacetylated and successively hydrolyzed to glucosamine. This is the first report that reveals the primary structure of GlcNase not only from an archaeon but also from any organism.

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MATERIALS AND METHODS

Bacterial strains, plasmid, and medium. Escherichia coli TG-1 and BL21-CodonPlus(DE3)-RI were used as hosts for the expression plasmid derived from pET-28b(+) (Novagen, Madison, Wis.) and were cultivated in Luria-Ber-
tani medium at 37°C. Ampicillin was added to the medium at a final concentration of 50 μg/ml when needed.

DNA manipulations and sequencing. DNA manipulations were performed by standard methods, as described by Sambrook and Russell (30). 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 minikit (Qiagen, Hilden, Germany). DNA sequencing was performed with BigDye terminator cycle sequencing ready reaction kit version 3.0 and a model 3100 capillary DNA sequencer (Applied Biosystems, Foster City, Calif.).

Construction of expression plasmid. The expression plasmid for the GlcNase gene from T. kodakaraensis (glmA<sub><i>K</i></sub>) was constructed by PCR as described below. Two oligonucleotides (sense, 5′-GTGATGCATATGGGAAAGGTTGAG-3′ and antisense, 5′-GGGGAATTCGCCGACGAGGATGTA-3′) were designed based on the GlmA<sub><i>K</i></sub> open reading frame and then ligated with the corresponding sites of the pET-32a (+) vector (Novagen, Madison, Wis.). The constructed plasmid was designated pET-glmA<sub><i>K</i></sub>.

Purification of recombinant GlmA<sub><i>K</i></sub>. E. coli BL21-CodonPlus(D3-RI) cells harboring pET-glmA<sub><i>K</i></sub> were cultured for overexpression with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at the mid-exponential growth phase and incubated for a further 5 h at 37°C. Cells were harvested by centrifugation (5000 x g for 15 min at 4°C) and then resuspended in buffer A (50 mM Tris-HCl [pH 7.5], 1 mM EDTA). The cells were disrupted by sonication, and the supernatant was obtained by centrifugation (14,000 x g for 30 min). Each fraction was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western blot analysis with specific rabbit antiserum against the recombinant GlmA<sub><i>K</i></sub>. A protein A-agarose conjugate was used to visualize the specific protein together with 4-chloro-1-naphthol and hydrogen peroxide.

Nucleotide sequence accession number. The nucleotide sequence data reported here are available in the EMBL/GenBank/DDBJ nucleotide sequence databases under accession number AB100422.

RESULTS

Search of candidate gene for GlcNAc catabolism in T. kodakaraensis KOD1 genome. As previously reported, the genes for chitinase (chIA<sub><i>K</i></sub>) and β-glycosidase (gβ-IA<sub><i>K</i></sub>) were located near each other in the T. kodakaraensis genome (Fig. 2, gray arrows) (4). Therefore we examined the ability of Gly<sub>250-300</sub> to hydrolyze GlcNAc and its deacetylated compound, GlcN<sub>2</sub>, because this enzyme has been determined to possess broad substrate specificity toward various β-linked sugars (4). However, the enzyme showed no activity toward GlcNAc and only faint activity toward GlcN<sub>2</sub> (data not shown). In addition, a homology search with the sequences of known GlcNAc-processing enzymes (GlcNAcase, GlcNAc2 phosphorylase, and GlcNAc2 phosphotransferase system) against the preliminary complete genome sequence of T. kodakaraensis did not lead to identification of any candidate gene.

We then addressed the possibility that a gene(s) for an unknown β-glycosyl hydrolase involved in chitinolysis may be clustered together with chIA<sub><i>K</i></sub> and analyzed the region around chIA<sub><i>K</i></sub> in detail. This attempt led to the identification of one gene, homologous to the genes for putative β-galactosidasises in the closely related genus Pyrococcus, located approximately 11 kbp upstream from chIA<sub><i>K</i></sub> in the opposite orientation (Fig. 2,

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black arrow). Since the gene turned out to encode a GlcNase, as described below, it was designated glmATk.

**Primary structure of glmATk.** The glmATk gene consisted of 2,358 bp, encoding a protein of 786 amino acids with a predicted molecular mass of 90,227 Da. The deduced amino acid sequence showed high overall identities to putative archaeal β-galactosidases, PAB1349 from “Pyrococcus abyssi” (64% identity), PH0511 from *Pyrococcus horikoshii* (61% identity), and PF0363 from *Pyrococcus furiosus* (57% identity). As shown in Fig. 3, the N-terminal region, spanning a quarter of the protein, showed homology to β-galactosidases of family 35 glycosyl hydrolases (41% identity to human β-galactosidase), while the central region of about 480 amino acid residues showed weak homology to the β-galactosidases of family 42 glycosyl hydrolases (20% identity to β-galactosidase from *Bacillus steaorthermophilus*). Both families are classified in the same superfamily, clan A of glycosyl hydrolases (<http://afmb.cnrs-mrs.fr/CAZY/index.html>), and their catalytic residues have been predicted by hydrophobic cluster analysis (8).

GlmA*tk* and its homologous archaeal proteins harbored the predicted catalytic proton donor and nucleophile residues in the homologous regions of family 35 and family 42 glycosyl hydrolases, respectively (Fig. 3). However, highly conserved motifs around these catalytic residues (QXENEY around the putative nucleophile in family 42) were not conserved (Fig. 3B and C). The C-terminal region of about 100 amino acid residues had no significant homology to other known enzymes besides the archaeal homologues. Typical signal peptides for secretion and transmembrane helices were not detected with the SOSUI transmembrane helix prediction software (<http://sosui.proteome.bio.tuat.ac.jp/~sosui/proteome/sosuiframe0.html>).

**Overexpression and purification of recombinant GlnmA*tk*.** To characterize the enzymatic properties of GlnmA*tk*, the recombinant protein was produced in *E. coli* cells with the PET expression system. The cells harboring the expression plasmid were induced with IPTG, and the recombinant protein was purified to apparent homogeneity in SDS-PAGE by heat treatment and column chromatographies, as described in Materials and Methods. Its N-terminal amino acid sequence was determined to be XKVEFSGKRY, suggesting elimination of the N-terminal Met residue (the predicted N-terminal amino acid sequence of GlnmA*tk* is MGKVEFSGKRYVID). The molecular mass of the recombinant GlnmA*tk* was estimated to be about 86 kDa by SDS-PAGE and 193 kDa by gel filtration chromatography. The results indicated that GlnmA*tk* was a homodimeric enzyme.

**Substrate specificity of GlnmA*tk*.** We then examined the hydrolytic activities of the purified recombinant GlnmA*tk* toward various β-disaccharides (GlcNAc₂, GlcN₂, lactose, cellobiose, and laminariobiose) as substrates. The reactions were monitored by TLC. As shown in Fig. 4A, GlnmA*tk* could hydrolyze neither lactose nor GlcNAc₂, but a remarkable activity was observed toward GlcN₂. This enzyme showed very weak hydrolytic activities toward cellobiose and laminariobiose in addition to the major GlcNase activity (Fig. 4A), and the β-galactosidase activity was also confirmed by fluorometric assay with 4-methylumbelliferyl β-D-glucoside as the substrate (1.77 × 10⁻² U/mg). It should be noted that GlnmA*tk* could not hydrolyze 4-methylumbelliferyl β-D-galactoside, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide, or 4-methylumbelliferyl N-acetyl-β-D-galactosaminide (<1.00 × 10⁻³ U/mg). Although the primary structure of GlnmA*tk* showed homology with β-galactosidases, as described above, the results obtained here clearly indicated that GlnmA*tk* was a GlcNase, not a β-galactosidase.

**Enzymatic properties of GlnmA*tk*.** The optimal pH and temperature of GlnmA*tk* for reduced chitobiose (GlcN₂OH) were 6.0 and 80°C, respectively. Activity levels at 37°C and 100°C were approximately 20% of that observed at the optimal temperature. For determination of the cleavage specificity of this enzyme, reaction products from various chain lengths of chitooligosaccharides (GlcN₃-h₂, GlcN₄-h₂) were analyzed with TLC (Fig. 4B). GlnmA*tk* showed hydrolytic activities toward all substrates examined, and at the early stages of the reactions, we detected spots corresponding to GlcN and oligosaccharides that were one unit shorter than the starting substrates. Subsequently, reduced chitooligosaccharides (GlcN₄-h₂OH) were applied as substrates in order to distinguish the direction of the oligosaccharides. In this experiment, the products were visualized separately with ninhydrin reagent (Fig. 4C) in addition to aniline-diphenylamine reagent (data not shown) because reduced chitooligosaccharides could not be detected with the latter reagent. When aniline-diphenylamine reagent was used, the only spots detected were those corresponding to GlcN. This...
Aspergillus niger mouse (AAA37292 [21]), tomato (P48980 [1]), apple (T17002 [29]), AAL80487; family 35/ H9252 thomonas manihotis (P48982 [34]), and

Apparently, GlmA \( \rightarrow \) arrowheads are presumably glucosaminitol (reduced GlcN).

shorter reduced chitooligosaccharide. The spots indicated by initial substrates were converted to GlcN and a one-unit-\( \rightarrow \) analyses revealed that at the early stages of the reactions, the indicated that all spots boxed in Fig. 4C, visualized with ninhydrin reagent, were reduced oligosaccharides. These TLC analyses revealed that at the early stages of the reactions, the initial substrates were converted to GlcN and a one-unit-shorter reduced chitooligosaccharide. The spots indicated by arrowheads are presumably glucosaminitol (reduced GlcN).

Apparent, GlmA\( _K \rightarrow \) was an exo-type enzyme which specifically hydrolyzed the first glucosaminide bond from the nonreducing end of chitooligosaccharides.

We then determined the kinetic constants of GlmA\( _K \rightarrow \) toward GlcN\( _{2-6} \). Among these substrates, GlmA\( _K \rightarrow \) showed the highest \( V_{\text{max}} \) and \( K_m \) values toward GlcN\( _2 \), while the lowest values were shown toward GlcN\( _7 \). The \( k_{\text{cat}}/K_m \) ratios toward all substrates were comparable. GlmA\( _K \rightarrow \) also released GlcN from chitosan polysaccharide (chitosan 10B, more than 98% deacetylated), but the specific activity (0.70 U/mg) was much lower than the \( V_{\text{max}} \) toward the chitooligosaccharides (12 to 99 U/mg). In contrast to the broad specificity of these substrates for chain length, this enzyme never accepted the N-deacetylated compounds colloidal chitin and GlcNAc\( _{2-4} \) as substrates regardless of their chain lengths (data not shown).

Expression profiles of GlmA\( _K \rightarrow \) in T. kodakaraensis. T. kodakaraensis was grown on various sugar-containing media, and the expression of GlmA\( _K \rightarrow \) was examined by Western blot analysis (Fig. 5). When the organism was grown at 85°C for 12 h, the expression of GlmA\( _K \rightarrow \) was induced only with the addition of GlcNAc\( _2 \), an end product from chitin by the extracellular chitinase ChiA\( _7 \) (lane 3). Indeed, significant GlcNase activity toward GlcN\( _2 \) was also detected in the extract from cells in the medium containing GlcNAc\( _2 \) (data not shown). The detection of GlmA\( _K \rightarrow \) protein and GlcNase activity within the cells also clarified that GlmA\( _K \rightarrow \) is an intracellular enzyme, as suggested from the primary structure. In contrast to GlcNAc\( _2 \), other disaccharides did not induce expression under these conditions (lanes 4 to 7). Expression of GlmA\( _K \rightarrow \) could not be detected in the presence of colloidal chitin after 12 h (lane 2), and a similar lack of induction was also observed with the GlcNAc\( _6 \) oligomer (data not shown). However, although to a lower extent, we clearly observed the induction of GlmA\( _K \rightarrow \) by colloidal chitin after prolonged incubation for 72 h (lane 9). These results indicate that GlmA\( _K \rightarrow \) expression can be induced under chitin degradation conditions through the intermediate, GlcNAc\( _2 \).

When GlcN\( _2 \) was added to the medium, serious browning of the medium was observed at 85°C, probably caused by a Maillard reaction of the amino sugar. In order to avoid the chemical reaction leading to the consumption of GlcN\( _2 \), we examined the effect of GlcN\( _2 \) on GlmA\( _K \rightarrow \) expression at 70°C. Although slight browning of the medium was still observed at the lower cultivation temperature, approximately half of the initial GlcN\( _2 \) remained in the medium even after cultivation. Nevertheless, no expression of GlmA\( _K \rightarrow \) was observed under this condition (lane 11), indicating that GlcN\( _2 \), although a substrate, was not an inducer of GlmA\( _K \rightarrow \) gene expression. Due to its inability to heat, a regulation system responding to GlcN\( _2 \) may not have evolved in the hyperthermophilic T. kodakaraensis. These expression profiles of GlmA\( _K \rightarrow \) against GlcNAc\( _2 \) and colloidal chitin supported the participation of GlmA\( _K \rightarrow \) in chitin catabolism in cooperation with ChiA\( _7 \) by T. kodakaraensis KOD1.

**DISCUSSION**

In this study, we identified a GlcNase gene from T. kodakaraensis KOD1 during our attempt to clarify the mechanism of archael chitin catabolism. This is the first report of a GlcNase gene not only from an archaean but also from any organism.

The \( glmA_7 \) gene was initially identified as a putative \( \beta \)-glycosyl hydrolase gene located near \( chiA_7 \) (11 kbp upstream) on the T. kodakaraensis genome (Fig. 2). We estimated that this gene product participated in the archael chitinolytic pathway and indicated that the recombinant GlmA\( _K \rightarrow \) indeed showed
GlcNase activity. Furthermore, this activity could actually be detected in the cell extract of T. kodakaraensis. Although GlmA_{Tk} can hydrolyze chitosan and chitooligosaccharides of various chain lengths, as described above, the deacetylated products of GlcNAc can be considered the physiological substrates in this organism for the following reasons. (i) The intracellular localization of GlmA_{Tk} prevents its direct interaction with polysaccharides existing outside the cells. In addition, the specific activity of GlmA_{Tk} for chitosan was much lower than those for chitooligosaccharides. (ii) ChiA_{Tk}, harboring both endo- and exo-type catalytic domains, accumulated only

**FIG. 4.** Hydrolysis of various β-disaccharides (GlcNAc, GlcN, lactose [Lac], cellulose [Glc], and laminaribiose [β-1,3-Glc]) (A), chitooligosaccharides (GlcN_{2-6}) (B), and reduced chitooligosaccharides (GlcN_{2-6}-OH) (C) with various chain lengths by GlmA_{Tk}. The reaction mixture (25 μl) containing 0.2 mg of the substrate and 25 pmol of GlmA_{Tk} in 50 mM MES-NaOH (pH 5.0) was incubated at 70°C. The reaction products were analyzed at the indicated times. TLC plates were visualized with aniline-diphenylamine reagent (A and B) and with ninhydrin reagent (C). The spots undetectable with the aniline-diphenylamine reagent in C are boxed. The spots pointed to by arrowheads are probable reduced GlcN. Lanes C, control without GlmA_{Tk} after each reaction; lanes S, various chain lengths of saccharides corresponding to the substrates used in each reaction; lanes rS, standard reduced chitooligosaccharides from GlcN_{2-6}-OH to GlcN_{6-6}-OH.

**TABLE 1.** Kinetic properties of GlmA_{Tk} toward various chitooligosaccharides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>V_{max} (U/mg)</th>
<th>K_{m} (mM)</th>
<th>k_{cat}/K_{m} (M^{-1} s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcN_{2}</td>
<td>98.7 ± 1.7</td>
<td>1.37 ± 0.06</td>
<td>1.08 × 10^{4}</td>
</tr>
<tr>
<td>GlcN_{3}</td>
<td>34.5 ± 2.6</td>
<td>0.270 ± 0.052</td>
<td>1.92 × 10^{5}</td>
</tr>
<tr>
<td>GlcN_{4}</td>
<td>41.7 ± 1.0</td>
<td>0.295 ± 0.021</td>
<td>2.12 × 10^{5}</td>
</tr>
<tr>
<td>GlcN_{5}</td>
<td>12.0 ± 0.2</td>
<td>0.0778 ± 0.0049</td>
<td>2.31 × 10^{5}</td>
</tr>
<tr>
<td>GlcN_{6}</td>
<td>35.1 ± 0.2</td>
<td>0.365 ± 0.0005</td>
<td>1.45 × 10^{5}</td>
</tr>
</tbody>
</table>

a GlcNase assays were performed as described in Materials and Methods.

**FIG. 5.** Western blot analysis of cell extract of T. kodakaraensis KOD1 grown on various sugar-containing media. The sugar content in each medium, cultivation time, and cultivation temperature are indicated. The amounts of protein applied were 20 μg (KOD1 cell extract) or 50 ng (recombinant GlmA_{Tk}, lane C).
GlcNAc₂ as an end product in the hydrolysis of colloidal chitin, and longer oligomers could not be detected (32, 33). These facts strongly suggested that in the chitin catabolic pathway of *T. kodakaraensis*, the substrate of GlmAₜₖ is intracellular GlcN₂. Furthermore, induction of GlmAₜₖ expression was observed after prolonged cultivation with chitin, and moreover, rapid induction occurred in the presence of GlcNAc₂ but not with GlcN₂. This implies that the generation of GlcN₂ occurs via Chiₜₖ-mediated degradation of chitin to GlcNAc₂. Our observations also raise the possibility of the presence of a novel GlcNAc unit-specific deacetylase in *T. kodakaraensis*, to provide the substrate for GlmAₜₖ from GlcNAc₂.

So far, among known organisms, chitin is degraded to dimer units by chitinas, followed by dimer processing with GlcNase, diacetylchitobiose phosphorylase, or GlcNAc₂ phosphotransferase system and 6-phospho-β-glucosaminidase. Alternatively, chitin can be degraded by chitosanase and GlcNase after the initial deacetylation of chitin (Fig. 1A) (6, 15, 16, 28). In *T. kodakaraensis* KOD1, as in many organisms, chitin is first degraded into GlcNAc₂ by Chiₜₖ (32, 33), but the pathway downstream from GlcNAc₂ was suggested to follow a distinct pathway, that is, deacetylation of GlcNAc₂ by an uncharacterized deacetylase and successive hydrolysis to GlcN by GlmAₜₖ (Fig. 1B). To our knowledge, a chitin catabolic pathway of this kind has not yet been reported and may be a novel pathway functioning in archaeal cells.

Until now, there have been only four reports on GlcNases. They have been identified from an actinomycete (Nocardia orientalis [22]) and fungi (*Trichoderma reesei* [25], *Penicillium funiculosum* [20], and *Aspergillus oryzae* [36]). As the corresponding genes have not been isolated, there is no information on their primary structures. The properties of these GlcNases and GlmAₜₖ are summarized in Table 2. These GlcNases cleave the nonreducing terminal glycosyl bond of chitooligosaccharides. Significant differences between GlmAₜₖ and the others were observed in terms of their subunit assembly and their localization. GlmAₜₖ was dimeric and an intracellular enzyme, while the other GlcNases were monomeric and extracellular enzymes. Therefore, GlmAₜₖ may not be related to the other GlcNases with respect to primary structure. The optimum temperature for GlmAₜₖ was the highest among these GlcNases.

The three archaeal proteins from *Pyrococcus* spp. that were homologous to GlmAₜₖ were originally assigned as putative β-galactosidases because the N-terminal and central regions in these proteins showed homologies toward family 35 and 42 β-galactosidases, respectively. Although these archaeal proteins also harbored the two putative catalytic glutamate residues conserved in these β-galactosidases, highly conserved motifs around these catalytic residues were not conserved, as described in Results (Fig. 3). An unrooted phylogenetic tree clearly showed that GlmAₜₖ and the putative archaean β-galactosidases were positioned far from either the family 35 or 42 β-galactosidases. It has also been reported that the putative β-galactosidase from *P. furiosus* (PF0363) did not possess β-galactosidase activity toward a chromogenic substrate (14). In light of these results, the putative archaean β-galactosidases can be predicted to be not β-galactosidases but GlcNases, as demonstrated in this study.

The glmAₜₖ gene was clustered with genes of a putative glucosamine-6-phosphate (GlcN6P) synthase and a putative ATP-binding cassette (ABC) transport system in the same direction on the *T. kodakaraensis* genome (Fig. 2). The transcription of these genes was estimated to be polycistronic because the flanking genes overlapped or were located within short interval regions (from −8 bp to 47 bp). The genes for previously studied chitinase and β-glycosidase were located in the region upstream of the putative ABC transport system in an opposite orientation. Chiₜₖ is an extracellular enzyme, but GlmAₜₖ was found within the cell, which indicates that GlcNₐ₄ produced by Chiₜₖ (or its deacetylated product) must be translocated across the cellular membrane to be further degraded. The putative ABC transport system found in the gene cluster (Fig. 2) may function as the amino disaccharide transporter. Generally, GlcN is converted to fructose-6-phosphate (Fru6P) by GlcN6P by phosphorylation and isomerization along with deamination (23). The resulting Fru6P is catalyzed by the glycolytic pathway. It has been reported that glucokinases from *P. furiosus* and *Thermococcus litoralis* can phosphorylate GlcN as well as glucose (17), and a gene homologous to these glucokinases is also present on the *T. kodakaraensis* genome (73% and 57% identity, respectively) at a different locus. GlcN6P synthase is an enzyme that converts Fru6P into GlcN6P, and there are two genes encoding products resembling this synthase on the genome. One of the genes for the synthase was located adjacently upstream of glmAₜₖ as described above (Fig. 2), raising the possibility that this gene product may be involved in the catabolism of GlcN produced from chitin.

Along with the first gene isolation of a β-glucosaminidase, the present study proposes the presence of a novel chitin catabolic pathway in *Archaea* (Fig. 1B). In order to confirm this pathway, isolation of GlcNAc₂ deacetylase as a key link between the already characterized enzymes is indispensable and is now under way.

**ACKNOWLEDGMENTS**

This work was supported by the Japan Science and Technology Corporation (JST) for Core Research for Evolutional Science and...
Technology (CREST) to T.I. and by a Grant-in-Aid from the Japan Society for the Promotion of Science (JSPS) fellows to T.T. from the Ministry of Education, Culture, Sports, Science and Technology.

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