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Molecular Analysis of the Gene Encoding a Novel Cold-Adapted Chitinase (ChiB) from a Marine Bacterium, *Alteromonas* sp. Strain O-7

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The chitinase B (ChiB) secreted by *Alteromonas* sp. strain O-7 was purified, and the corresponding gene (chiB) was cloned and sequenced. The open reading frame of the chiB gene encodes a protein of 850 amino acids with a calculated molecular mass of 90,223 Da. ChiB is a modular enzyme consisting of two reiterated domains and a catalytic domain belonging to chitinase family 18. The reiterated domains are composed of chitin-binding domain (ChiBD) type 3 and two fibronectin type III (Fn3)-like domains. Expression plasmids coding for ChiB or deletion derivatives thereof were constructed in *Escherichia coli*. Deletion analysis showed that the ChiBD domain of ChiB plays an important role in efficient hydrolysis of insoluble chitin. The optimum pH and temperature of ChiB were 6.0 and 30°C, respectively. The enzyme showed relatively high catalysis, even at low temperatures close to 0°C, and remarkable thermal lability compared to ChiA and ChiC, which are the mesophilic chitinases of the same strain. The $k_{cat}/K_m$ value for the ChiB reaction at 10°C was about 4.7 times higher than that of ChiC. These results suggest that ChiB is a cold-adapted enzyme. The RNA transcript of chiB was induced by 1% GlcNAc, and along with a rise in temperature, the RNA transcript showed a tendency to decrease. Thus, among the ChiA, ChiB, and ChiC chitinases, production of ChiB may be advantageous for the strain, allowing it to easily acquire nutrients from chitin and to survive in cold environments.

ChiB, a cold-adapted chitinase from *Alteromonas* sp. strain O-7, was purified and sequenced. The gene encoding ChiB was cloned and sequenced. The enzyme showed high activity at low temperatures, making it advantageous for survival in cold environments. The RNA transcript of chiB was induced by GlcNAc, and its expression decreased with an increase in temperature. This suggests that ChiB is a cold-adapted enzyme.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Alteromonas* sp. strain O-7 was grown at 27°C in Bacto Marine Broth 2216 (Difco) and used as the source of chromosomal DNA. *E. coli* JM109 and BL21(DE3) were grown at 37°C in Luria-Bertani medium supplemented with appropriate antibiotics for selection of transformants and for production of recombinant proteins, respectively. For agar medium, Luria-Bertani medium was solidified with 1.5% (wt/vol) agar (Nacalai Tesque).
The chiB gene was amplified by PCR with the primers and with chromosomal DNA digested with BamHI as the template. PCR was performed for 30 cycles consisting of a 30-s denaturation step at 94 °C, a 30-s annealing step at 50 °C, and a 1-min extension step at 72 °C for 2 min. The amplified DNA was digested with ScaI and SfuI, and the resulting fragment (2.0 kb) was cloned into the corresponding sites of pET-20b (+). The truncated ChiB protein (ChiBAN1) lacking the N-terminal region (residues 1 to 261) and the truncated ChiB protein (ChiBAN1N2) lacking the region corresponding to residues 1 to 512 were also amplified by PCR in the same manner as pET-ChiB. The primers used to encode ChiBAN1N2 were P-8 and P-9 (Table 1), and those used to encode ChiBAN1N2 were P-8 and P-10 (Table 1). The PCR products were inserted in frame between the SalI and SacI sites. The resulting plasmids, encoding the regions including residues 262 to 839 and residues 513 to 839 of ChiB, were purified with a HisTrap column (Amersham Bioscience). With the buffer (300 ml) and then with a linear gradient of NaCl (0 to 1 M) at a flow rate of 24 ml/h. ChiB activity was eluted at about 0.4 M NaCl. The active fractions were pooled and concentrated by ultrafiltration with a Q1000 membrane (Advantec, Tokyo, Japan). The concentrated sample was applied to a Sephadex G-100 column (1.9 by 50 cm; Amersham Bioscience) equilibrated with the same buffer containing 0.1 M NaCl. For further purification, the active fraction was chromatographed by using a fast protein liquid chromatography Resource Q (6 ml; Amersham Bioscience) column equilibrated with buffer and then the enzyme was eluted with a linear gradient of NaCl (0 to 0.6 M). Active fractions were eluted at a concentration of about 0.15 M and used as the purified enzyme solution.

General recombinant DNA techniques. Alteromonas chromosomal DNA was isolated as described previously (34). Plasmids pUC18 and -19 were used as cloning vectors. Plasmid pET-20b (+) (Novagen) was used as the expression vector. Agarose gel electrophoresis, plasmid DNA preparation, transformation of E. coli, and Southern hybridization were performed as described by Sambrook and Russell (30). Restriction enzymes and other modifying enzymes were purchased from Toyobo (Osaka, Japan).

Cloning of chiB. Chromosomal DNA of Alteromonas sp. strain O-7 was digested with various restriction enzymes and electrophoresed on a 0.6% agarose gel. The fragments from 1.0 to 3.0 kb were excised from the gel and puriﬁed with a GenElute gel extraction kit (Sigma). These fragments were self-ligated and used as template DNAs. Degenerate reverse primers P-1 and P-2 (Table 1) were synthesized on the basis of the N-terminal amino acid sequence of ChiB. PCR ampliﬁcation was performed with KOD-Plus-DNA polymerase (Toyobo) for 30 cycles consisting of 94 °C for 30 s, 55 °C for 15 s, and 68 °C for 5 min. The 2.3-kb fragment was ampliﬁed by using the HindIII-digested template and phosphorylated by T4 DNA polymerase kinase. The phosphorylated fragment was cloned into the dephosphorylated Smal site of pUC18. Analysis of the entire nucleotide sequence of the inserted DNA indicated that the 5′ upstream region of the chiB gene was missing. To clone the 5′ upstream region of the chiB gene, a second inverse PCR was performed. Two primers, P-3 and P-4 (Table 1), were synthesized on the basis of the nucleotide sequence of the ﬁrst inverse PCR product. The 1.1-kb fragment was ampliﬁed by using the Hpall and EcoRV-digested template. However, analysis of the nucleotide sequence indicated that the 5′ upstream region of the chiB gene was still missing. Thus, a third inverse PCR was performed by using two primers, P-5 and P-6 (Table 1), synthesized on the basis of the nucleotide sequence of the second PCR product. The 2.2-kb fragment was ampliﬁed by using the HindIII-digested template.

Construction of expression plasmids. Expression plasmids pET-ChiB, encoding the chiB residues 25 to 839, was constructed as follows. We synthesized two oligonucleotide primers, P-7 and P-8 (Table 1), that were modiﬁed to contain SacI and SfuI restriction sites to facilitate cloning in frame into pET-20b (+). The

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequencea</th>
<th>Locationa</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1</td>
<td>5′-TGTGTTTTT(A/G)CCATAT(A/T)GGAAA(A/G)TC-3′</td>
<td>1163–1734</td>
</tr>
<tr>
<td>P-2</td>
<td>5′-ATTAGGACAT(A/T)GAATTC(A/G)CTTT-3′</td>
<td>1777–1799</td>
</tr>
<tr>
<td>P-3</td>
<td>5′-AAATGGATGAGATTACCCAGGT-3′</td>
<td>1538–1551</td>
</tr>
<tr>
<td>P-4</td>
<td>5′-TCCGCAATCCAGTTCAAGCATG-3′</td>
<td>1893–1886</td>
</tr>
<tr>
<td>P-5</td>
<td>5′-TCCGAATTCTGCTGACGTTGAC-3′</td>
<td>1186–1163</td>
</tr>
<tr>
<td>P-6</td>
<td>5′-GGTACAGGTTGTTGGATCAAGTGCACCC-3′</td>
<td>1234–1257</td>
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<tr>
<td>P-7</td>
<td>5′-GGGACCCGCGGATCCTGACAGC-3′</td>
<td>244–264</td>
</tr>
<tr>
<td>P-8</td>
<td>5′-CTGTGTTTTCACGAGTTGCGCTGACAG-3′</td>
<td>2718–2698</td>
</tr>
<tr>
<td>P-9</td>
<td>5′-AAGGTTGACGTTGCAAGCATGTCGACAGT-3′</td>
<td>1551–1579</td>
</tr>
<tr>
<td>P-10</td>
<td>5′-AACCACATATCTCTGATCGACGTC-3′</td>
<td>1704–1732</td>
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<td>5′-CTAAGGTTGTCTTATGTTGCGCTG-3′</td>
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<td>P-12</td>
<td>5′-CTAACTCAGGTTGTCTTATGTTGCGCTG-3′</td>
<td>1361–1338</td>
</tr>
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</table>

a The mutagenized restriction sites are underlined.

b Nucleotide numbering of the chiB gene (accession no. AB086847).
serum diluted to 1:1,000 in phosphate-buffered saline containing 2.0% skim milk (Difco). Bound antibody was detected as described previously (21).

Real-time quantitative PCR analysis of the \( \text{chiB} \) transcript. \( \text{Alteromonas} \) sp. strain O-7 was cultured in Bacto Marine Broth 2216 or the same medium containing 1.0% GlcNAc at 17, 27, or 37 °C until the optical density at 600 nm reached 1.2. Total RNA was extracted from 1.0-ml suspensions of \( \text{Alteromonas} \) sp. strain O-7 cells with an SV total RNA isolation system (Promega) in accordance with the manufacturer’s instructions. Total RNA (5.0 \( \mu \)g) and primer P-12 (Table 1) were used to reverse the \( \text{chiB} \) transcripts. The reaction was carried out at 55 °C for 60 min with Moloney murine leukemia virus reverse transcriptase (RNase H minus; Promega) and terminated by heating at 70 °C for 15 min. The amount of the reverse transcript was measured by real-time quantitative PCR. P-11 and P-12 (Table 1) were the primers used. PCR amplification was monitored with a QuantiTect SYBR Green PCR kit (Qiagen) in a LightCycler (Roche Diagnostics). For each sample, a log-linear line was fitted automatically by selecting two points above the threshold band to determine the fractional cycle number of the crossing point. The data were calculated automatically by the LightCycler software (version 3.53; Roche Diagnostics).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank databases under accession no. AB086847.

RESULTS

Purification of ChiB. When \( \text{Alteromonas} \) sp. strain O-7 was grown in medium supplemented with chitin, zymogram analysis (48) showed four activity bands corresponding to proteins of 85 (ChiA), 65 (truncated form of ChiA), 45 (ChiC), and 35 (ChiB) kDa in the culture supernatant after 36 h (data not shown). To characterize ChiB, the 35-kDa protein was purified from the culture supernatant of the strain by successive column chromatographies on DEAE-Toyopearl 650 M, Sephadex G-100, and Resource Q. The purification procedure is summarized in Table 2. By the procedure described, the enzyme was purified about 22.0-fold with a yield of 3.5%. The molecular mass of the enzyme estimated by SDS-PAGE was 35 kDa (data not shown), and the N-terminal amino acid sequence was DKPIGKKHLIGYWHNFVNP. The optimum pH and temperature of purified ChiB were 6.0 and 30°C, respectively, when pNP-(GlcNAc)\(_2\) was used as the substrate.

Cloning and nucleotide sequence of the \( \text{chiB} \) gene. The nucleotide sequences of three amplified DNAs were determined and connected by overlapping the sequences to analyze the full-length \( \text{chiB} \) gene. The \( \text{chiB} \) gene consists of 2,550 nucleotides encoding a protein of 850 amino acids with a predicted molecular mass of 90,223 Da (Fig. 1). The open reading frame has an ATG start codon at position 187 that is preceded by a possible ribosome-binding site (AGGGA) at a distance of 11 nucleotides. An inverted repeat composed of a 6-bp stem and a loop of seven bases was located 11 bp downstream of the \( \text{chiB} \) gene.

![FIG. 1. (A) Deduced amino acid sequence encoded by the \( \text{chiB} \) gene. The signal peptide sequence is underlined. The type 3 ChtBD is boxed, and the Fn3-like domain is shaded. The regions that are homologous to the consensus sequence of the type 3 ChtBD are indicated by white letters on a black background. The N-terminal amino acid sequence of ChiB purified from the culture supernatant is shown by bold letters. Solid circles above amino acid residues represent the consensus sequences of family 18 chitinases. (B) Diagram of the domain structure of ChiB: signal peptide:■; ChtBD, □; Fn3-like domain, □; catalytic domain, □.](http://jb.asm.org/ on March 4, 2014 by PENN STATE UNIV)
The deduced N-terminal 24-amino-acid sequence showed the typical features of signal peptides, which are composed of a positively charged region, a hydrophobic region, and a signal sequence cleavage site (27). From the characterization of the cleavage sites, it is presumed that the putative site of cleavage might be between alanine 24 and tyrosine 25, which is compatible with the −3, −1 rule of von Heijne (43). The molecular mass of the translated protein was much larger than that of the enzyme purified from the culture supernatant of Alteromonas sp. strain O-7 (35 kDa). The N-terminal amino acid sequence of the enzyme purified from the culture supernatant coincided precisely with the sequence starting from Asp519 of the deduced amino acid sequence (Fig. 1). These results suggest that ChiB purified from the culture supernatant appears to be generated by the action of a protease(s) present in the culture supernatant after the initial gene product is secreted into the culture medium. In order to detect the initial gene product in the culture supernatant, Western blot analysis with a polyclonal rabbit antibody to ChiB was performed (Fig. 2). When the strain was grown at 27°C in Bacto Marine Broth 2216 containing 1% GlcNAc, a band corresponding to a protein of 90 kDa was detected after 5 h. On the other hand, a band corresponding to a protein of 35 kDa appeared after 7 h of cultivation and as the amount of the 35-kDa protein increased, the amount of the 90-kDa protein decreased. These results demonstrate that the 35-kDa protein is a truncated form of the 90-kDa protein that is the initial product of the chiB gene.

**Domain structure of ChiB.** The BLAST analysis program was used to compare ChiB with proteins in databases. Most of the structural elements could be readily identified by sequence comparison. Analysis of the amino acid sequence of ChiB derived from the nucleotide sequence revealed that the enzyme consists of a multidomain structure (Fig. 1). An N-terminal signal peptide was followed by a fragment of 42 amino acids (Pro32 to Trp73) that showed significant homology to chitin-binding domain (ChtBD) type 3, as classified by the National Center for Biotechnology conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The domain showed sequence identities of 54% to the ChtBD of Alteromonas sp. ChiC (37), 44% to the ChtBD of Alteromonas sp. ChiD (41), 43% to the ChtBD of Pseudoalteromonas sp. ChiA (31), and 38% to the ChtBD of Vibrio cholerae ChiA (8). Sequence alignment of the ChtBD of ChiB with other type 3 ChtBDs revealed a consensus sequence (AKWWTQ) that is well conserved in bacterial ChtBDs (6). The duplicated domains located between Pro87 and Gly164 and between Pro182 and Ser260 were adjacent to the ChtBD and showed similarity to a fibronectin type III (Fn3)-like domain that is quite prevalent in bacterial sequences (26). Furthermore, the similar domain organization, consisting of ChtBD and two Fn3-like domains, was reiterated in a 228-amino-acid-residue region corresponding to residues 281 to 508 of ChiB (Fig. 1). Although a sequence identity of 31% between the first and second ChtBDs was found, the consensus sequence of the type 3 ChtBDs was not perfectly conserved in the second ChtBD (Fig. 1). However, residue Trp308, which seems to be essential for chitin binding, was identified.

The catalytic domain of ChiB was next to the reiterated sequences. The amino acid sequence from Asp519 to Asn850 was homologous to a number of chitinases belonging to glycosyl hydrolyase family 18 (14, 15). This region showed sequence identities of 79% to Vibrio cholerae ChiA (8), 52% to Xanthomonas sp. strain AK ChiA (29), 42% to Bacillus circulans ChiD (46), 39% to Streptomyces lividans ChiB (22), and 33% to Streptomyces thermoviolaceus Chi30 (40). Family 18 bacterial chitinases contain the consensus sequences SXGG and DXDXE, which are composed of a positively charged region, a hydrophobic region, and a signal sequence cleavage site (27). Two consensus sequences DXDXE are substrate-binding and active sites, respectively. These consensus sequences were also perfectly conserved in the catalytic domain of ChiB, indicating that Glu648 of ChiB is involved as the proton donor in the catalytic double-displacement mechanism during hydrolysis (15, 42). Among the seven domains, there were small Gly-, Ala-, Pro-, Ser-, and Thr-rich sequences that typically resemble domain linker regions (32).

**Expression and purification of recombinant proteins.** To elucidate the function of the region consisting of ChtBD and two Fn3-like domains, we constructed the recombinant ChiB protein and deletion derivatives thereof (ChiBΔN1 and ChiBΔN1ΔN2). ChiB, ChiBΔN1, and ChiBΔN1ΔN2 were purified by a combination of HisTrap affinity chromatography and Resource Q ion-exchange chromatography. The molecular masses of these proteins calculated from the deduced amino acid sequence with no signal peptide (ChiBΔN1, 67 kDa; ChiBΔN1ΔN2, 41 kDa) are in reasonable agreement with those estimated by SDS-PAGE (ChiBΔN1, 70 kDa; ChiBΔN1ΔN2, 43 kDa) (Fig. 3). However, the apparent molecular mass of the purified ChiB protein determined by SDS-PAGE was higher than that calculated on the basis of the deduced amino acid sequence of ChiB (92 kDa). These purified proteins were used for further enzymatic characterization.

**Characterization of recombinant proteins.** The pH and temperature profiles of ChiB, ChiBΔN1, and ChiBΔN1ΔN2 were examined by using pNP-(GlcNAc)2, a chromogenic substrate for chitinase. The pH and temperature profiles of these enzymes were almost identical, and the optimal pH and temperature of each enzyme were 6.0 and 30°C, respectively (data not shown).
shown). The optimum pH of ChiB (pH 6.0) was identical to those of ChiA and ChiC, whereas the optimum temperature of ChiB was much lower than those of ChiA and ChiC from the same strain, and the respective activities of ChiB at 10, 10, and 20°C were 28, 41, and 92% of that at the optimal temperature (Fig. 4). Figure 4 shows the effect of temperature on the stability of ChiA, ChiB, and ChiC. The denaturation curves of these enzymes clearly illustrated the low thermal stability of ChiB. The half-time of inactivation of ChiB at 40°C was 7 min, and the enzyme retained only 15% of its original activity after incubation at 40°C for 60 min. On the other hand, after preincubation for 60 min at 40°C, ChiA and ChiC retained 100 and 80% of their original activities, respectively. These results indicate that ChiB has features typical of a cold-adapted enzyme: relatively high catalytic activity at low temperature and remarkable thermosensitivity (9, 16).

The kinetic properties of ChiA, ChiB, and ChiC are shown in Table 3. The $k_{cat}/K_m$ value for the ChiB reaction at 20°C was 22.8- and 4.2-fold higher than those for the ChiA and ChiC reactions, respectively. The value for the ChiB reaction at 10°C was 4.7-fold higher than that for the ChiC reaction. The activity of ChiA at 10°C was too low for measurement of the kinetic constant of ChiA.

**Function of reiterated sequences.** To clarify the function of the reiterated sequences found in ChiB, the hydrolytic activities of ChiB, ChiBΔN1, and ChiBΔN1ΔN2 were measured at various temperatures (10, 20, and 30°C) (Table 4). When colloidal chitin was used as the substrate, the respective activities of ChiBΔN1 at 10, 20, and 30°C were 70, 75, and 76% of those of ChiB. The respective activities of ChiBΔN1ΔN2 at 10, 20, and 30°C were 32, 24, and 22% of those of ChiB. On the other hand, when glycol chitin was used as the substrate, ChiBΔN1 and ChiBΔN1ΔN2 showed slightly higher activity than ChiB at the assay temperatures tested. These results indicate that both reiterated sequences in the N-terminal region (residues 25 to 508) of ChiB are essential for efficient hydrolysis of insoluble chitin but not soluble chitin. However, the reiterated domains do not contribute to the cold adaptation of ChiB.

![FIG. 3. SDS-PAGE of recombinant ChiB, ChiBΔN1, and ChiBΔN1ΔN2. Lanes: M, molecular size standards; 1, ChiB; 2, ChiBΔN1; 3, ChiBΔN1ΔN2.](image)

![FIG. 4. (A) Temperature dependence of ChiA, ChiB, and ChiC. Reactions were carried out at various temperatures for 30 min with 50 mM citrate buffer, pH 6.0. The ChiA, ChiB, and ChiC protein concentrations were 1.7, 1.1, and 1.9 ng/μl, respectively. Symbols: □, ChiA; ●, ChiB; △, ChiC. (B) Thermal stabilities of ChiA, ChiB, and ChiC.](image)
ChiB cultured with no inducer, production of the RNA transcript of chiB by 1% GlcNAc at the cultivation temperatures examined, °C 2216 with or without 1.0% GlcNAc at various temperatures performed. The strain was cultured in Bacto Marine Broth growth temperatures, real-time quantitative PCR analysis was the presence of 1% GlcNAc.

The chitinolytic system of the strain consists of three chitinases bacterium that grows in the range of 10 to 37°C (33). To examine whether expression of the chiB gene is regulated by growth temperatures, real-time quantitative PCR analysis was performed. The strain was cultured in Bacto Marine Broth 2216 with or without 1.0% GlcNAc at various temperatures (17, 27, and 37°C). The RNA transcript of chiB was induced by 1% GlcNAc at the cultivation temperatures examined, and along with a rise in temperature, the RNA transcript of chiB showed a tendency to decrease (Fig. 5). The respective amounts of RNA transcript of chiB at 27 and 37°C were 70 and 41% of that at 17°C. On the other hand, when the strain was cultured with no inducer, production of the RNA transcript of chiB did not depend on the temperature. These results suggest that the chiB gene is regulated by the growth temperature in the presence of 1% GlcNAc.

**Gene expression.** Alteromonas sp. strain O-7 is a marine bacterium that grows in the range of 10 to 37°C (33). To examine whether expression of the chiB gene is regulated by growth temperatures, real-time quantitative PCR analysis was performed. The strain was cultured in Bacto Marine Broth 2216 with or without 1.0% GlcNAc at various temperatures (17, 27, and 37°C). The RNA transcript of chiB was induced by 1% GlcNAc at the cultivation temperatures examined, and along with a rise in temperature, the RNA transcript of chiB showed a tendency to decrease (Fig. 5). The respective amounts of RNA transcript of chiB at 27 and 37°C were 70 and 41% of that at 17°C. On the other hand, when the strain was cultured with no inducer, production of the RNA transcript of chiB did not depend on the temperature. These results suggest that the chiB gene is regulated by the growth temperature in the presence of 1% GlcNAc.

**DISCUSSION**

Alteromonas sp. strain O-7 is an efficient degrader of chitin in the marine environment (33). We have already shown that the chitinolytic system of the strain consists of three chitinases (ChiA, ChiB, and ChiC), three β-N-acetylglucosaminidasases (GlcNAcase A, GlcNAcase B, and GlcNAcase C), a chitinase-like enzyme (ChiD), a transglycosylative enzyme (Hex99), a chitin-binding protein (Cbp1), and a chitin-binding protease (AprIV) (21, 34–39, 41). In this paper, we report that ChiB is a modular enzyme with a high level of catalytic activity at low temperatures compared with ChiA and ChiC. The chiB gene encodes a protein of 890 amino acids that has a novel domain architecture. The region consisting of one type 3 ChtBD and two Fn3-like domains was reiterated in the amino-terminal half of the protein. To clarify the function of the ChtBD and the Fn3-like domain, truncated forms of the enzyme (ChiBΔN1 and ChiBΔN1ΔN2) were constructed and the enzyme activities toward soluble and insoluble chitin were examined. When insoluble chitin was used as the substrate, the activities of the truncated enzymes decreased remarkably in comparison with that of full-length ChiB. On the other hand, when soluble chitin was used as the substrate, the truncated enzymes showed slightly higher activities than ChiB. The type 3 ChtBD was found in ChiA, ChiC, ChiD, Cbp1, and AprIV of Alteromonas sp. strain O-7 as a common structural unit. We have already demonstrated that the type 3 ChtBD strongly binds α- and β-chitin and is essential for efficient hydrolysis of insoluble chitin (21, 37, 41). These results indicate that the type 3 ChtBD of ChiB directly participates in the binding to chitin molecules and plays an important role in the hydrolysis of the insoluble substrate. On the other hand, the reiterated Fn3-like domains of ChiB were located between the first and second type 3 ChtBDs and between the second type 3 ChtBD and a catalytic domain. Fn3-like domains are structurally characterized as β-sheets containing an immunoglobulin-like fold (7). Fn3-like domains are found in various prokaryotic chitinases in widely different arrangements (11, 28, 37, 45). Watanabe et al. reported that removal of the Fn3-like domains within chitinase A1 from Bacillus circulans WL-12 resulted in lower chitin-hydrolyzing activity, although the domain had no effect on chitin binding (47). Therefore, Fn3-like domains of ChiB may function as linkers to maintain the optimal distance between and orientation of the domains, although the true function of these Fn3-like domains remains to be elucidated (23).

The recombinant ChiB protein was purified to homogeneity by employing both affinity and conventional chromatographies. ChiB belongs to chitinase family 18 and shares most of its properties with other bacterial chitinases (14, 15). However, this enzyme showed a relatively high catalysis level, even at low temperatures close to 0°C, and remarkable thermal lability compared to ChiA and ChiC, which are the mesophilic chitinases of the same strain. ChiB showed properties similar to those of ArChiA and ArChiB, which are produced by a psychrophilic marine bacterium, Arthrobacter sp. strain TAD20 (17), which is a solitary instance of cold-adapted chitinase regardless of the increase in the number of bacterial chitinases. However, ChiB differs from ArChiA and ArChiB in the following points. (i) ChiB is an enzyme produced by the mesophilic marine bacterium Alteromonas sp. strain O-7 together with mesophilic chitinases ChiA and ChiC. (ii) The domain structure of ChiB differs from those of ArChiA and ArChiB. (iii) ChiB retained only 15% of the original activity after incubation at 40°C for 60 min, whereas ArChiA and ArChiB retained 18 and 30% of their original activity after incubation at 50°C for 60 min. In general, cold-adapted enzymes display an apparent optimal activity shifted toward low temperatures and manifest pronounced heat lability (9, 16, 18). The temperature-activity and temperature-stability relationships of ChiB were clearly different from those of ChiA and ChiC (Fig. 4). Furthermore, the kinetic parameters were determined for ChiA, ChiB, and ChiC at three different temperatures. The kcat/Km ratio of ChiB was 12.32 at 10°C, which is approximately 4.7-fold higher than that of ChiC. However, the activity of ChiA at 10°C was too low for measurement of the kinetic constant of the enzyme. These findings suggest that ChiB has properties in common with other cold-adapted enzymes. There are usually multiple chitinase genes in chitinolytic bacteria; however, the contributions of individual enzymes to chitin degradation have not been elucidated in detail. Alteromonas sp. strain O-7 is a marine bacterium that grows at temperatures ranging from 10 to 37°C (33). The amounts of chiB RNA transcript showed a
tendency to decrease with a rise in the temperature, although the mode of regulation has not been clarified. Thus, among chitinases ChiA, ChiB, and ChiC, production of ChiB may be advantageous for the strain, allowing it to easily acquire nutrients from chitin and to survive in cold environments. It has been proposed that increased flexibility is the most important factor for the catalytic efficiency of cold-adapted enzymes at low temperature (9). Analyses of the crystal structure and primary structure of cold-adapted enzymes have suggested that these enzymes have lower proline and arginine contents than their mesophilic counterparts (1–3, 10, 24, 25, 44). A structural role for arginine in multiple hydrogen bonds to backbone carbonyl and side chain oxygens has been proposed (4, 20, 24). On the other hand, proline residues are thought to modulate the entropy of protein unfolding by affecting backbone flexibility (19). The model structure of class C β-lactamase from the Antarctic psychrophile Psychrobacter immobile A5 indicated that the enzyme possesses the lowest arginine and proline contents of any class C enzyme (10). Furthermore, Galkin et al. have reported a clear relationship between arginine residue content and thermostability in the three NAD+–dependent alanine dehydrogenases from psychrophilic and mesophilic bacteria (12). Thus, we compared the arginine and proline contents of ChiA, ChiB, and ChiC as shown in Table 3. The ratio of arginine residues to the total number of amino acid residues of ChiB or its catalytic domain was lower than those of ChiA and ChiC. However, there was no clear relationship between Arg content and adaptation to cold because ChiA, which had unmeasurable activity at 10°C, had the next lowest Arg content and nearly the same Arg content in its catalytic domain as ChiB. Therefore, the Arg content of ChiB may not contribute to the thermal lability of the enzyme. Moreover, like that of NAD+–dependent alanine dehydrogenases (12), the thermal flexibility of ChiB could not be explained by the proportion of proline residues. Interestingly, ChiB also has a low proportion of tyrosine residues, which could provide polar interactions between aromatic rings (9). Unlike the Arg content, there was a clear relationship between the tyrosine contents in the catalytic domains of ChiA, ChiB, and ChiC and their thermostabilities. These results suggest that the increased flexibility related to the increased heat lability of ChiB could be explained by its tyrosine residue content. To clarify the strategy that ChiB uses to adapt to low temperatures, crystallization of ChiB is now being performed in our laboratory.

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