Overexpression and characterization of a novel chitinase gene from a marine bacterium Pseudomonas sp. BK1

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The chitinase A (ChiA)-coding gene of Pseudomonas sp. BK1, which was isolated from a marine red alga Porphyra dentata, was cloned and expressed in Escherichia coli. The structural gene consists of 1602 bp encoding a protein of 534 amino acids, with a predicted molecular weight of 55,370 Da. The deduced amino acid sequence of ChiA showed low identity (less than 32%) with other bacterial chitinases. The ChiA was composed of multiple domains, unlike the arrangement of domains in other bacterial chitinases. Recombinant ChiA overproduced as inclusion bodies was solubilized in the presence of 8 M urea, purified in a urea-denatured form and re-folded by removing urea. The purified enzyme showed maximum activity at pH 5.0 and 40°C. It exhibited high activity towards glycol chitosan and glycol chitin, and lower activity towards colloidal chitin. The enzyme hydrolyzed the oligosaccharides from (GlcNAc)₄ to (GlcNAc)₆, but not GlcNAc to (GlcNAc)₃. The results suggest that the ChiA is a novel enzyme, with different domain structure and action mode from bacterial family 18 chitinases.

Keywords: Pseudomonas, chitinase, cloning, overexpression, refolding

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Chitin, an insoluble linear β-1,4-linked polymer of N-acetylglucosamine (GlcNAc) is one of the most abundant polysaccharides in nature. It is a major component of most fungal cell walls, insect exoskeletons, and the shells of crustaceans. Chitinolytic bacteria play an important role in the degradation of chitin, resulting in the recycling of a carbon and nitrogen source into a readily usable form in the marine ecosystem. A number of bacterial chitinases and chitinase genes from various species have been characterized. In general, chitinases are classified in two major categories — endochitinases that cleave chitin polymer randomly at internal sites, and exochitinases that catalyze the progressive release of diacetylchitobiose starting at the non-reducing ends of chitin chains. Based on the sequence and structural similarities of the catalytic domains, chitinases are grouped into two distinct families (families 18 and 19) of glycosyl hydrolases. Most bacterial chitinases known to date belong to family 18, usually with a modular organization comprising the catalytic domain and the carbohydrate-binding modules (CBM).

Although considerable information is available on bacterial chitinase genes, reports about the chitinase gene from Pseudomonas species are lacking. In the present study, we have isolated a marine bacterium that possesses the chitinase activity from a marine red alga Porphyra dentata and have identified this strain as a member of Pseudomonas species. We also report the cloning and overexpression of the novel chitinase A (ChiA) gene in E. coli, and biochemical characterization of the recombinant ChiA that was produced as inclusion bodies and then recovered as active enzyme by refolding procedure.

Materials and Methods

Isolation of chitinase-producing microorganism

To isolate chitinase-producing microorganisms Porphyra dentata was collected from a farm of laver in Haenam, Korea. P. dentata tissue was excised and then suspended in filtered seawater with shaking for 2 hr. After centrifugation at 5,000 × g for 10 min, the supernatant was diluted and spread on the synthetic medium containing 1.2% agar. The synthetic medium...
consists of the following components per seawater liter (pH 7.5); 10 g polypeptone, 1 g yeast extract, 0.1 g K₂HPO₄, 0.6 mg FeCl₃ and 1 g Tris. Of agar-decomposing strains obtained from the agar plate, the strains showing the extracellular chitinase activity in the culture for 3 days at 23°C were isolated by the enzyme assay using 0.2% glycol chitin as substrate. Analysis of 16S rDNA sequence as well as biochemical and physiological characterization of the isolated strain were performed as described.

**Cloning of Chi A gene**

Chromosomal DNA of *Pseudomonas* sp. BK1 was isolated by the procedure described earlier with slight modification. Briefly, the chromosomal DNA was partially digested with *Sau3AI* and separated on 0.7% agarose gel. DNA fragments with sizes from 3 to 5 kb were then purified by using the GeneClean kit (Bio101, CA, USA), and ligated to *BamHI*-digested and dephosphorylated pBluescript SK (+) vector. *E. coli* JM109 cells were transformed with the ligated DNA. The screening of chitinase-producing clones was performed as described using LB agar plates containing 0.05% (w/v) ethylene glycol glitin, 0.01% (w/v) trypan blue, and 100 µg/ml of ampicillin.

Inserted DNA fragment from the halo-forming recombinant was subcloned into the corresponding sites of pUC 119 or pBluescript SK (+) vector and then sequenced. The analysis of sequence data and sequence similarity searches were performed using the BLAST (N) program of the National Center for Biotechnology Information (NCBI). Homology alignment was performed with the CLUSTAL W program using MacVector 6.5 software (Oxford Molecular Group).

**Expression of Chi A gene in *E. coli***

DNA fragment containing the Chi A gene was amplified by PCR using Pyrobest DNA polymerase (Takara Biomedicals, Japan) and a combination of forward (5′-CCCATATGAAACGCACCCATTAGATACTGTAAATTAC-3′, nucleotides 1-28) and reverse (5′-AACGATCTTACAGTTGTCACGCTAAACCGT-3′, complementary to nucleotides 1580-1606) primers, where the underlines represent the *NdeI* and *BamHI* sites, respectively, and the ATG codon for the initiation of translation is shown in italic. The plasmid pBluescript SK (+) containing the Chi A gene was used as a template.

The amplified 1.6 kb fragment was digested with *NdeI* and *BamHI* and then inserted into the corresponding sites of pET-29a (+), resulting in expression plasmid pET-chiA. *E. coli* BL21(DE3) cells harboring pET-chiA were grown in 100 ml of LB medium containing 50 µg/ml of kanamycin at 37°C with shaking until the absorbance at 600 nm was approximately 0.6. Subsequently, protein production was induced by adding isopropyl-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.5 mM and incubating at 37°C for further 3 hr. After centrifugation at 5000 × g for 30 min at 4°C, the pellets were suspended in 50 mM Tris-HCl buffer (pH 7.5) and treated with lysozyme (0.1 mg/ml), and then disrupted by sonication for 5 min with a 30 sec pulse. The cell components were separated into soluble and insoluble fractions by centrifugation at 12,000 × g for 20 min. The supernatant was recovered as extracellular fraction after 90% saturation of ammonium sulfate, followed by dialysis against the same buffer.

**Refolding and purification of recombinant ChiA**

*E. coli* BL21 (DE3) harboring pET-chiA was cultured in 500 ml of LB medium containing 50 µg/ml of kanamycin at 37°C with shaking. Protein expression and cellular fractionation were carried out as described above. Insoluble fraction containing inclusion bodies was suspended in 50 ml buffer A (50 mM Tris-HCl, pH 7.5, 2% TritonX-100, and 10 mM EDTA) and kept for 10 min at room temperature with occasionally mixing. Insoluble inclusion bodies were recovered as a pellet after centrifugation at 25,000 × g for 10 min. After washing three times with the same buffer A, the pellet was suspended in 30 ml buffer B (50 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol, 5% glycerol, 1 mM EDTA, and 8 M urea) and kept at room temperature for 2 hr to dissolve the inclusion bodies.

The supernatant of the solubilized inclusion bodies obtained by centrifugation at 12,000 × g for 15 min was applied at 1 ml/min to a 5 ml HiTrap Q XL column (Amersham Bioscience) equilibrated with buffer B. After washing the column with the same buffer B to remove unbound proteins, elution was performed by a linear gradient of 0 to 1 M NaCl at a flow rate of 1 ml/min. The fractions with a single peak of protein were collected and run on SDS-PAGE to check the purity of the eluted protein. The denatured purified protein was diluted with 2 volumes of buffer C (50 mM Tris-HCl, 1 mM dithiothreitol, and 2 M urea, pH 7.5) and then refolded by dialysis against 50 mM Tris-HCl buffer (pH 7.5) at 4°C for
48 hr through very slow dropping (approximately 12 drops/min) using peristaltic pump. No precipitate was generated during this refolding process. After centrifugation at 30,000 × g for 1 hr, the supernatant was applied at 1 ml/min to FPLC Mono Q HR 5/5 column (Amersham Bioscience) equilibrated with 50 mM Tris-HCl buffer (pH 7.5). After washing of the column with the same buffer at 1 ml/min, proteins were eluted with a linear gradient of NaCl from 0 to 1 M in the buffer at the flow rate of 0.5 ml/min. The purified enzyme was used for biochemical characterization.

Enzyme assay and SDS-PAGE
ChiA activity was measured by the colorimetric method as described8 using 0.2% colloidal chitin and glycol chitin as substrates. Activity was calculated with a standard curve obtained from known concentration of GlcNAc (0-0.05 mg). One unit of the enzyme activity was defined as the amount of the enzyme that released 1 µmol of GlcNAc per min. The enzymatic hydrolysis of N-acetylchitooligosaccharides was analyzed by thin-layer chromatography (TLC) as described13. Protein concentration was determined using the Bio-Rad protein assay kit according to the manufacturer's instructions, with bovine serum albumin (Sigma) as the standard for the calibration curve. SDS-PAGE was performed on 12% running gel as described by Laemmli14, and the resolved proteins were visualized by Coomassie staining. A low range protein standard (Bio-Rad) was used as molecular mass marker. ChiA activity in SDS-PAGE was detected as previously described15.

Effect of pH and temperature on enzyme activity
The following buffers were used to investigate the effect of pH on ChiA activity: 50 mM NaH2PO4/Na2HPO4 (pH 6.0 to 7.6), 50 mM Tris/HCl (pH 7.5 to 9.0) and 50 mM glycine/NaOH (pH 9.0 to 11.0). The effect of pH on protease stability was determined by incubating aliquots of the purified enzyme in buffers with different pH values for 30 min at 37°C. The optimum temperature for ChiA activity was determined over the range of 20-80°C and assay mixtures were equilibrated at the required temperature before adding the enzyme.

Nucleotide sequence accession number
The nucleotide sequence of ChiA gene reported in this study has been deposited in the GenBank database under accession number AY249148.

Results and Discussion
Isolation and identification of microorganism
The microorganism having chitinase activity was isolated from a marine red alga Porphyra dentata and designated as strain BK1. The strain was closely related to the Pseudomonas subgroup, based on its morphology, physiological properties, fatty acid composition and quinine component (data not shown). The phylogenetic analysis of strain BK1 using its 16S rDNA sequence data also supported the isolate closely related to the Pseudomonas subgroup (similarity, 89.7-98.7%). Accordingly, the isolated bacterium BK1 was named as Pseudomonas sp. BK1.

Cloning and sequence analysis of the ChiA gene from Pseudomonas sp. BK1
Approximately 1700 transformants were obtained by a single transformation experiment and screened for chitinase activity. An E. coli clone harboring the chitinase gene exhibited a clear zone on the blue background of the medium, indicating that the chitinase gene of Pseudomonas sp. BK1 was functionally expressed. The recombinant plasmid isolated from this clone contained a 3.5 kb insert DNA. The open reading frame (ORF) of ChiA gene consists of 1602 nucleotides encoding a protein of 534 amino acids with a predicted molecular weight of 55,370 Da (data not shown).

Comparison of the deduced amino acid sequence of ChiA with those of other chitinases in DNA and protein data banks showed a similarity to the chitinases of Pseudomonas aeruginosa PAO1 (NP_250990) and Bacillus circulans WL-12 (BAA13974) with 35% and 25% identities, respectively, whereas no similarity was found between ChiA and other known chitinases in the protein database of the National Center for Biotechnology Information (NCBI). Despite the lower identity, ChiA contained the conserved sequence D-X-D-X-E at positions 150-154 that is involved in the catalytic activity of family 18 chitinases16.

While this work was in progress, the sequence of a chitinase (pchA) gene from a marine bacterium Pseudomonas sp. PE2, showing very remarkable sequence similarity to that of ChiA described here, with an identity of 90% at amino acid level was deposited in GenBank database with accession no. AB063185. Although cloning and expression of the pchA gene has been recently reported7, biochemical characterization of the purified enzyme, including substrate specificity has not been investigated.

Nucleotide sequence accession number
The nucleotide sequence of ChiA gene reported in this study has been deposited in the GenBank database under accession number AY249148.
Analysis of the putative cleavage site of signal peptide by using the SignalP program was located between Ala-27 and Ala-28.

In general, almost all of the bacterial chitinases belonging to family 18 glycoside hydrolases are composed of multiple domains, such as a catalytic domain, a chitin-binding domain, a fibronectin type III-like domain, or a cadherin-like domain. Computer analysis with the deduced mature amino acid sequence of ChiA from Pseudomonas sp. BK1 using CDART program of NCBI revealed a novel-type enzyme, composed of three discrete domains in the following order: a family 18 chitinase catalytic domain (Lys-30 through Asp-326), a chitin-binding domain (Ala-355 through Pro-395), and a CBM (Glu-414 through Gln-533). Like the arrangement of domains in Bacillus circulans ChiA, this enzyme has its catalytic domain in the N-terminal portion and chitin-binding domain in the C-terminal portion (Fig. 1). However, the CBM is located at the C-terminus in this enzyme, unlike the arrangement of domains in other bacterial chitinases (Fig. 1), indicating that this enzyme has a novel domain structure. The function of each domain remains to be characterized.

Overexpression and refolding purification of ChiA in E. coli

When E. coli BL21 (DE3) cells carrying pET-chiA were induced for 3 hr with 0.5 mM IPTG at 37°C, chitinase activity was clearly detected in both extracellular and intracellular fractions of the cells. However, a considerable amount of protein with molecular mass of about 55 kDa was present as inclusion bodies in the insoluble fraction (Fig. 2, lane 2), but the corresponding protein was not detected clearly in other fractions (data not shown). Although several attempts including variations of IPTG concentration, induction time, and growth temperature were done to increase the amount of recombinant protein with ChiA activity in soluble fraction, the visibly increased production of soluble protein was not observed.

It is known that high-level or overexpression of recombinant chitinases in soluble fraction of E. coli cells is difficult like our result. Thus, the recombinant ChiA produced as inclusion bodies was solubilized by the buffer containing 8 M urea and then purified by HiTrap Q column chromatography in the presence of 8 M urea (Fig. 2, lanes 2 and 3). The active enzyme was obtained by successive refolding and finally purified by Mono Q column chromatography. The purified enzyme showed a single band with the molecular mass of about 55 kDa on SDS-PAGE and exhibited chitinase activity on gel activity assay with glycol chitin as substrate (Fig. 2, lanes 4 and 5). This size was in good agreement with 55370 Da calculated from the amino acid sequence. The purification yield was 12% and the specific activity was 1.29 U/mg towards glycol chitin.

Characterization of the recombinant ChiA

The purified recombinant ChiA showed maximum activity at pH 5.0 and 40°C, when glycol chitin was used as the substrate (data not shown). These temperature and pH optima for the activity were similar to those of chitinase (ChiC) from Pseudomonas aeruginosa. The substrate specificity of the purified enzyme was investigated with the
various substrates. As shown in Table 1, the enzyme exhibited high activity towards glycol chitosan and glycol chitin, whereas low activity was detected towards colloidal chitin and glucan. This substrate specificity was similar to those of chitinases from Aeromonas sp. 10S-24 and Aspergillus fumigatus YJ-40721. The enzyme showed no activity towards carboxymethyl cellulose (CMC), lichenan and pullulan.

When N-acetylchitooligosaccharides from monomer to hexamers were used as a substrate, the enzyme hydrolyzed oligosaccharides from (GlcNAc)4 to (GlcNAc)6, but not GlcNAc to (GlcNAc)3 (Fig. 3). These results indicate that ChiA is an endochitinase, with unique action mode on N-acetylchitooligosaccharides. The action mode also suggests that the enzyme requires substrates with four or more N-acetylglucosamine residues for the expression of its activity. To our knowledge, no bacterial chitinase has so far been reported to possess activity towards N-acetylchitooligosaccharides with more N-acetylglucosamine residues than (GlcNAc)4.

The marine red alga Porphyra, including P. dentata and P. yezoensis, is abundantly cultivated in eastern Asia including Japan, Korea and China for food use. Its farming has frequently suffered great economic damage due to diseases, such as red rot and green spot diseases, caused by fungus and bacteria22. The development of a transgenic Porphyra species tolerant to fungal infection is considered as one of the most promising tools to resolve these problems22. The genes encoding antifungal proteins such as chitinase and β-1,3-glucanase are excellent candidates for that purpose, like in the case of the higher terrestrial plant23,24. Therefore, the ChiA gene of Pseudomonas sp. BK1 may be also useful for developing a transgenic Porphyra species resistant against fungal pathogens.

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References
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