Overexpression and characterization of thermostable chitinase from Bacillus atrophaeus SC081 in Escherichia coli

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The chitinase-producing strain SC081 was isolated from Korean traditional soy sauce and identified as Bacillus atrophaeus based on a phylogenetic analysis of the 16S rDNA sequence and a phenotypic analysis. A gene encoding chitinase from B. atrophaeus SC081 was cloned in Escherichia coli and was named SCChi-1 (GQ360078). The SCChi-1 nucleotide sequences were composed of 1788 base pairs and 596 amino acids, which were 92.6, 89.6, 89.3, and 78.9% identical to those of Bacillus subtilis (ABG57262), Bacillus pumilus (ABI15082), Bacillus amyloliquefaciens (ABO15008), and Bacillus licheniformis (ACF40833), respectively. A recombinant SCChi-1 containing a hexahistidine tag at the amino-terminus was constructed, overexpressed, and purified in E. coli to characterize SCChi-1. H6SCChi-1 revealed a hydrolytic band on zymograms containing 0.1% glycol chitin and showed the highest lytic activity on colloidal chitin and acidic chitosan. The optimal temperature and pH for chitinolytic activity were 50°C and pH 8.0, respectively. [BMB reports 2011; 44(3): 193-198]

INTRODUCTION

Chitinase is an enzyme that catalyzes the hydrolysis of chitin, the linear polymer of β-1, 4-linked N-acetylglucosamine (GlcNAc). Chitin, a highly insoluble biopolymer, is one of the most abundant organic compounds in nature (1). The enormous amounts of chitin that are continuously generated require disposal and recycling on a formidable scale. Thus, considerable attention has been focused on chitin hydrolysis. Additionally, chitin oligosaccharides generated by chitin hydrolysis possess versatile functional properties (2). Industrially, chitin oligosaccharides have been processed by chemical methods. For example, GlcNac is produced by the acid hydrolysis of chitin with concentrated HCl at high temperature. However, this chemical process has some problems, including the production of acidic wastes, low yield, and high cost. Therefore, it is important to isolate chitinase, which is essential for producing chitin oligosaccharides from chitin.

Chitinases have been found in a wide range of organisms including bacteria, fungi, higher plants, insects, crustaceans, and some vertebrates. In particular, bacteria produce various chitinases to digest chitin and utilize it as an energy source (3), and most of these chitinases have been isolated and characterized from Bacillus species (1, 4, 5). Bacillus species secrete a large number of chitinases into the medium, which complicate downstream processing and affect product stability (1, 4). Therefore, it would be advantageous to overexpress Bacillus chitinase intracellularly in an Escherichia coli expression system. Secretion of recombinant proteins into the periplasm has several advantages over intracellular production, such as facilitated downstream processing, higher product stability and solubility, increased biological activity, and correct folding and processing (3, 6, 7). Although these advantages are known in E. coli expression systems, these systems are limited in that not every chitinase can be expressed efficiently.

In this report, we generated a high level chitinase using an E. coli expression system. We isolated the chitinase from Bacillus atrophaeus, and it was characterized to hydrolyze colloidal chitin as well as chitosan. Additionally, the enzyme showed its highest activity at 60°C compared with the other chitinases.

RESULTS AND DISCUSSION

Screening and identification of a strain producing chitinase

SC081, isolated from Korean traditional soy sauce, had the highest chitinolytic activity, as it had the largest clear zone around the colony on a plate containing 1.0% colloidal chitin. SC081 is a Gram-positive rod-shaped Bacillus that produces a soluble brown pigment in a medium containing an organic nitrogen source such as Bacillus atrophaeus. B. atrophaeus is a Gram-positive, aerobic, endospore-forming, rod-shaped bacterium that produces a pigment on media containing an organic nitrogen source (8). To date, SC081 and B. atrophaeus had similar properties. SC081 was affiliated with the genus Bacillus, closely related to B. atrophaeus (99% sequence identity), and formed a robust clade with B. atrophaeus based on a
Phylogenetic analysis of its 16S rDNA gene sequence (data not shown). Based on these data and previous reports, SC081 was classified as *B. atrophaeus* and named *B. atrophaeus* SC081. Although a large numbers of chitinases have been identified in *Bacillus* sp., chitinases have not been found in *B. atrophaeus*.

**Cloning and sequencing of chitinase SCChi-1**

To clone the chitinase gene from *B. atrophaeus* SC081, subclones were obtained using PCR, and the plasmid DNA was isolated and analyzed by DNA electrophoresis. Subsequently, the 2.0 kb lengths of DNA were sequenced, and a gene coding chitinase was selected. The complete nucleotide sequences and the deduced amino acid sequences are presented in Fig. 1A. The entire open reading frame consisted of 1786 nucleotides, encoding a putative protein of 596 amino acids with a theoretical molecular mass of 63.5 kDa and designated as SCChi-1 (GQ360078). SCChi-1 was also characterized against known chitinases using a comparative sequence analysis and was 92.6, 89.6, 89.3, and 78.9% identical to that of *Bacillus subtilis* (ABG57262), *Bacillus pumilus* (ABI15082), *Bacillus amyloliquefaciens* (ABO15008), and *Bacillus licheniformis* (ACF40833), respectively (Fig. 1B-D). Similar to these chitinases, SCChi-1 is composed of three domains in the following order: a glycosyl hydrolase domain from Ser-34 to Pro-448 (1, 5, 9), a fibronectin 3 (Fn3) domain from Ser-458 to Thr-539 (1, 3, 10), and a chitin binding domain (ChIBD) from Lys-549 to Leu-582 (3, 9). These domains contain consensus residues, as indicated in Fig. 1. In the glycosyl hydrolase domain (Fig. 1B), the conserved Phe190, Asp194, Glu198, and Leu205 residues are associated with chitin hydrolysis (9, 11). In the Fn3 domain (Fig. 1C), the conserved Pro, Ser, and Thr residues are important for the degradation of insoluble and crystalline polysaccharides such as chitin. Several well-conserved Pro residues implies a proline-specific protease, and hydroxyamino acid residues such as Ser and Thr connect the catalytic domain and the chitin binding domain as a domain linker (1, 3). Therefore, the Fn3 domain is found in several enzymes such as chitinases, cellulases, amylases, poly (3-hydroxybutyrate) depolymerases, and bacterial glycosyl hydrolases (10). The C-terminal region of SCChi-1 contained the well-conserved ChIBD (Fig. 1D). The importance of ChIBD has been demonstrated for some bacterial chitinases in the degradation of insoluble chitin (11). In particular, Tyr and Trp residues and several polar hydrogen bonding groups, which are involved in the ability of chitinase to stack directly against the pyranose rings of N-acetylglucosamine residues in chitin, are known in ChIBD (11). Therefore, the SCChi-1 sequence analysis suggested that it may be able to function as a chitinase. However, a functional chitinase activity study of SCChi-1 is needed to determine whether it plays a role as a chitinase.

**Expression and purification of SCChi-1 in E. coli**

The SCChi-1 coding region was inserted into the *E. coli* expression vector pBADNH to produce SCChi-1 for the func-

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**Fig. 1.** Sequence analysis of SCChi-1 from *B. atrophaeus* SC081. (A) The deduced amino acid sequence of SCChi-1 is given below the nucleotide sequence. The stop codon is indicated by an asterisk. The glycosyl hydrolase domain is indicated by a box, and the fibronectin type 3 (Fn3) domain is underlined. The chitin/cellulose-binding domain homologs are written in bold. (B, C, D) Alignment of the chitinase/catalytic domain (B), the Fn3 domain (C), and the chitin/cellulose binding domain (D) of SCChi-1. The domains were compared with those of *B. subtilis* ABG57262, *B. pumilus* ABI15082, *B. amyloliquefaciens* ABO15008, and *B. licheniformis* ACF40833. Dark background represents the identity, and arrows indicate the residues identified as being conserved among bacterial and fungal chitinases. * indicates conserved aromatic residues.
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Identification and purification of H6SCChi-1. (A) Overexpressed H6SCChi-1 was indicated on SDS-PAGE (lanes 1 and 2). H6SCChi-1 chitinase activity after induction was detected on zymograms with 0.1% glycol chitin (lanes 3 and 4). H6SCChi-1 purified on a Ni2+ affinity chromatography (lane 5). Lane M, marker proteins; lanes 1 and 3, non-induced cell lysate containing H6SCChi-1; lanes 2 and 4, cell lysate containing overexpressed H6SCChi-1. (B) MALDI-TOF MS spectrum of purified SCChi-1. (B) The picks show fragments cleaved by trypsin, and the numbers indicate mass values of the fragment. Circle indicates matched peptides compared with chitinase from Bacillus sp. DAU101. In Fig. 2C, the detected amino acid sequence corresponded to the expected amino acid sequence of SCChi-1. These data suggest that the expected amino acid sequence based on the SCChi-1 gene sequence isolated from B. atrophaeus SC081 is correct. Furthermore, it shows that the H6SCChi-1 fusion protein was expressed exactly and purified in E. coli.

Determination of SCChi-1 chitinase activity
To test a potential function for SCChi-1, the same amount of crude protein extracted from the recombinant E. coli clone and extracellular protein including chitinase from B. atrophaeus SC081 was tested on agarose plates containing colloidal chitin, glycol chitin, soluble-acidic chitosan, and glycol chitosan, respectively. As a result, the crude extracts from cells overproducing H6SCChi-1 showed the highest chitinase activity with colloidal chitin and soluble-acidic chitosan among all the substrates tested (Fig. 3A-C). In contrast, the crude protein including non-expressed H6SCChi-1 and extracellular protein from B. atrophaeus SC081 containing putative chitinase did not hydrolyze these substrates (Fig. 3A-D). The chitinase activity of the extracellular protein from B. atrophaeus SC081 was identified by zymography with glycol chitin after an overnight reaction (data not shown), but activity was not detected during this measurement (2-h reaction). This means that the relative activity of E. coli cell lysates containing H6SCChi-1 was faster.
than the extracellular protein from *B. atrophaeus* SC081 (Fig. 3E), suggesting that the *E. coli* expression system is efficient for degrading chitin. Additionally, H$_6$SCChi-1 hydrolyzed acidic chitosan. Chitinase activity has been associated only with chitin and has not been demonstrated for chitosan (1, 4). Our study showed that SCChi-1 isolated from *B. atrophaeus* SC081 functions as a chitinase hydrolyzing chitin and chitosan (Fig. 3C and E). Recent studies have focused on converting chitin and chitosan to oligosaccharides, because the oligosaccharides are not only water-soluble but also possess versatile functional properties such as antitumor activity, immunoenhancing effects, and antibacterial and antifungal activity (2, 14). Therefore, SCChi-1 hydrolyzing chitin as well as chitosan is applicable to numerous industrial applications. Although more experiments are needed to obtain evidence for the above supposition, our work has shown that chitinase can hydrolyze chitin and chitosan.

**Effect of temperature and pH on SCChi-1 activity**

Because chitin hydrolysis is accelerated by high temperature and is conducted in the presence of acid, enzymes activity under these conditions would be of particular interest (3). In this study, SCChi-1 showed optimal activity at 50°C and was thermally stable by retaining more than 80% of its initial activity between 40°C and 60°C for 1 h (Fig. 4A). The optimal pH for activity was pH 8.0, and activity was stable between pH 4-10 for 1 h, retaining more than 60% of the initial activity (Fig. 4B). Enzyme stability has been reported for chitinases from *B. cereus* TKU006, *Chaetomium globosum*, and *Pseudomonas* sp. PE2. In *B. cereus* TKU006, chitinase is stable at pH 3-11 and 50% of its initial activity is retained at 60°C, after reactions at various pHs and temperatures for 1 h (4). *Chaetomium globosum* and *Pseudomonas* sp. PE2 show stability similar to SCChi-1 when incubated at pH 3-10 or at 40-55°C and at pH 5.5-9 or 20-40°C for 30 min, respectively (3, 15). These reports along with our data demonstrate that SCChi-1 has a higher temperature and broader pH stability range than most other chitinases. Therefore, SCChi-1 may effectively hydrolyze chitin, which is performed at high temperatures and under acidic conditions.

**MATERIALS AND METHODS**

**Materials and chemicals**

All chemicals were of reagent grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Difco Laboratories (Franklin Lakes, NJ, USA). Transformed bacterial cells were induced with L (+)-arabinose (Sigma). Colloidal chitin was prepared from powdered chitin, and glycol chitin was obtained by acetylation of glycol chitosan (Sigma), according to
the method of Lee et al. (1). Soluble-acidic chitosan (pH 5.0) was made from powdered chitosan by the method of Choi et al. (16).

**Bacterial strains, plasmids, and media**

Chitinolytic bacteria were selected on LB agar plates containing 1% colloidal chitin. The plasmid pGEM-T Easy Vector (Promega Co., Madison, WI, USA) was used as a cloning vector, and pBADNH (17) was used as an expression of fusion protein, which contained a hexahistidine tag at the amino-terminus of SCChi-1. Ni-NTA matrices from Qiagen (Valencia, CA USA) and imidazole (Sigma) were employed to purify 6× His-tagged proteins. E. coli DH5α and MC1061 were used for the transformation and as the expression host, respectively. The transformants were selected from LB agar plates with ampicillin (50 μg/ml).

**Cloning of the chitinase SCChi-1 gene**

To clone chitinase genes from *B. atrophaeus* SC081 by PCR, the amino acid sequence of chitinase (AF069131) from *B. subtilis* was searched using the tBLASTn program to find the most similar DNA sequences. As a result, amplification primers were designed as follows, F: 5'-ATGAAAAAAGTGTTTTCA-3'; R: 5'-TTATTTGCAATCACCAAT-3'. Genomic DNA was extracted from *B. atrophaeus* SC081 with the G-spin™ for Bacteria kit (Intron Biotechnology, Seoul, South Korea) and was used as the PCR template. The resulting PCR products were cloned into pGEM-T Easy Vector, transformed into *E. coli* DH5α, and MC1061 were used for the transformation and as the expression host, respectively. The transformants were selected from LB agar plates with ampicillin.

**Expression and purification of H6SCChi-1**

To prepare an *E. coli* SCChi-1 expression vector, the SCChi-1 gene was ligated into the pBADNH expression vector between the *Sac I* and *Sph I* restriction sites to produce H6SCChi-1. Subsequently, the expression construct was transformed into *E. coli* DH5α for sequencing. Recombinant SCChi-1 was expressed and purified according to the method of Cho et al. (17). To prepare H6SCChi-1, exponentially growing cells were induced by adding 0.2% (w/v) arabinose, grown for 4 h at 28°C, and harvested by centrifugation at 10,000 × g for 5 min. The pellet was resuspended in protein-extraction lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole), then sonicated (total processing time, 10 min; pulse-on time, 3 s; pulse-off time, 12 s), and centrifuged at 15,000 × g for 30 min. The supernatant containing H6SCChi-1 was dialyzed in protein buffer (25 mM Tris-Cl pH 7.5, 1 mM EDTA). The amount of protein was determined using a Bio-Rad (Richmond, CA, USA) Protein Assay kit with bovine serum albumin as the standard.

**SDS-PAGE and chitinase zymograms**

SDS-PAGE was conducted on 10% SDS-polyacrylamide gels with the Multiphor II system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After SDS-PAGE, the gel was stained with 0.05% Coomassie Brilliant Blue R-250. Zymogram analysis was performed as follows. A protein sample was added to SDS-PAGE sample buffer and heated at 80°C for 5 min. The protein was separated with a 10% polyacrylamide gel containing 0.1% glycol chitin, and the gel was immersed overnight in refolding buffer (50 mM Tris-HCl [pH 7.5], 2.5% Triton X-100) at 37°C. The gel was washed with distilled water, and then stained with Coomassie Brilliant Blue R-250. After 2 h, the gel was destained with distilled water, including methanol and acetic acid, and the lytic zones were visualized.

**MALDI-TOF mass spectrometry**

Purified SCChi-1 was digested for 18 h at 37°C using trypsin (10 ng/μl, sequencing grade, Promega). The digested peptides were extracted from the gel matrix and analyzed with a MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). The tandem mass (MS/MS) data were acquired and analyzed using the MASCOT Spectra Mass Fingerprinting online software tool (available at http://www.matrixscience.com/cgi/search_form.pl?FORM-VER = 2&SEARCH = PMF), and further searched using the Firmicutes (Gram-positive bacteria) database.

**Substrate specificity of SCChi-1**

The modified agar diffusion method was used to visualize SCChi-1 chitinase activity (18, 19). First, the colloidal chitin, glycol chitin, glycol chitosan, and soluble chitosan plates were prepared. Ten mL of 0.1% (w/v) colloidal chitin, glycol chitin, or glycol chitosan in 25 mM Tris-Cl (pH 7.5) and 0.1% of soluble-acidic chitosan solution (pH 5.0) containing agarose (1% w/v) was poured into 5-cm diameter Petri dishes and allowed to solidify at room temperature for 2 h. Then, 20 μg of *E. coli* cell lysates containing or lacking H6SCChi-1 and the same amount of extracellular protein from *B. atrophaeus* SC081 was spotted on the plates. After incubating the plates for 1 and 2 h at 37°C, they were stained with 0.01% Calcofluor white M0R in 25 mM Tris-Cl pH 7.5. After 5 min, the brightener solution was removed, and the plates were washed with distilled water.
Clearing zones were visualized by placing the plates on a UV-transilluminator (1). The relative activities of *E. coli* cell lysates containing H$_6$SCChi-1 and extracellular protein from *B. atrophaeus* SC081 lyzing colloidal chitin (pH 7.5) and soluble-acidic chitosan (pH 5.0) were calculated by measuring the amount of reducing sugar using the modified dinitrosalicylic acid method.

**Effect of pH and temperature on chitinase activity of purified SCChi-1**

Chitinase activity was measured with colloidal chitin as a substrate and assayed in a reaction mixture containing 1.0% colloidal chitin in 25 mM Tris-Cl (pH 7.5) and the purified SCChi-1. The mixture was incubated at 37°C for 1 h and then boiled for 5 min to stop the reaction. After cooling, the mixture was centrifuged and the amount of reducing sugar in the supernatant was determined using the modified dinitrosalicylic acid method. Enzyme activity was obtained from a calibration curve prepared by following the same procedure with D-glucose as the standard. The effect of pH on chitinase activity was assayed from pH 2.0 to 10.0, and the reaction mixtures were incubated for 1 h at 37°C. Maximum activity was expressed as 100%, and others were compared to maximum activity. The effect of temperature on chitinase activity was studied at various temperatures and pH 7.5. The reaction mixture, in 25 mM Tris-Cl at pH 7.5, was treated for 1 h at different temperatures ranging from 20 to 60°C (20, 30, 37, 40, 50, and 60°C). The relative activities were calculated as a percentage of maximum activity.

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