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Enzymatic and molecular characterization of an acidic and thermostable chitinase 1 from *Streptomyces thermodiastaticus* HF3-3

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Chitinase 1 (Chi1) is an acidic and thermostable hydrolytic enzyme capable of the breakdown of chitin, a resilient biopolymer that is the primary building block of fungi cell walls and marine exoskeletons. In this study, Chi1 was purified from the bacterium *Streptomyces thermodiastaticus* HF3-3, and its properties were carefully characterized. The molecular mass of Chi1 was estimated to be approximately 46 kDa and, through sequencing, its *N*-terminal amino acid sequence was identified as ADSGKVKL. Although the optimal operating temperature and pH for Chi1 were determined to be 65°C and pH 5.5, respectively, the purified enzyme was stable over wide pH (1.5–9) and temperature ranges. Moreover, Chi1 retained 87% of its activity in the presence of 15% NaCl. While Chi1 activity was inhibited by Ag^{+} and Mn^{2+}, other chemicals tested had no significant effect on its enzymatic activity. The K_m and V_{max} values of Chi1 for the substrate colloidal chitin were 1.23 ± 0.7 mg/mL and 6.33 ± 1.0 U/mg, respectively. Thin-layer chromatography analysis of the enzymatic reaction end products mainly detected diacetylchitobiose. We also cloned the Chi1 gene and purified the recombinant protein; the properties of the recombinant enzyme were nearly identical to those of the native enzyme. Therefore, Chi1 purified from *S. thermodiastaticus* HF3-3 is unique, as it is highly stable under broad range of pH values, temperatures, and chemical exposures. Combined, these properties make this enzyme attractive for use in the industrial bioconversion of chitin.

Key Words: acidic; chitinase; *Streptomyces thermodiastaticus*; thermal stability

Introduction

Chitin, a polymer composed of β-(1,4)-linked *N*-acetyl-D-glucosamine, is the second most abundant carbohydrate polymer, and is a source of biomass. It is the primary component of exoskeletons and connective tissues in arthropods and fungal cell walls, and is also found, to a limited extent, in marine organisms. About 10% of the global landing of aquatic products comprises organisms rich in chitinous materials (10–55% based on dry weight), including the following: shrimp, crabs, squids, oysters, and cuttlefish. Every year, approximately 80,000 metric tons of chitins accumulate from marine waste (Makino et al., 2006; Patil et al., 2000). These waste materials have become a global challenge and remain an area of active interest, as they generate pollution, unnecessarily occupy space, and require excessive costs for proper management and disposal. Thus, the use of these waste materials to produce value-added products, such as oligosaccharides and antifungal chitinases, offers an attractive solution (Pradeep and Yun, 2014).

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Chitinases (EC.3.2.1.14) are enzymes that catalyze the hydrolysis of chitin and are used for the bioconversion of chitin. Chitinases can be sub-classified into two types based on their chitin hydrolyzing capabilities: exochitinase and endochitinase. Endochitinases cleave chitin chains at random locations generating low molecular weight oligomers, such as chitotetraose, chitotriose, and diacetylchitobiose. On the contrary, exochitinases release chitobiose from either the reducing or non-reducing end of the chitin chain (Brzezinska et al., 2013).

Chitinases are produced by a broad range of organisms, including viruses, bacteria, fungi, insects, plants, and mammals. Most organisms express a wide range of chitinases with distinct functions, including digestion, pathogen defense, cuticle turnover, and cellular differentiation (Brzezinska et al., 2013; Khoushab and Yamabhai, 2010). Chitinases can be further subdivided into three families based on their amino acid sequence similarities: chitinase families 18, 19, and 20. Family 18 chitinases are found largely in bacteria, fungi, viruses, mammals, insects, and some plants (class III and V plant chitinases). Family 19 chitinases were initially isolated from plants (class I, II, and V plant chitinases), and can also be derived from bacteria. Family 20 includes *N*-acetylglucosaminidase and *N*-acetylhexosaminidase (Brzezinska et al., 2013). Bacterial chitinase are widely used to inhibit fungal growth and have recently been implemented to treat pathogenic fungal diseases in plants. Another functional application of chitinases is in the bioconversion of chitin into pharmacologically-active products, such as *N*-acetylglucosamine and chito-oligosaccharides. These bioactive molecules are effective antimicrobials that can enhance the immune-response, activate the host defense system, assist with drug delivery, and act as antioxidants in a wide range of processes, including cellular homeostasis, wound healing, blood cholesterol control, and food preservation (Dahiya et al., 2006; Khoushab and Yamabhai, 2010). Additionally, acetylglucosamine and chito-oligosaccharides can be used for the preparation of protoplasts from filamentous fungi, and the production of proteins in single cells (Dahiya et al., 2006; Yano et al., 2004). With this in mind, many industrial processes take place at extreme pH levels and temperatures. Therefore, it is critical that the enzymes used for these processes can remain stable under these extreme conditions.

In this study, we describe the purification and characterization of chitinase 1 (Chi1) from the bacterium *Streptomyces thermodiastaticus* HF3-3, which produces thermostable glycolytic enzymes. The expression of the *Chi*1 gene, which encodes the enzyme, and its nucleotide sequence are also described. This enzyme is unique because it is stable over a broad range of pH values and in the presence of some denaturants, as well as being relatively stable at elevated temperatures. These properties suggest that this chitinase can be used for various industrial applications.

Materials and Methods

Substrates and chemicals. Chitin from crab shells and 3,5-dinitrosalycikic acid (DNS) were purchased from Nacalai tesque (Kyoto, Japan). Analytical or best commercially available grades of all other chemicals and reagents were used, unless otherwise specified.

Microorganism and culture. For the production of chitinase1 (Chi1), *S. thermodiastaticus* HF3-3 was cultivated for 120 h at 50°C while shaking at 100 rpm in a medium containing 1.0% chitin powder, 0.05% K_2HPO_4, 0.05% KH_2PO_4, 0.3% ammonium sulfate, 0.1% yeast extract, and 0.03% $MgSO_4 \cdot 7H_2O$ at pH 7.0. For the construction of the recombinant plasmid, Escherichia coli XL 10 GOLD was grown at 37°C in LB medium containing 100 μg/mL of ampicillin.

Purification of Chi1 from S. thermodiastaticus HF3-3. *S. thermodiastaticus* was cultured as described above. Crude enzyme was purified from culture by filter paper-mediated filtration. Eight hundred mL of crude enzyme was then further concentrated to 150 mL using PEG #20000. The concentrated crude enzyme was dialyzed against a buffer containing 10 mM Tris-HCl (pH 8.0) at 4°C. The dialysate was then applied to a DEAE-Cellufine column (1.5 × 15 cm; JNC; Japan) equilibrated with 10 mM Tris-HCl (pH 8.0). The column was subsequently washed with the same buffer containing 50 mM NaCl, and the enzyme was eluted in a stepwise fashion with a buffer containing 75 mM NaCl. The active fractions were then dialyzed against a 10 mM potassium phosphate buffer (K.P.B, pH 7.0) and concentrated by ultrafiltration using the Amicon ultra-15 centrifugal filter unit (10,000 MWCO; Millipore, USA). The concentrated sample was then loaded into a Hitrap Q-HP column (1 mL; GE healthcare, Sweden) equilibrated by 10 mM K.P.B. (pH 7.0). The enzymes were eluted with a linear gradient of 0–300 mM NaCl. The active fraction was eluted as a symmetrical peak at a concentration of about 120 mM NaCl. The active fractions were collected and dialyzed against 10 mM K.P.B. (pH 7.0). The purified enzyme was stored at 4°C.

Cloning and expression of Chi1 gene. The complete *Chi1* gene was sequenced by whole genome sequence analysis of *S. thermodiastaticus* HF3-3 (data not published). The Chi1 gene including its signaling peptide was identified based on *N*-terminal amino acid sequencing of purified Chi1 protein (ADSGKVKL). First, chromosomal DNA was purified from *S. thermodiastaticus* HF3-3 by phenol/chloroform extraction. The gene was then amplified using PCR. The oligonucleotide primers used to amplify the *Chi1* gene fragment were 5′-GACCCGCCATATGGCCGACAGCGGCAAGG-3′, which contains a unique *Nde*I restriction site (underlined) overlapping the 5′ initiation codon, and 5′-CTCGAATTCAACGCCAGACCGTTG-3′, which contains a unique *Eco*RI restriction site (underlined) overlapping the 3′ end of the termination codon. The PCR reaction mixture consisted of 40 ng of chromosomal DNA, 30 pmol of oligonucleotide primers, 200 μM of each deoxynucleoside triphosphates (dNTPs), and 1 unit of KOD-plus-Neo polymerase (TOYOBO, Japan). Thermocycling was done as follows: one cycle at 94°C for 2 min followed by 35 cycles at 98°C for 10 s and 68°C for 1 min. A DNA fragment of 1.2-kb was successfully amplified, digested with the *Nde*I and *Eco*RI, and ligated

with the pET21a (Novagen, Germany) expression vector, which was linearized with *Nde*I and *Eco*RI. This generated a pET21-Chi1 vector, which was then used to transform the *E. coli* XL-10 GOLD (TAKARA Bio).

Plasmid DNA was isolated from the host *E. coli* XL-10 GOLD strain and transformed into the *E. coli* expression strain Rosetta-Gami B DE3 (Millipore). The transformed bacteria were inoculated into 5 mL LB medium containing ampicillin (100 μg/mL) and chloramphenicol (10 μg/mL). After an overnight incubation at 37°C with shaking, the culture was transferred to a 500-mL Sakaguchi flask containing 100-mL of LB medium supplemented with 100 μg/mL of ampicillin and 10 μg/mL of chloramphenicol, which was incubated at 37°C on a reciprocal shaker (100 strokes/min). After 3 h incubation, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.4 mM, and the culture was incubated at 20°C for 12 h.

Purification of recombinant Chi1. Cells were harvested by centrifugation (10,000 × *g* for 15 min), suspended in 10 mL buffer A (50 mM sodium phosphate buffer, pH 7.1, 200 mM NaCl, 30 mM imidazole), and disrupted by sonication on ice. Cell debris was then removed by centrifugation (10,000 × *g* for 15 min). The supernatant (3 mL) was directly applied to a Co^{2+} column (Ni sepharose 6 Fast Flow column was regenerated by 0.1 M $CoSO_4$) equilibrated with buffer A. The enzyme was eluted in a stepwise fashion with buffer A containing 100 mM imidazole. Active fractions were analyzed by 12.5% SDS-PAGE and subsequently pooled. Active fractions were desalted by PD-10 columns (GE healthcare, UK) and further applied to a Q-HP column (1 mL) equilibrated with 10 mM K.P.B. Chi1 was eluted with a linear gradient 0–300 mM NaCl using the same buffer.

Assay for chitinase activity. Chitinase activity was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959), which detects the amount of reducing sugars released from colloidal chitin. Unless otherwise stated, the standard reaction mixture contained 100 μL of enzyme solution with 100 μL of 0.2 M citrate buffer (pH 5.5) and 200 μL of 1% colloidal chitin (a final colloidal chitin concentration of 0.5%). The mixture and the appropriate blanks were incubated at 30°C for 30 min. The reaction was terminated by the addition of 640 μL of 3,5-dinitrosalicylic acid reagent dissolved in a 1% NaOH solution and 80 μL of 10 mM glucose, followed by boiling for 15 min. Subsequently, 320 μL of 40% (w/v) potassium sodium tartrate solution was added. The colored solution was centrifuged at 12,000 rpm for 1 min and the absorbance of the supernatant was measured at 575 nm. One unit of chitinase activity was defined as the amount of enzyme needed to release 1 μmol of *N*-acetyl-D-glucosamine per min under standard assay conditions.

Protein analysis. The protein concentration was determined using Lowry's method; egg albumin acting as the standard. SDS-PAGE (12.5% acrylamide slab gel, 1-mm thick) was carried out using the Laemmli procedure. The molecular mass of the purified enzyme was determined using Pre-stained Protein Markers (Nacalai tesque, Japan). After SDS-PAGE, the gel was stained with coomassie brilliant blue. In addition, chitin zymography (activity staining) was performed using a 12% (w/v) Native-PAGE gel that contained 0.1% colloidal chitin. After electrophoresis, the gel was incubated in 10 mM citrate buffer (pH 5.5) for 1 h at 60°C. The gel was then stained with 0.01% Calcofluor white M2R in 10 mM citrate buffer (pH 5.5) for 1 h, and de-stained with water. The lytic zones were photographed under a UV-transilluminator. The *N*-terminal amino acid sequence was determined using PPSQ-51A (Shimadzu, Japan).

Effects of pH, temperature, and salinity on the activity and stability of the enzyme. Optimal pH for the Chi1 hydrolytic reaction was determined by performing the standard assay using buffers over a range of pH levels. To determine the pH stability, the enzyme was incubated at 30°C for 5 h with buffers with pH levels ranging from 1.0 to 11.0. Residual enzyme activity was determined under standard assay conditions. The buffers used were as follows: HCl-KCl (pH 1.0–2.0); citrate (pH 3.0–6.0); potassium phosphate buffer (pH 6.0–8.0); Tris-HCl (pH 8.0–9.0); carbonate-bicarbonate (pH 9.0–11.0), and acetate (pH 4.0–5.0). The optimal reaction temperature was determined by performing the standard assay for 3 h at temperatures ranging from 20°C to 80°C. The effect of NaCl on enzyme activity was determined by performing the standard assay method with buffers containing various concentrations of NaCl.

Effects of various chemicals on enzyme activity. The effect of metal ions on enzyme activity was also examined. Metal ions, Ag^{+}, K^{+}, Na^{+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Fe^{3+} as well as EDTA, a chelating agent, were added to the reaction mixture at a final concentration of 1 mM. Moreover, the effects of various organic solvents on enzyme activity were also measured. Acetic acid, acetone, butanol, chloroform, DMSO, ethanol, isoamyl alcohol, methanol, and toluene were added to the reaction mixture at final concentrations of 10% (v/v). Furthermore, the effects of various protein denaturants on the activity of the enzyme were investigated. Urea, β-mercaptoethanol, and dithiothreitol (DTT) were added to the reaction mixture at a final concentration of 10 mM, whereas surfactants, including sodium dodecyl sulfate (SDS), Tween 80, and Triton X 100, were added at a final concentration of 1% (v/v). Chitinase enzyme activity, in response to these manipulations, was expressed as a relative percentage of maximal activity.

Substrate specificity and enzyme kinetics. Purified chitinase was incubated with various substrates such as colloidal chitin, chitin powder, chitosan powder, cellulose powder, and carboxymethyl cellulose. These substrates were added to the standard assay mixture at a concentration of 1% (w/v), and the degree of substrate hydrolysis was analyzed by the DNS method, mentioned above. Enzyme kinetics was evaluated using colloidal chitin as substrate (at final concentrations ranging from 0.05 to 5 mg/mL). Enzymatic activity was determined by the standard assay method. The kinetic constants, K_m and V_{max} were calculated using KareidaGraph (Synergy Software).

Table 1. Purification summary.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
(a) Purification summary of Chi1					
Culture filtrate	277.9	93.8	0.3	0.0	100.0
PEG concentration	272.2	106.7	0.4	1.2	113.8
DEAE-Cellufine	29.4	19.7	0.7	2.0	21.0
Hitrap Q-HP	5.7	13.5	2.4	7.0	14.3
(b) Purification summary of recombinant Chi1					
Culture filtrate	10.1	2.7	0.3	1.0	100.0
Co^{2+}-NTA	0.9	2.0	2.2	8.2	73.6
Hitrap Q-HP	0.6	1.4	2.2	8.4	50.7

Table 2. Comparison of Chi1 with other chitinases from *Streptomyces* sp.

Strain name	Mol. wt.	Optimal		Stability		Reference
		pH	Temp.	pH	Temp.	
S. sp. CS495	41	12	60	5–12.5	60	Pradeep and Yun (2014)
S. cyaneus SP-27	27	7	60	5.5–8.5	60	Yano et al. (2008)
S. thermoviolaseus Chi40	40	8	70–80	8–10	70–80	Tsujibo et al. (1993a)
S. coelicolor Chi18aC	67	2	60			Kawase et al. (2006)
S. sp.	40	2, 6	50	2–7	50	Narayanan et al. (2015)
S. roseolus	40	6	60	6–8	60	Xiayun et al. (2012)
S. sp. NK52	44	10	70			Prakash et al. (2013)
S. violaceusniger	56.5	5	50	3–10	50–60	Nagpure and Gupta (2013)
S. sp. DA11	34	8	45–50	6–9	45	Han et al. (2009)
S. thermodiastaticus	46	5.5	65	1.5–9	65	Current study

Thin-layer chromatography. The end products of Chi1 hydrolytic reaction were analyzed by silica gel thin-layer chromatography (TLC) (Tanaka et al., 1999). Aliquots (1 μL) of the reaction mixtures were chromatographed on a silica gel plate (Merck, Darmstadt, Germany) with 1-butanol/methanol/28% ammonia solution (5:3:2 (v/v/v)), and the end products were detected by spraying the plate with aniline-diphenylamine reagent (1 mL of aniline, 1 g of diphenylamine, 50 mL of acetone, and 7.5 mL of 85% phosphoric acid) and baking it at 180°C for 3 minutes.

Results

Purification of chitinase

Chitinase enzyme was purified from cultured filtrate of *S. thermodiastaticus* HF3-3 as described in detail in the methods section and summarized in Table 1(a). The enzyme Chi1 was extracted at a 7.0-fold purification, with a specific activity of 2.4 U/mg, and a yield of 14.3%.

Gel electrophoresis

The purified enzyme was analyzed by SDS-PAGE and activity staining (Fig. 1). SDS-PAGE showed a single band for Chi1, with a molecular mass of about 46 kDa. The purified enzyme exhibited activity towards colloidal chitin on the gel in the zymogram (data not shown).

The N-terminal amino acid sequence of Chi1

Sequencing of Chi1 revealed that the *N*-terminal amino acid sequence of this enzyme is ADSGKVKL. We searched for chi1 homologues, which possessed *N*-terminal sequence similarities, by entering the protein sequence into protein-protein BLAST. The sequence was highly homologous with that of other family 18 chitinases, especially Chi40 from *S. thermoviolaseus* OPC-520 (Tsujibo et al., 1993a) (75% identity).

Characterization of Chi1

We determined that the optimal pH for Chi1 enzyme activity is 5.5, but the enzyme maintained an activity level of over 80% over a wide pH range (1.5–6.0). Indeed, Chi1 remained stable when incubated at 30°C for 5 h with pH levels between 1.5 and 9.0 (Fig. 2). The optimal temperature for enzymatic activity was determined to be approximately 65°C; however, Chi1 maintained high levels of activity at temperatures as high as 100°C (relative activity of over 50%). When the enzyme was incubated at 65°C for 3 h, no loss of enzymatic activity was observed (Fig. 3). However, 30 min incubation at 70°C resulted in a 55% reduction in Chi1 activity levels. The effects of various metal ions, salinity, denaturing reagents, and solvents on chitinase activity were investigated. As summarized in Table 3, Co^{2+} enhanced, while Ag^{+} and Mn^{2+} inhibited, Chi1 activity. Chitinase maintained over 90% and 87% relative activity at NaCl concentrations of 10% and 15%, respectively. Supplementing the reaction buffer with Tween 80 and Triton X-100, significantly augmented chitinase activity. On the contrary, the addition of SDS, EDTA, dithiothreitol, or organic solvents slightly inhibited Chi1 activity.

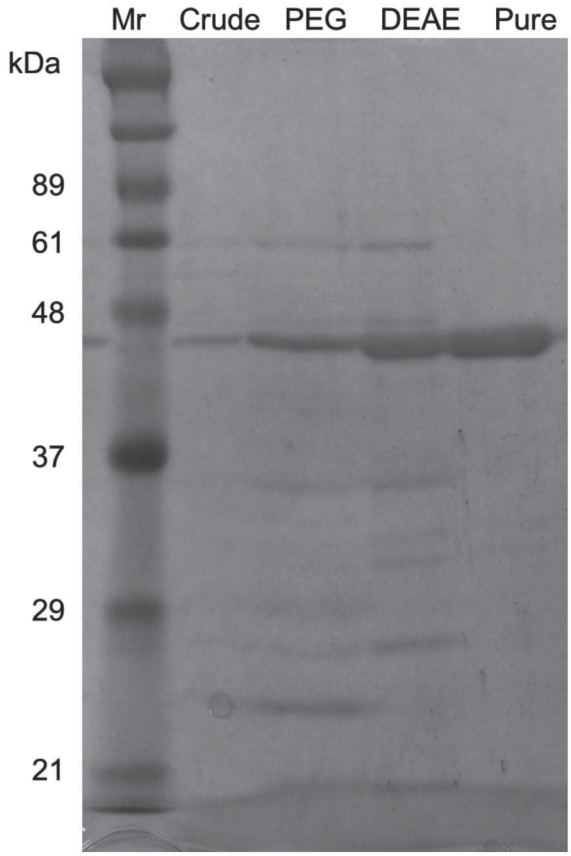

Fig. 1. SDS-PAGE and zymography of Chi1 from *S. thermodiastaticus* HF3-3.

Lanes Mr, marker proteins; Crude, crude enzymes; PEG, polyethylene glycol concentration; DEAE, DEAE-cellulofine; Pure, purified enzyme.

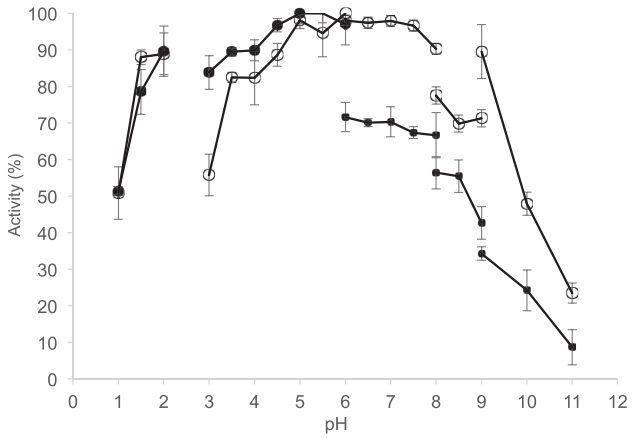

Fig. 2. The optimal pH for Chitinase activity (○) and pH stability of the enzyme (●).

HCl-KCl (pH 1.0–2.0), citrate (pH 3.0–6.0), phosphate (pH 6.0–8.0), Tris-HCl (pH 8.0–9.0), and carbonate buffer (pH 9.0–11.0) were used to determine the optimal pH and pH stability for enzyme activity at 30°C

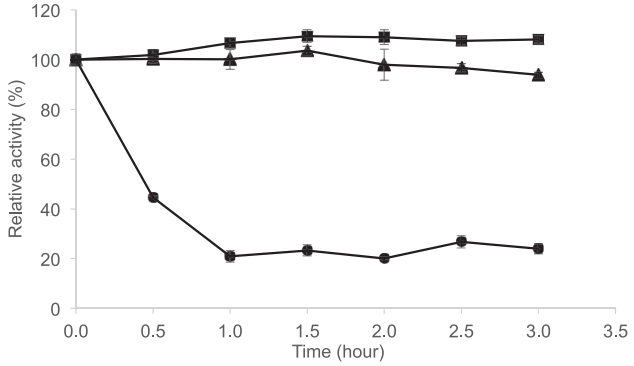

Fig. 3. Temperature stability of chitinase activity. 60°C (Filled box), 65°C (Filled triangle), and 70°C (Filled circle)

Substrate specificity and enzyme kinetics

We investigated the substrate specificity of purified chitinase1 by testing its affinity to various substrates (Table 4). Chi1 demonstrated the highest affinity toward colloidal chitin. When chitin powder, chitosan powder, cellulose powder, or carboxymethyl (CM) cellulose were used as substrates, Chi1 enzymatic activity was reduced in comparison to when colloidal chitin was added. We then investigated Chi1 enzyme kinetics by varying the concentration of colloidal chitin (0.05–5 mg/mL). The apparent K_m and V_{max} values for colloidal chitin were 1.23 ± 0.7 mg/mL and 6.33 ± 1.0 U/mg, respectively.

Chitin hydrolysis

The end products of Chi1-mediated hydrolysis of colloidal chitin were analyzed by TLC (Fig. 4). Chi1 hydrolysis of colloidal chitin produced *N*-acetyl-D-glucosamine (GlcNAc), diacetylchitobiose $(GlcNAc)_2$, and triacetylchitobiose $(GlcNAc)_3$. $(GlcNAc)_2$ was the major oligosaccharide produced, and very small amounts of GlcNAc and $(GlcNAc)_3$ were also produced. These results suggest that Chi1 is an endo-type chitinase as the major product of its hydrolysis was $(GlcNAc)_2$ (Tanaka et al., 1999).

Cloning and sequence analysis of the S. thermodiastaticus Chi1 gene

The Chi1 gene was identified by *N*-terminal amino acid sequencing of the purified enzyme and genomic DNA sequencing of *S. thermodiastaticus* (Fig. 5). The complete ORF of Chi1 (1248 bp) showed high sequence similarities with other known chitinase genes. The ORF encodes for a protein of 415 amino acids, with a calculated molecular mass of 43,815 Da, and a theoretical isoelectric point (pI) of 4.87. The G/C content of this ORF was found to be 69.5%, and the start and terminal codons were identified as ATG and TAA, respectively. We also noted a Shine-Dalgarno sequence (AGGAGG) situated 6 bp upstream of the putative transcription start site. Two promoter motifs were detected upstream of the Shine-Dalgarno sequence, one at -10 (TATATT) and another at -35 (TTGACC).

The *N*-terminal amino acid sequence of mature Chi1 purified from *S. thermodiastaticus* HF3-3 was found to begin at residue Ala44, as deduced by the amino acid sequence encoded by the *Chi1* gene (Fig. 5). This finding demonstrates that the protein contains a putative signaling peptide of 43 amino acids. Thus, mature Chi1 is a protein of 372 amino acids, with a calculated mass of 39,568 Da and a deduced pI of 4.56. A BLAST search demonstrated

Table 3. Effects of metal ions and other reagents on chitinase activity of Chi1.

Reagents	Concentration	Relative activity (%)
Ag^{+}	1 mM	41 ± 5.1
Ca^{2+}	1 mM	101 ± 3.4
Co^{2+}	1 mM	113 ± 2.1
Cu^{2+}	1 mM	106 ± 1.5
Fe^{2+}	1 mM	94 ± 5.5
Fe^{3+}	1 mM	97 ± 1.3
K^{+}	1 mM	90 ± 3.6
Mg^{2+}	1 mM	90 ± 1.6
Mn^{2+}	1 mM	66 ± 0.6
Na^{+}	1 mM	98 ± 1.3
Zn^{2+}	1 mM	104 ± 2.1
EDTA	1 mM	89 ± 2.2
Acetone	10% (v/v)	96 ± 1.1
Butanol	10% (v/v)	92 ± 2.4
DMSO	10% (v/v)	91 ± 2.6
Ethanol	10% (v/v)	96 ± 2.5
Isoamyl alcohol	10% (v/v)	112 ± 1.0
Methanol	10% (v/v)	87 ± 0.9
Toluene	10% (v/v)	98 ± 2.8
Urea	10 mM	90 ± 1.5
2-Mercaptoethanol	10 mM	108 ± 6.0
DTT	10 mM	85 ± 1.2
SDS	1% (v/v)	81 ± 0.8
Tween 80	1% (v/v)	125 ± 3.2
Triton X-100	1% (v/v)	132 ± 2.4
None	—	100 ± 2.1

Table 4. Substrate specificity.

Substrate	Relative activity (%)
colloidal chitin	100.0 ± 3.9
powder chitin	21.2 ± 4.5
chitosan	13.9 ± 5.1
cellulose	12.9 ± 2.9
CM-cellulose	2.8 ± 1.2

that the Chi1 sequence coincides with one unique domain classified to family 18 glycoside hydrolases. The mature peptide sequence of Chi1 showed the highest degree of sequence similarity with Chi40 from *S. thermoviolaceus* (93% identity) (Tsujibo et al., 1993b, 1995). The gene and protein sequence data reported in this manuscript will appear in the DDBJ under the accession number LC312712.

Properties of recombinant Chi1

Recombinant Chi1 (ReChi1) was purified from a cell-free extract with Co^{2+}-NTA affinity chromatography and Hitrap Q-HP column chromatography. Purification of ReChi1 is summarized in Table 1(b). ReChi1 was extracted at an 8.3-fold purification with a specific activity of 2.2 U/mg and a yield of 50.7%. The recombinant enzyme was stable under a range of pH values between 1.0 and 5.0, when incubated at 30°C for 5 h. The optimal operating pH and temperature for the recombinant protein were found to be 4.5 and 60°C, respectively. Chi1-mediated hydrolysis of colloidal chitin produced diacetylchitobiose $(GlcNAc)_2$ as the main end product. These properties were almost identical to those found with Chi1 purified from *S. thermodiastaticus*.

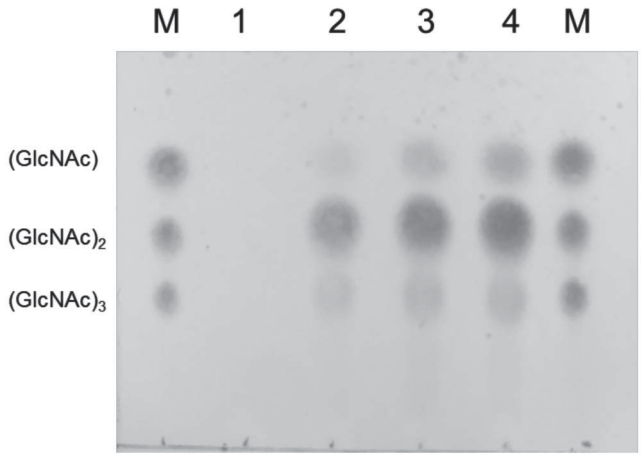

Fig. 4. TLC analysis of the colloidal chitin degradation products (30°C and pH 5.5 with Chi1).

Lanes M, mixture of *N*-acetyl-D-glucosamine (GlcNAc), diacetylchitobiose $(GlcNAc)_2$ and triacetylchitotriose $(GlcNAc)_3$; 1, 0 min; 2, 60 min; 3, 12 h; 4, 24 h.

Discussion

In this study, we identified and characterized an acid- and thermostable chitinase isolated from *S. thermodiastaticus* HF3-3. Chi1 was purified from *S. thermodiastaticus* using 830 mL of the culture, with a specific activity of 2.4 U/mg and recovery yield of 14.3%. Recombinant Chi1 was obtained from 100 mL culture, with a specific activity of 2.2 U/mg and yield of 50.7%. The specific activity of both enzymes was similar. SDS-PAGE analysis was used to determine the molecular weight of Chi1 to be approximately 46 kDa. It was similar to that of *Streptomyces* sp. AJ9463 chitinase (Suzuki et al., 2006). The effects of pH and temperature alterations on the activity of Chi1 and other chitinases from *Streptomyces* sp. are shown in Table 2. Chi1 remained stable over a wide pH range and demonstrated a high level of activity in an acidic environment. The stability of Chi1 at varying pH levels was comparable to the acidic chitinases reported for *Streptomyces coelicolor* (Kawase et al., 2006) and *Streptomyces* sp. (Narayanan et al., 2015). Furthermore, Chi1 exhibited high thermal stability and high activity at 65°C. The optimal temperature and thermal stability of Chi1 were similar to those of chitinases previously isolated from other species such as *S. cyaneus* SP-27, *Streptomyces*. sp. CS495, and *S. roseolus* (Pradeep and Yun, 2014; Xiayun et al., 2012; Yano et al., 2008) (Table 2). Chitinases from *S. thermoviolaceus* (Tsujibo et al., 1993a) and *Streptomyces* sp. NK52 (Prakash et al., 2013) exhibit a higher optimal temperature of 70°C. In general, many industrial decomposition processes using chitins operate at extreme pH levels (either acidic or alkaline) and high temperatures; thus, a chitinase that would be useful for these processes must maintain a high level of stability over wide pH and temperature ranges. Because Chi1 has a high tolerance to prolonged exposure to extremely acidic pHs, high optimal temperatures, and thermal stability, this enzyme stands out from previously reported chitinases isolated from *Streptomyces* sp. and has a high commercial

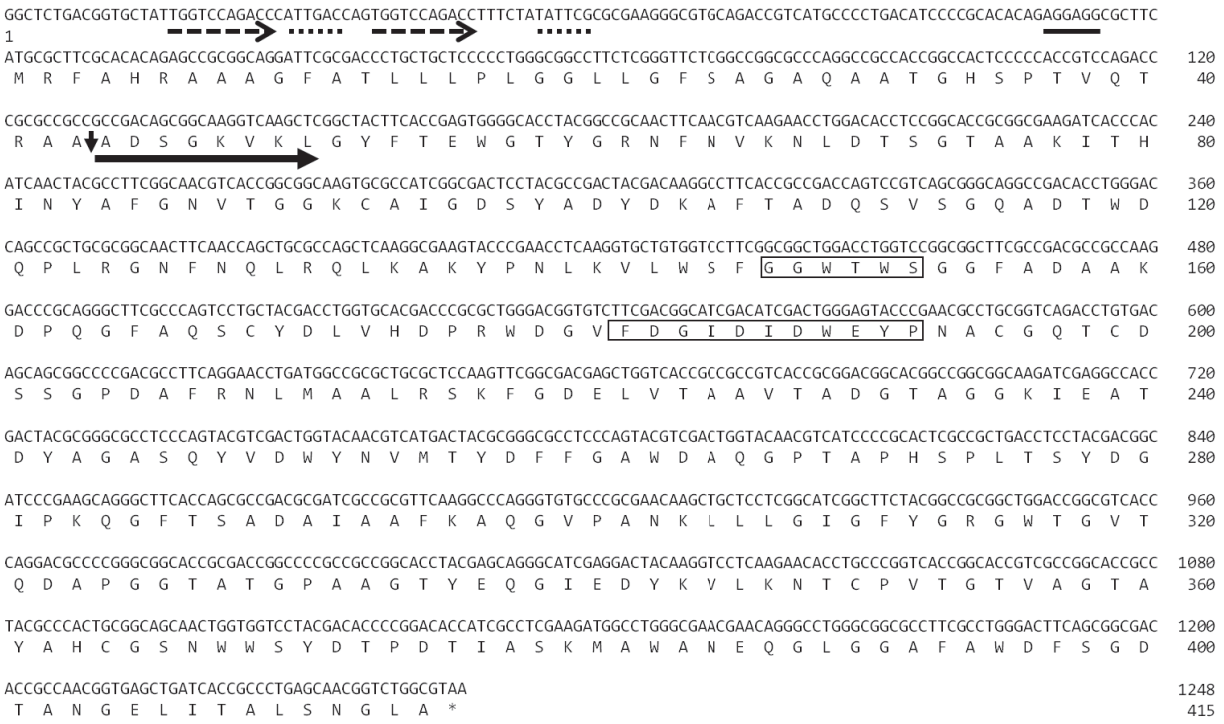

Fig. 5. Nucleotide sequence of coding *Chi1* gene and the deduced amino acid sequence of Chi1.

The putative ribosome binding site (AGGAGG) is underlined with solid line. The -10 and -35 regions of a possible promoter sequence are underlined with dotted line. The direct repeat sequences are shown by dashed arrows. The signal peptide cleavage site and the *N*-terminal amino acid sequence of ST Chi1 shown by solid arrow. The stop codon is indicated by asterisk. The specific motifs in catalytic domain of chitinase 1 are boxed.

potential for the bioconversion of chitin. Similar to the chitinases isolated from *Streptomyces* sp. and *Streptomyces violaceusniger* (Nagpure and Gupta, 2013; Narayanan et al., 2015), Chi1 from *S. thermodiastaticus* HF3-3 was strongly inhibited by Ag^+. Likewise, Chi1 from *S. thermodiastaticus* HF3-3 and chitinases from *Streptomyces* sp. CS195 and *Streptomyces* sp. (Narayanan et al., 2015; Pradeep and Yun, 2014) were all inhibited by Mn^{2+}. Chi1 exhibited 87% of its original activity in the presence of 15% w/v NaCl. Chitinase isolated from *Streptomyces* sp. DA11 showed the highest level of activity (45% psu salinity) (Han et al., 2009). This finding suggests that Chi1 isolated from *S. thermodiastaticus* HF3-3 has an advantage over other chitinases in the degradation of chitin materials from marine waste as it is able to maintain a high level of activity even at high salinity levels. The K_m value for Chi1 was lower than that of the other chitinases; 1.34 mg/mL from *Streptomyces* sp. CS195 and 6.74 mg/mL from *Streptomyces* sp. (Narayanan et al., 2015; Pradeep and Yun, 2014), respectively. This suggests that the substrate affinity of the enzyme obtained in this study was different from that of other extreme pH-tolerant chitinases. As with most endo-type family 18 chitinases, the major end product released during Chi1-mediated hydrolysis was $(GlcNAc)_2$ (Pradeep and Yun, 2014; Tanaka et al., 1999; Tsujibo et al., 1993a). These findings suggest that Chi1 is closely related to endo-type family 18 glycosyl hydrolases.

The catalytic domains of family 18 chitinases can be classified into three groups (A, B, and C) based on their amino acid sequence similarities (Suzuki et al., 1999; Watanabe et al., 1993). Chi1 purified from *S. thermodiastaticus* HF3-3 contains a catalytic domain most homologous with group A, where both GGWTWS and FDGxDxDWEYP motifs are found (Hobel et al., 2005). Although the amino acid sequence of Chi1 is highly homologous with that of Chi40 from *S. thermoviolaceus* (93% identity), their temperature and pH properties clearly differ. It has been reported that Chi40 is stable and most active at an alkaline pH (Tsujibo et al., 1993a, 1993b, 1995). Therefore, the differences between Chi1 and Chi40 properties warrants further investigation, with specific consideration of the heterogeneity of the structures of the enzymes. Further analysis of the molecular structure and function of this enzyme from *S. thermodiastaticus* should provide us with much more information, and these analyses are presently on going.

Conclusions

A 46-kDa extracellular chitinase (Chi1) produced by *Streptomyces thermodiastaticus* HF3-3 was isolated and purified. Optimal temperature and pH for Chi1 were 65°C and 5.5, respectively. Moreover, this enzyme proved to be stable in a wide pH range (1.5–9.0), temperatures (65°C for 3h), and salinity (87% activity in the presence of 15% w/v NaCl). The *N*-terminal amino acid sequence of Chi1 was found to be ADSGKVKL. Chi1 activity was inhibited by Ag^+ and Mn^{2+}, but DTT, SDS, EDTA, and other organic solvents had no significant effect on enzyme activity. Chi1-mediated hydrolysis of chitin produced mainly

diacetylchitobiose and trace amounts of *N*-acetyl-D-glucosamine and triacetylchitobiose, indicating that Chi1 is an endo-chitinase. Chi1 is stable over broad range of pH and temperatures, highlighting its potential applications in the biofuel, food, and textile industries, as well as in chitinous waste management.

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