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Cloning and characterization of a chitinase from *Thermobifida fusca* reveals Tfu_0580 as a thermostable and acidic endochitinase

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ABSTRACT

Being capable of hydrolyzing chitin, chitinases have various applications such as production of *N*-acetylchitooligosaccharides (COSs) and *N*-acetylglucosamine (GlcNAc), degrading chitin as a consolidated bioprocessing, and bio-control of fungal phytopathogens. Here, a putative chitinase in *Thermobifida fusca*, Tfu_0580, is characterized. Tfu_0580 was purified by homogeneity with a molecular weight of 44.9 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Tfu_0580 displayed a clear activity against colloidal chitin, which is comparable to a commercial *Streptomyces griseus* chitinase. Enzyme activities against *p*-nitrophenyl β-D-*N*,*N*′,*N*′′-triacetylchitotriose (*p*-NP-$(GlcNAc)_3$), *N*,*N*′-diacetyl-β-D-chitobioside (*p*-NP-$(GlcNAc)_2$) and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (*p*-NP-(GlcNAc)) showed that Tfu_0580 exhibited highest activity against *p*-NP-$(GlcNAc)_3$. Further optimization of the enzyme activity conditions showed: 1) an optimum catalytic activity at pH 6.0 and 30 °C; 2) activity over broad pH (4.8–7.5) and temperature (20–55 °C); 3) stimulation of activity by the metallic ions Ca^{2+} and Mn^{2+}.

1. Introduction

In the last decades, fundamental studies have examined an interesting research field concerned with exploitation of efficient bioprocess for lignocellulose biomass. As the world's second most abundant biopolymer, chitin shares similar features to cellulose: 1) high abundance in nature; 2) homopolymer of a simple sugar (e.g. *N*-acetylglucosamine); 3) specialized enzymes for degradation (e.g. chitinase) [1–3]. Annually, around 6–8 million tons of waste crab, shrimp, and lobster shells are produced globally where chitin is the main component of the seafood waste. The seafood waste poses potentially social and economic issues to the world. Recently, a concept of "chitin refinery" was proposed in 2015 aiming at converting the seafood waste into value-added chemicals. Enzymatic hydrolysis of chitin is one of the most efficient approaches for chitin degradation and has been developed into various applications, such as production of *N*-acetylglucosamine [4] or *N*-acetylchitooligosaccharides [5], bioconversion of chitinolytic biomass to value-added chemicals [6–8], and bio-control of fungal phytopathogens [9].

To date, a number of native chitinolytic organisms have been isolated such as *Serratia marcescens* [10], *Bacillus circulans* [11], *Acinetobacter parvus* [12], and *Aeromonas* sp [13]. Their chitinase activities, functionalities, secretion and regulation mechanisms have been widely characterized and investigated [8,14–16]. These organisms can be potentially developed for a chitin-based consolidated bioprocessing due to the complete chitinase expression, regulation and secretion system [8,17]. To date, many of these organisms and their chitinases can be found in patents for various applications [18–20].

An essential aspect of discovering chitinases with novel properties is to target enzymes from extremophiles (i.e. thermophile, acidophile, halophile). Exploring chitinases from these organisms can be advantageous: 1) chitinases from the special organism tends to have a similar particular property; 2) chitin degradation often requires multi-step chitinases in a synergistic manner, chitinases with active functions at wide pH or temperature ranges can be easily modulated and cooperated with other enzymes; 3) chitinases that can tolerate high temperature or low pH conditions have received particular research interest. For instance, Staufenberger et al. reported a chitinase, BAB65950 from *Sulfolobus tokodaii* within the crenarchaeotes [21]. The optimum

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activity of the enzyme was measured at pH 2.5 and 70 °C. Garcia-Fraga et al. charcterized a chitinase HsChiA1p from a marine halophile organism, *Haloarcula japonica* [22]. The enzyme prefers a pH of 7.3 and a temperature of 40 °C but a high concentration of metallic ions (1.5 M NaCl). Fu et al. isolated a novel chitinase, PbChi74 from a newly found thermophilic marine bacterium, *Paenibacillus barengoltzii*, that prefers a high temperature (65 °C) and low pH condition (4.5) [23]. The PbChi74 was determined as an exochitinase that can produce *N*-acetylglucosamine from colloidal chitin.

Within *Actinomycetes*, *Thermobifida fusca* is known for a highly expressed cellulolytic system as well as its high growth temperature (46 °C) and pH stability (pH below 5.5). In addition, many useful and functional proteins (i.e. cutinases and cellulases) from *T. fusca* have been reported with a preference of a high temperature and acidic environment [24,25]. Thus, chitinases from *T. fusca* may be a potential interesting target to obtain functional proteins with novel properties. After search in the *T. fusca* genomic information, two open reading frames (*tfu_0580*, GI: AAZ54618 and *tfu_0868*, GI: AAZ54906) are found with an annotation of chitinases. Recently, Tfu_0868 has been cloned and overexpressed in *Escherichia coli* [26]. The enzyme was found to be relatively thermostable at 57.5 °C and the optimum temperature being 40–45 °C. In addition, Tfu_0868 was observed with minimal chitinase activity against chitin but with some chitin-binding activity.

In this study, the chitinase gene *tfu_0580* was cloned, expressed, and purified in *E. coli* with an 8-his tag on its N-terminal end. IPTG concentration and induction time were investigated to obtain a maximum protein production. Chitinase activities against various substrates (colloidal chitin, *p*-NP-$(GlcNAc)_3$, *p*-NP-$(GlcNAc)_2$ and *p*-NP-(GlcNAc)) were examined and Tfu_0580 exhibits a chitinase activity against colloidal chitin. Effects of reaction buffer, temperature, pH and metallic ions on Tfu_0580 activities were determined. Overall, this study presented a general method of characterizing chitinase functionality from *T. fusca* and Tfu_0580 is the first reported functional chitinase in *T. fusca.*

2. Materials and methods

2.1. Strains and cultivation conditions

Thermobifida fusca ATCC BAA-629 was grown in Hagerdahl medium containing 10 g/L glucose [27]. 50 mL pre-cultures of *T. fusca* YX were grown at 55 °C and 250 rpm for 24 h in a 500 mL Erlenmeyer flask. *Escherichia coli* NEB10β was purchased from New England Biolabs (Ipswich, MA, USA) and was used for both DNA cloning and protein expression. *E. coli* strains were cultured at 37 °C and 250 rpm in LB or SOB medium supplemented with 50 μg/mL streptomycin or 50 μg/mL kanamycin.

2.2. Colloidal chitin preparation

Colloidal chitin was prepared using the modified method of Roberts and Selitrennikoff as follows [28]. Briefly, 5 g of chitin (Sigma-Aldrich, St. Louis, MO, USA) were added slowly to 90 mL of 12 M HCl. The mixture was vigorously stirred for 2 h. 500 mL of 0.82 g/mL ethanol was added to this suspension and then centrifuged at 6000 rpm and 4 °C for 20 min. The pellets were washed with distilled water until the supernatant reached a neutral pH. The prepared colloidal chitin was stored at 4 °C until use.

2.3. PCR amplification and cloning of T. fusca tfu_0580 gene

Standard DNA manipulations were performed according to the method described in Molecular Cloning [29]. The genomic DNA of *T. fusca* YX was isolated using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the user's instruction manual and stored at -20 °C. The *tfu_0580* gene (GenBank AAZ54618) was PCR-amplified by primers tfu_0580-f (5′-GAAATCCCGCCTGTCCGTTA-3′) and tfu_0580-r (5′-AGGAACTGTCTCGTTTCGGC-3′) using *T. fusca* genome as a template. The cloning plasmid backbone was PCR-amplified by primers pUN-f (5′-GGCACCCCGACAACTGGCCGAAAC-GAGACAGTTCCTCTCTAACGGACTTGAGTGAGG-3′) and pUN-r (5′-GGTAACGGACAGGCGGGATTTCCGAGTAGTTCAGTAGCGGA-3′) using pJ251-GERC as a template (Addgene, Cambridge, MA, USA). The expression plasmid backbone was PCR-amplified by primers pJ401-f (5′-TGGTCCGCGCCATGCACGGGTAAGGTCTCACCCCAAGG-3′) and pJ401-r (5′-AAGTAGCCGATGATACGCACATGATGGTGGT-GATGGTGATGATGCATATGTTTTACCTCCTAAGGTCTC-3′) using pJ401 as a template (Addgene, Cambridge, MA, USA). An 8×histidine peptide was added at N-terminus of Tfu_0580. The primers were designed using an online primer design software based on the Q5 High-Fidelity DNA Polymerase (https://tmcalculator.neb.com/).

The amplified PCR products were recovered and purified from 10 g/L agarose gels using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). The PCR-amplified DNA fragments were assembled into cloning or expression vectors using the isothermal assembly method [30] according to the instruction manual (New England Biolabs, Ipswich, MA, USA). Around 2 μL assembled plasmid was added into 50 μL competent cells and was transformed at 12.5 kV/cm^2 using a Bio-Rad Gene Pulser (Hercules, CA, USA). The recombinant *E. coli* strains were verified by both colony PCR and Sanger sequencing by primers tfu_0580-cds-f (5′-CTTAGGAGGTAAAACATATGCATCATCACCATCACCACCAT-CATGTGCGTATCATCGGCTAC-3′) and tfu_0580-cds-r (5′-GCCCTTGGGGTGAGACCTTACCCGTGCATGGCG-3′). The Tfu_0580 expression *E. coli* strain was designated as *E. coli* pJ401-tfu_0580.

2.4. Tfu_0580 expression and purification

The *E. coli* pJ401-tfu_0580 pre-cultures were inoculated from a freezer stock and were grown in a test tube containing 3 mL LB medium and 50 μg/mL kanamycin. 2.5 mL pre-cultures of *E. coli* pJ401-tfu_0580 were added to 50 mL SOB medium in a 250 mL Erlenmeyer flask and incubated at 37 °C and 250 rpm. After the Absorbance ($A_{600\ nm}$) of the culture broth reached around 0.4, isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA) was added to final concentration of 0–10 mM, and the culture was further grown at 30 °C and 250 rpm for 22 h.

The *E. coli* pJ401-tfu_0580 cell pellets were harvested by centrifuging at 8000 rpm and 4 °C for 10 min. Subsequently, the cell pellets were re-suspended in 4 mL of sonication lysis buffer (50 mM sodium phosphate buffer pH 7.7, 300 mM sodium chloride, 10 mM imidazole and 0.03% Triton X-100) and the cell suspensions were pulsar sonicated at 30% of power for 10 min in an ice bath. Tfu_0580 was extracted from the crude enzymes using a His-Spin Protein Miniprep™ kit (Zymo Research, Irvine, CA, USA) according to the instruction manual. The eluted fractions showing high chitinase activity were collected and checked for homogeneity by SDS-PAGE (Bio-Rad, Hercules, CA, USA). The SDS-PAGE gel was stained and de-stained using a SYPRO™ Ruby Protein Gel Stain kit (Molecular Probe, Eugene, OR, USA) according to the basic protocol of the user's manual. The protein concentration was determined using the Bradford method and bovine serum albumin as a standard [31].

2.5. Computational analysis of protein sequence

The chitinase sequences were analyzed using the Blast 2.0 program from the NCBI database (https://blast.ncbi.nlm.nih.gov/