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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/

Blast.cgi). Signal peptide and signal peptidase cleavage sites were predicted using SignalP version 3.0 [32]. The theoretical isoelectric point (pI) and molecular weight (MW) were predicted using calculated pI/MW on the ExPASy Proteomics Server [33]. The presence and delimitation of protein domains was accomplished using the conserved domain database (CDD) [34]. Multiple amino acid sequence alignments were generated using ClustalW [35]. The identity between a pair of aligned sequences was calculated as the number of identical residues divided by the number of aligned positions, excluding the sites with gaps, and expressed as a percentage. Structural alignments based on comparisons of 3D structures deposited in the protein data bank (PDB) were obtained from Dali database [36]. The functional domains were named following the nomenclature adopted by the carbohydrate active enzymes (CAZy) database [37].

2.6. Enzyme assay using colloidal chitin as a substrate

The assay mixture consisting of 15 μL 25 g/L (w/v) colloidal chitin and 15 μL enzyme solution was incubated at 40 °C for 20 h. Then, the assay mixture was centrifuged at 12,000 rpm for 10 min and the supernatant was used to quantify released reducing sugars by the Somogyi-Nelson method [38]. *N*-Acetylglucosamine was used as a standard to create a linear standard curve. One unit (U) of chitinase activity was defined as the amount of enzyme releasing 1 μmol of *N*-acetylglucosamine equivalent reducing sugars per minute under the assay condition. Specific enzyme activity was defined as the enzyme activity (mU/mL) over the amount of protein (mg/mL). Assays were carried out in duplicate, and the results were expressed as mean ± SD.

2.7. Enzyme assay using p-*NP-(GlcNAc)$_n$* as a substrate

Chitinase activity against *p*-NP-$(GlcNAc)_n$ was assayed using a chitinase assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to the instruction manual. The assay mixture containing 10 μL enzyme solution and 90 μL substrate solution was incubated at 37 °C for 30 min and was stopped by adding 200 μL 0.4 M Na_2CO_3 solution. The substrate solution contained 0.2 mg/mL *p*-nitrophenyl β-D-*N*,*N*′,*N*″-triacetylchitotriose, or 1 mg/mL *p*-nitrophenyl *N*,*N*′-diacetyl-β-D-chitobioside, or 0.5 mg/mL *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide. The release of *p*-nitrophenol during each reaction was measured immediately at the adsorption of 405 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1.0 μmol of *p*-nitrophenol from the substrate at pH 7.0 and 37 °C per minute per each milligram of protein. The *E. coli* NEB10β crude lysate was used as a negative control. *Trichoderma viride* chitinase (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control. Assays were carried out in duplicate, and the results were expressed as mean ± SD.

2.8. Effect of buffer, temperature, pH, and metallic ion on chitinase activity

Optimum reaction buffer for the chitinase Tfu_0580 was determined using 20 mM Tris−HCl, 20 mM phosphate-buffer saline (PBS) or 20 mM NaAc-HAc. Optimum temperature for the chitinase Tfu_0580 was tested between 20 and 80 °C. Optimum pH was tested from pH 4.8 to pH 9.0. Enzyme activity was also tested after adding some metallic ions, such as Na^+ (NaCl), K^+ (KCl), Ca^{2+} ($CaCl_2$), Mg^{2+} ($MgCl_2$), Zn^{2+} ($ZnCl_2$), or Mn^{2+} ($MnCl_2$), at a final concentration of 5 mM. Assays were carried out in duplicate, and the results were expressed as mean ± SD.

2.9. Statistical analysis

A two-tailed student *t*-test was used for the statistical analysis of significance of two independent datasets in this study according to an online software (http://www.socscistatistics.com/tests/studentttest/Default.aspx). A significant difference between two independent experimental data is defined as $P < 0.05$.

3. Results and discussion

3.1. Cloning and sequence analysis of *Tfu_0580 from* T. fusca

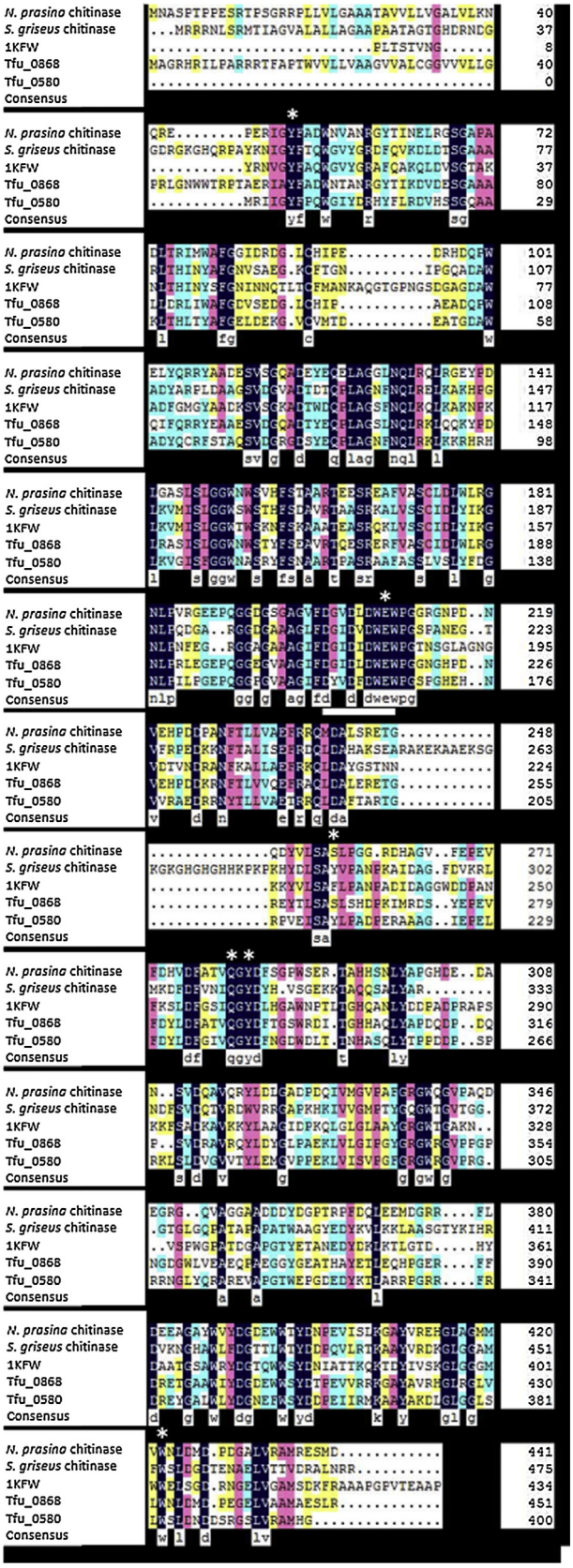
The amino acid sequence similarities of Tfu_0580 compared to other chitinases were aligned in Fig. 1. The *tfu_0580* has an open reading frame (ORF) of 1203 bp encoding 400 amino acids with a theoretical molecular mass of 44.9 kDa and pI of 5.78. The protein is predicted to have a modular structure and is composed of a glyco_18 domain (residues from 3 to 388), which is an eight-stranded alpha/beta barrel with a pronounced active-site cleft at the C-terminal end of the beta-barrel (DXXDXDXE). Multiple amino acid sequence alignments revealed that Tfu_0580 showed the highest identity of 46% with a chitinase from *Nocardiopsis prasina* [39], followed by *Streptomyces griseus* (WP_030711259, 43% identity) [40], and by *Arthrobacter* Tad20 (1KFW_A, 41% identity) [41]. In terms of the conserved motif and active sites, Tfu_0868 amino acid sequence is similar to Tfu_0580 but with an obvious amino acid discrepancy of active sites (Ser263 of Tfu_0868 vs Phe213 of Tfu_0580).

The Tfu_0580′s glycosyl_GH18 domain shows a high or moderate identity with different sequences of GH18 chitinase deposited in the PDB database. The conserved motif enables binding to the substrate through hydrophobic interactions between certain aromatic residues and the sugar molecules, allowing the catalytic domain to hydrolyze the substrate in an effective way. The consensus active sites of glycosyl_GH18 domain found in Tfu_0580 are Tyr^6, Phe^{38}, Asp^{162}, Asp^{164}, Glu^{166}, Glu^{239}, Tyr^{241}, Asp^{242}, Phe^{295}, Trp^{383}, which help stabilize the C-terminal substrate-binding cleft [42]. The crystal structure of ChiA (PDB 1NH6) from *Serratia marcescens* QMB1466 shows that ten amino acids form the substrate-binding cleft. In particular, six aromatic residues of those ten favor the catalytic activity and stabilize the structure: Tyr^{418} interacts with the *N*-acetyl group of the reducing-end GlcNAc; Phe^{396} and Trp^{275} are on opposite sides of the cleft and stack against the hydrophobic faces of GlcNAc sites +2 and +1; Trp^{539}, Trp^{167} and Tyr^{170} are at the bottom of the cleft and are positioned to interact with the hydrophobic faces of the GlcNAc at sites -1, -3 and -5 [42].

3.2. Protein expression and purification

Tfu_0580 from the crude enzyme was purified based on the eight-histamines peptide at N-terminus of Tfu_0580 by loading the crude enzyme into a nickel-charged his-affinity gel column, washing contaminant proteins using washing buffer and eluting Tfu_0580 using elution buffer. During the protein purification, it was observed that elution using 250 mM imidazole can achieve higher Tfu_0580 yield compared to elution using 150 mM or less imidazole (data not shown). An apparently homogeneous band of slightly more than 44.9 kDa was seen on an SDS-PAGE due to addition of an 8-histidine tag at the N-terminal end, indicating successful cloning, expression and purification of Tfu_0580 at homogeneity (Fig. 2A).

In order to maximize Tfu_0580 production from the *E. coli* pJ401-tfu_0580, the effects of IPTG concentrations on inducing Tfu_0580 production were investigated by monitoring the time



course of Tfu_0580 production post-induction using the Bradford protein assay and SDS-PAGE gel imaging. An optimum induction condition for *E. coli* pJ401-tfu_0580 overexpression of Tfu_0580 was feeding 2 mM IPTG at around Absorbance $A_{600\ nm}$ 0.35 and continuing post-induction for 36 h (Fig. 2B and C). Compared to the cell growth of the *E. coli* NEB10β strain, *E. coli* pJ401-tfu_0580 did not show apparent differences at growth rate and biomass yield, which have been reported in other gene cloning and expression cases [43].

3.3. Enzyme activities

In order to evaluate chitinase activities against chitin, colloidal chitin was used as a substrate and the activity was assayed by measuring the release of reducing sugar equivalents from colloidal chitin after the enzyme reaction. Table 1 showed the measured activity of both crude and purified Tfu_0580, as well as a commercial chitinase from *Streptomyces griseus*. Both crude and purified Tfu_0580 were found to have obvious chitinase activities against colloidal chitin. The purified Tfu_0580 activity (2.75 ± 0.32 mU/mg protein) against colloidal chitin is comparable to that of the *Streptomyces griseus* chitinase (4.41 ± 0.72 mU/mg protein). In contrast to the finding of no obvious chitinase activity of Tfu_0868 against colloidal chitin reported in a recent study [26], our results showed the first evidence of chitinase activity against colloidal chitin in *T. fusca*. Compared to the reported predicted functional active sites (Gln289 and Ser263) of Tfu_0868 [26], the equivalent active sites of Tfu_0580 are Gln and Tyr. It was found that Gln289 and Ser263 of Tfu_0868 lead to weak chitinase activity, however, the *Arthrobacter* sp. Tad20 chitinase (PDB: 1KFW) whose equivalent active sites are Gln and Phe, exhibits highly active chitinase activity. Since Tyr is similar to Phe in terms of structure, it may help the active site to process colloidal chitin compared to Tfu_0868.

In order to understand the acting manner of Tfu_0580 on chitin, *p*-Nitrophenol β-D-*N*,*N*′,*N*″-triacetylchitotriose, *p*-Nitrophenol *N*,*N*′-diacetyl-β-D-chitobioside, and *p*-Nitrophenol *N*-acetyl-β-D-glucosamine were tested for endochitinase, chitobiosidase, and β-*N*-acetylglucosaminidase activities by quantifying the amount of releasing *p*-Nitrophenol, respectively. The chitinase from *Trichoderma viride* was tested as a positive control, mainly because the *T. viride* chitinase is a known exochitinase. Table 2 showed that Tfu_0580 exhibited both endochitinase (782 ± 10 mU/mg) and chitobiosidase (465 ± 30 mU/mg) activities but no β-*N*-acetylglucosaminidase activities. The commercial *Trichoderma viride* chitinase showed endochitinase (98 ± 28 mU/mg), chitobiosidase (536 ± 35 mU/mg), and β-*N*-acetylglucosaminidase (411 ± 23 mU/mg) activities. Therefore, Tfu_0580 tends to act in an endochitinase manner.

To the fact that Tfu_0580 is able to hydrolyse *p*-NP-$(GlcNAc)_2$ and *p*-NP-$(GlcNAc)_3$ but unable to hydrolyze *p*-NP-(GlcNAc), and in addition to the Tfu_0580 catalytic domain structure, the enzyme tends to act in an endochitinase manner and the presence of an alpha + beta insertion in the GH18 catalytic domain may favor the endo-type activity by forming a deep substrate-binding cleft on the top of the TIM barrel, with a conserved motif of DXXDXDXE [26].

Fig. 1. Sequence alignment of Tfu_0580 from *T. fusca* with other bacterial chitinase. Numbers on the RIGHT are the positions of the last amino acids in each line. Protein sequences from *T. fusca* includes Tfu_0580 (Accession Number: AAZ54618) and Tfu_0868 (Accession Number: AAZ54906). The other listed sequences include the chitinases from *Nocardiopsis prasina* (Accession Number: WP_026129548), *Streptomyces griseus* (Accession Number: WP_030711259), *Arthrobacter* Tad20 (PDB number: 1KFW). The black area represents 100% identity, green and pink area represents > 50% homology; yellow area represents > 30% identity. The conserved TIM barrel motif (DXXDXDXE) is marked in white line and the active binding and catalysis sites were marked as "*".

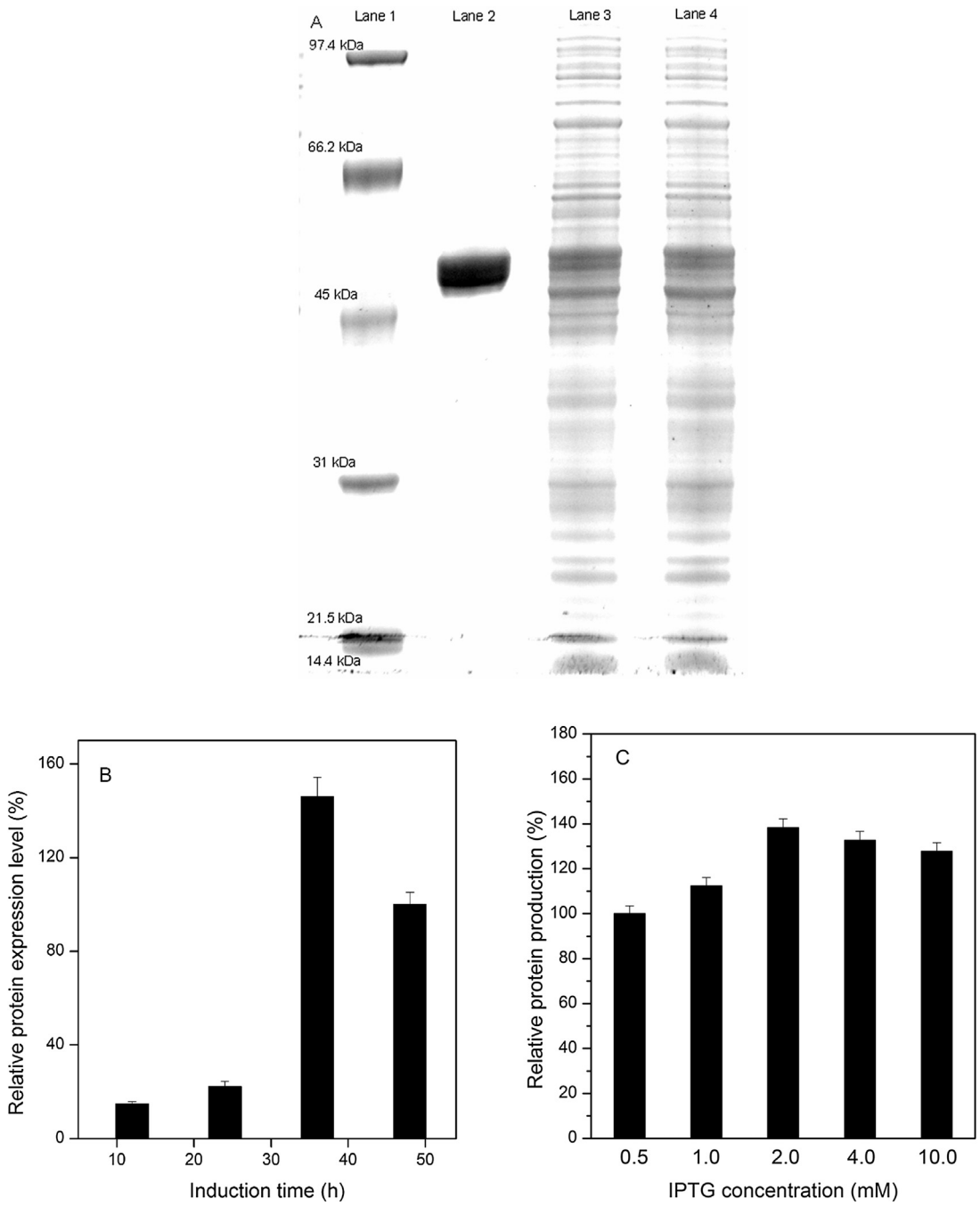


Fig. 2. (A) SDS-PAGE analysis of proteins during the purification process of Tfu_0580 from *T. fusca* expressed in *E. coli*. Lane 1, low molecular weight protein standards; lane 2, after purification, lane 3, crude enzyme (pJ401-tfu_0580 supernatant, 24 h after induction), lane 4, crude enzyme (*E. coli* NEB10β without plasmid, supernatant); (B and C) Effects of induction time and IPTG concentrations on the Tfu_0580 production from the recombinant *E. coli* at 30 °C. (B) Tfu_0580 production with 12 h intervals after 0.5 mM IPTG induction; (C) relative protein production at various IPTG concentrations.

3.4. Effects of reaction buffer, pH and metallic ion

In order to optimize Tfu_0580 activity against colloidal chitin, reaction conditions including buffer system, pH, temperature and metallic ions were investigated. The specific reaction conditions can be found in the Materials and Methods. It was found that reaction buffer of Tris-HCl and PBS performed similar ($P > 0.1$) in terms of chitinase activity, while a buffer system of NaAc-HAc showed 90% ($P < 0.01$) of that from Tris-HCl, shown in Fig. 3A.

The effect of metallic ion on Tfu_0580 activity was measured in 20 mM Tris-HCl buffer at pH 6.0 with 5 mM of Na^+ (NaCl), K^+ (KCl), Ca^{2+} ($CaCl_2$), Mg^{2+} ($MgCl_2$), Zn^{2+} ($ZnCl_2$), and Mn^{2+} ($MnCl_2$). It is noted that the purified Tfu_0580 solution was dialyzed using a sealed membrane filter and incubated in nanopure water for overnight before conducting the metallic ion characterization. It was observed that two divalent Ca^{2+} and Mn^{2+} increased activity to 125% ($P < 0.01$) and 119% ($P < 0.05$), respectively, while there was almost no activity difference among K^+, Na^+, Mg^{2+}, and Zn^{2+} ($P > 0.1$), compared to the activity without addition of metallic ions, shown in Fig. 3B.

The effect of temperature on Tfu_0580 activity was determined from a range of 20 to 80 °C. Tfu_0580 was generally active

Table 1
Chitinase activity of Tfu_0580 against colloidal chitin as a substrate.

Enzyme	Total activity [a] (mU/mL)	Total protein [b] (mg/mL)	Specific activity [c] (mU/mg)
Crude Tfu_0580	6.71 ± 0.12	5.6 ± 0.3	1.20 ± 0.34
Purified Tfu_0580	1.08 ± 0.98	0.39 ± 0.03	2.75 ± 0.32
Streptomyces griseus chitinase	2.21 ± 0.93	0.50 ± 0.03	4.41 ± 0.72

[a] Total activity is defined as mU crude or purified chitinase activity according to the chitinase activity test described in the Material and Methods section.
[b] Total protein is defined as mg/mL crude or purified chitinase according to the Bradford method described in the Material and Methods section.
[c] Specific activity is defined as mU crude or purified chitinase activity per mg protein.

Table 2
Substrate specificities of Tfu_0580.

	Tfu_0580 mU/mg	*Trichoderma viride* chitinase mU/mg
p-NP *N*-acetyl-β-D-glucosamine	–	411 ± 23
p-NP *N*,*N*′-diacetyl-β-D-chitobioside	465 ± 130	536 ± 35
p-NP β-D-*N*,*N*′,*N*′′-triacetylchitotriose	782 ± 10	98 ± 28
Colloidal chitin	2.75 ± 0.32	2.8 ± 0.22

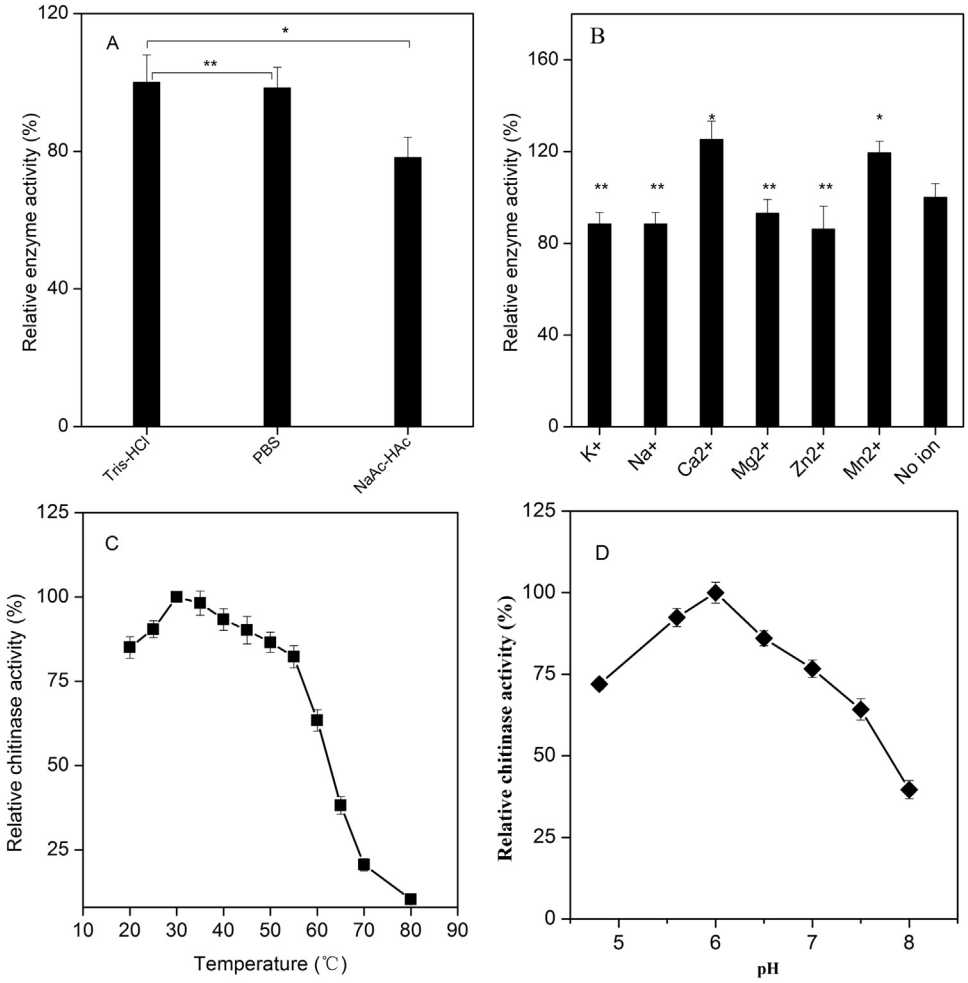


Fig. 3. Effects of reaction buffer, metallic ion, temperature, and pH on chitinase activity of Tfu_0580. (A) relative Tfu_0580 activity at different assay buffer (20 mM Tris-HCl, 20 mM PBS, and 20 mM HAc-NaAc) at pH 6.0, 30 °C, normalized by 20 mM Tris-HCl activity; (B) relative Tfu_0580 activity with addition of metallic ions at 20 mM Tris-HCl pH 6.0, 30 °C, normalized by no metallic ion activity; (C) relative Tfu_0580 activity at different temperature at 20 mM Tris-HCl pH 6.0, normalized by 30 °C activity; (D) relative Tfu_0580 activity at different pH at 20 mM Tris-HCl 30 °C, normalized by pH 6.0 activity. The statistical analysis was marked "*" as $P < 0.05$ (significance) and "**" as $P > 0.1$ (insignificance).

(remains more than 80% activity) at temperature between 20 and 55 °C, while the chitinase activity dropped to less than 40% when temperature was above 65 °C, shown in Fig. 3C. The effect of pH for chitinase activity of Tfu_0580 was measured at a range of pH from 4.8 to 8.0. When pH was at 8.0, Tfu_0580 activity dropped to 40% of its highest activity, shown in Fig. 3D. Tfu_0580 tends to be active under an acidic condition, which remain more than 60% highest activity between pH 4.8 and 7.5, with an optimum pH of 6.0.

4. Conclusions

In this study, Tfu_0580 was expressed in *E. coli* and purified by affinity purification. The chitinase functions were studied using various chitinolytic substrates. Tfu_0580 acts as an endochitinase that is capable of degrading colloidal chitin, while Tfu_0868 shows no clear activity with colloidal chitin. Further investigation of enzymatic conditions showed that Tfu_0580 is stable at high temperature (80% active at 55 °C) and prefers to an acidic condition (pH 6.0), and Ca^{2+} and Mn^{2+} can help boost the chitinase activity. Overall, it is demonstrated that Tfu_0580 has a positive activity on chitin and a broad spectrum of information was given regarding substrate, pH, metallic ion, reaction buffer, temperature, and this information should be helpful and valuable for further applications and mechanistic studies.

Conflict of interest

Dr. Qiang Yan and Dr. Stephen S Fong declare that they have no conflict of interest.

Author agreement

Dr. Qiang Yan declares that he has no conflict of interest. Dr. Stephen S Fong declares that he has no conflict of interest. All authors declare that this paper is not submitting elsewhere. All authors agree to submit this manuscript to the Journal "Biotechnology Reports".

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