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Applicability of endochitinase of *Flavobacterium johnsoniae* with transglycosylation activity in generating long-chain chitooligosaccharides

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ABSTRACT

Chitin and its derivatives are used for a variety of applications. *Flavobacterium johnsoniae* UW101 is an aerobic Gram-negative bacterium. Genome analysis of *F. johnsoniae UW101* revealed the presence of 10 glycoside hydrolases (GHs) that may degrade or modify chitin. The gene encoding chitinase B (*Fj*chiB), which encodes a single catalytic GH18 domain has been cloned and heterologously expressed in *Escherichia coli*. *Fj*ChiB was optimally active in 50 mM sodium citrate buffer (pH 6.0) at 40 °C. *Fj*ChiB was salt-tolerant and catalytically versatile, with substrate specificity towards 75% DDA (degree of de-acetylation) chitosan, followed by colloidal chitin. Chitotetraose (DP4) was the shortest of the oligomeric substrates used by *Fj*ChiB. The K_m and V_{max} values of *Fj*ChiB for colloidal chitin were 49.38 mg/ml and 11.2 nanokat mg^{-1}, respectively. The overall catalytic efficiency (k_{cat}/K_m) of *Fj*ChiB was 1.40×10^3 mg^{-1} ml s^{-1}. *Fj*ChiB exhibited transglycosylation (TG) with chitopentaose (DP5) and chitohexaose (DP6) substrates. The TG by *Fj*ChiB was fine-tuned by introducing a tryptophan (G106W) and asparagine (D148N) in the highly conserved catalytic groove and catalytic center, respectively. Hydrolytic products profile and homology modelling indicated that *Fj*ChiB is an endochitinase that holds promise for the conversion of chitin into useful products through both TG and/or hydrolysis.

1. Introduction

Chitin and cellulose biomass are inexpensive and renewable staple fibers for bioethanol production following their conversion into fermentable sugars [1,2]. Chitin is the second most abundant natural polysaccharide after cellulose. It forms the external protective material of arthropods and crustaceans as well as a major component of the fungal cell wall. Chitin is composed of β-(1, 4)-linked units of the amino sugar *N*-acetyl-glucosamine (NAG). Chito-oligosaccharides (CHOs) with different degree of polymerization (DP) can be generated by chitinases, chemical synthesis and also by controlled acid hydrolysis. The CHOs have attracted attention in recent years due to their versatile biological activities with biomedical [3], food/feed [4] and agricultural applications [5,6]. Chitinases that generate a variety of CHOs are therefore of great interest for a variety of industrial applications.

Chitinases (EC 3.2.1.14) hydrolyze chitin by cleaving β-1, 4-*N*-glycosidic bonds into oligomeric and monomeric components. They represent several glycoside hydrolase (GH) families, namely GH18, GH19, GH23, and GH48. Chitinases facilitate various physiological processes such as nutrition, parasitism, morphogenesis and immunity, in many different species, ranging from bacteria to plants and animals. Multiple chitinases produced by members of Enterobacteriaceae and other sources have been discovered and characterized [7,8].

A few of the GH18 family chitinases show transglycosylation (TG) activity, i.e. they transfer the released CHOs to a suitable acceptor, rather than using a water molecule to break the glycosidic bond. The products of TG activity are therefore longer than the substrate and donor oligosaccharides. The TG activity of chitinases has gained much attention due to the agricultural applications of long-chain CHOs [5]. Bacterial GH18 chitinases with TG activity were identified in *Stenotrophomonas maltophilia* [7], *Coccidioides immitis* [9], *Serratia marcescens* [10], and *S. proteamaculans* [11]. *Stm*ChiA was an endochitinase with TG and antifungal activity, while *Sm*ChiD and *Sp*ChiD were processive hyper TG chitinases [7,10]. Structure-based three-dimensional models have also been generated to understand and/or to improve the TG activity of chitinases [12].

CHOs have received increasing attention due to their spectrum of remarkable properties, which include biocompatibility, biodegradability, and bioadhesivity, as well as their antimicrobial activity and the absence

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of allergenicity and non-toxicity [13]. The industrial production of chitinases using either bacteria or fungi has been studied in detail. The extensive use of CHOs for biomedical and cosmetic application is fueling the growth of chitin and chitinase research [14]. Several chitinases with potential applications have already been structurally and functionally characterized.

Gram-negative bacterium *Flavobacterium johnsoniae* (DSMZ 2604) is commonly found in soil and fresh water. *F. johnsoniae* produces an array of carbohydrate-degrading enzymes including those required for chitin utilization [15]. The genome of *F. johnsoniae* encodes at least 10 GHs involved in chitin utilization. ChiA is an extracellular chitinase exuded *via* a type IX secretion system (T9SS) [16]. We cloned Fjoh_4560 (*Fj*ChiB) that encodes a 35.5 kDa catalytic domain GH18 chitinase. Due to the similarity with ChiA of cattle (*Bos taurus*), i.e. 25% identity over 244 amino acids [15], *Fj*ChiB is designated as an "animal-type family 18 bacterial chitinase" [17]. In this paper, the biochemical characterization of *Fj*ChiB and its ability to retain enzymatic activity at high salt concentration are being reported. Due to the inherent feeble TG activity of *Fj*ChiB on oligomeric substrates, improvement in TG was assessed by generating single amino acid substitutions in the highly conserved catalytic motif of the enzyme.

2. Materials and methods

2.1. Bacterial strains, plasmids, culture conditions and chemicals

The genomic DNA of *F. johnsoniae* DSMZ 2604 strain UW 101 (the type strain) was used as a template to clone the *Fj*chiB gene. The plasmid pET-28a (+) (Novagen, Darmstadt, Germany) and the host *Escherichia coli* Rosetta-gami 2(DE3) (Novagen, Madison, USA) were used for heterologous expression. *F. johnsoniae* was grown in Casitone-yeast extract (CYE) medium at 30 °C with shaking. *E. coli* was grown in lysogeny broth (LB) at 37 °C. Kanamycin (50 μg/ml) and chloramphenicol (25 μg/ml) was added to the LB when needed. Oligonucleotide primers were purchased from Eurofins (Bangalore, India). All the enzymes required for cloning were purchased from MBI Fermentas (Ontario, Canada). Isopropyl-β-D-thiogalactoside (IPTG), antibiotics and all other chemicals were purchased from Calbiochem or Merck (Darmstadt, Germany), or Hi-media labs (Mumbai, India). The polymeric chitin substrates α and β-chitin, and chitosan were provided by Dr. Dominique Gillete, Mahatani Chitosan (Veraval, India). Colloidal chitin and glycol chitin were prepared as described [18] and stored at 4 °C for further use. CHOs $(GlcNAc)_n$ (n = 1–6; DP1-DP6) were purchased from Seikagaku Corporation (Tokyo, Japan) through CapeCod, USA. *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K, WRK) was purchased from TCI chemicals (Japan).

2.2. Molecular cloning and site-directed mutagenesis of FjchiB

Gene-specific oligonucleotide primers were designed for *Fj*chiB sequence (GenBank: ABQ07559.1). The genomic DNA from *F. johnsoniae* was isolated using a DNA isolation kit (Macherey Nagel, Düren, Germany). The *Fj*chiB gene was amplified using high fidelity Phusion DNA polymerase with specific primers (Table 1) with *F. johnsoniae* genomic DNA as the template. The PCR thermal profile comprised an initial denaturation at 94 °C for 4 min, followed by 32 cycles of 94 °C for 45 s, 42 °C for 45 s, and 72 °C for 50 s, and a final extension step at 72 °C for 10 min. The amplicon was transferred to pET-28a at the *Bam*HI and *Xho*I sites and introduced into *E. coli* DH5α. The recombinant plasmids were sequenced and checked for frame shifts. Positive plasmids (pET 28a-*Fj*chiB) were then introduced into *E. coli* Rosetta-gami 2 (DE3) for protein expression. The transformants were selected on LB with suitable antibiotics. Two point mutations were generated in the catalytic region of *Fj*chiB using the *Dpn*I method [4] with Q5 high fidelity DNA polymerase (NEB, Ipswich, MA, USA) and the primers (Eurofins, Bangalore, India) listed in Table 1.

Table 1
Primers used for wild type (WT) gene cloning and site-directed mutagenesis.

Gene	DNA template	Primer	Primer sequence
*Fj*ChiB WT	gDNA	Forward	5′ -ATCATGGATCCATGTGTACCAGTGAAAAAG-3′
		Reverse	5′ -ATTATCTCGAGATTACCGCACATGCCTGAAG-3′
G106W	*Fj*ChiB WT	Forward	5′ -CTTGCCGGCTGGGTCATTTCAACC- 3′
		Reverse	5′ -TGAAATGACCCAGCCGGCAAGTGA- 3′
D148N	*Fj*ChiB WT	Forward	5′ -GTAGATGTTAATCTCGAATGGGAT- 3′
		Reverse	5′ -CCATTCGAGATTAACATCTACACC- 3′

2.3. Expression and purification of FjChiB and mutants

*Fj*ChiB and its two mutants were overexpressed as His_6-tagged proteins adding 0.3 mM IPTG and incubating for 12 h at 27 °C and was purified from the cell lysate by Ni^{2+}-NTA affinity chromatography according to the manufacturer's recommendations (Clontech) as described earlier [10]. The fractions containing pure protein were pooled, concentrated and buffer exchanged using 10 kDa Amicon filter units (Sartorius-Stedim Biotech, Goettingen, Germany). The protein concentration was measured using a BCA assay kit (Novagen, USA) with BSA as a standard.

2.4. Characterization of FjChiB

2.4.1. Zymogram analysis

Dot blot assays were carried out to determine the activity of purified *Fj*ChiB. A polyacrylamide gel appended with 0.1% soluble glycol chitin was prepared and spotted with 6 μg *Fj*ChiB. The gel was placed in a humid chamber at 37 °C for overnight and then stained with 0.01% calcofluor white M2R solution for 20 min at 25 °C. The gel was washed twice with sterilized distilled water. Lytic zones were visualized by placing the gel on a UV transilluminator.

2.4.2. Chitinase assay

Chitinase activity was measured colorimetrically by determining the number of reducing groups released from colloidal chitin substrate [19] with slight modifications as described [10]. *Fj*ChiB was incubated with colloidal chitin in 50 mM sodium citrate pH 6.0 at 40 °C, 200 rpm for 1 h, followed by centrifugation at 13,000 ×g at 4 °C for 20 min to remove insoluble chitin. Forty μl of the clear supernatant containing the reducing groups was mixed with 300 μl of the freshly prepared color reagent (0.5 M sodium carbonate, 0.05% potassium ferricyanide), and boiled the mixture for 20 min at 120 °C. The loss of color indicated chitinase activity. The absorbance of the reaction mixture (200 μl) was recorded in 96-well microtiter plates at 420 nm. The total reducing end groups were quantitatively determined with a standard curve of GlcNAc. One unit was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per min under standard assay conditions. All experiments were carried out in triplicates.

2.4.3. pH, and temperature optima, and steady-state kinetics

The optimal pH of *Fj*ChiB was determined by measuring chitinase activity in buffers ranging from pH 2.0 to pH 10.0 at 40 °C. The following buffer systems were used at 50 mM: glycine-HCl buffer (pH 2.0), citrate buffer (pH 3.0–6.0), sodium acetate buffer (pH 4.0–5.6), sodium phosphate buffer (pH 6.0–8.0), Tris-HCl buffer (pH 8.0–9.0), and glycine-NaOH buffer (pH 9.0–10.0). To determine the optimal temperature, chitinase was assayed at different temperatures ranging from 10 to 70 °C in 50 mM of the sodium citrate buffer pH 6.0, at intervals of 10 °C.

The kinetic parameters (V_{max}, K_m, k_{cat} and k_{cat}/K_m) of *Fj*ChiB were determined by carrying the reaction under optimal conditions as described by Purushotham and Podile [11] using different concentrations