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Multiple chitinases of an endophytic *Serratia proteamaculans* 568 generate chitin oligomers

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ABSTRACT

Serratia proteamaculans 568 genome revealed the presence of four family 18 chitinases (*Sp* ChiA, *Sp* ChiB, *Sp* ChiC, and *Sp* ChiD). Heterologous expression and characterization of *Sp* ChiA, *Sp* ChiB, and *Sp* ChiC showed that these enzymes were optimally active at pH 6.0–7.0, and 40 °C. The three *Sp* chitinases displayed highest activity/binding to β-chitin and showed broad range of substrate specificities, and released dimer as major end product from oligomeric and polymeric substrates. Longer incubation was required for hydrolysis of trimer for the three *Sp* chitinases. The three *Sp* chitinases released up to tetramers from colloidal chitin substrate. *Sp* ChiA and *Sp* ChiB were processive chitinases, while *Sp* ChiC was a non-processive chitinase. Based on the known structures of ChiA and ChiB from *S. marcescens*, 3D models of *Sp* ChiA and *Sp* ChiB were generated.

1. Introduction

Chitin is the second most abundant insoluble biopolymer, next only to cellulose, composed of β-1, 4-linked subunits of the acetylated amino sugar *N*-acetylglucosamine (NAG). Based on packing of the chitin chains two crystalline polymorphs are described. The dominant polymorph α-chitin has antiparallel chains, and β-chitin shows parallel chains. Unlimited bioresource like chitin is useful for environmentally friendly and biocompatible products, as well as biofuels. Chitinases are mainly responsible for chitin degradation and modification. The development of effective enzymes for the conversion of insoluble recalcitrant polysaccharides is one of the key issues for biomass conversion. To access the crystalline and inaccessible substrates like cellulose and chitin, enzymes have developed special tactics to ensure efficient degradation. In addition to their catalytic domains, cellulases and chitinases often have one or multiple carbohydrate-binding modules. These domains are beneficial for enzyme efficiency because they adhere to, and sometimes disrupt, the substrate. Further, chitin degrading organisms produce accessory proteins that disrupt the crystalline substrate, increasing the efficiency of chitinases (Vaaje-Kolstad et al., 2010).

Microbes have developed efficient strategies for the depolymerization, transport, and metabolism of chitin and its derivatives. Such systems involve multiple enzymes usually encoded as separate polypeptides. Marine bacterium *Alteromonas* spp. strain O-7 produces four chitinases (Orikoshi et al., 2005b). Soil bacteria like *Serratia marcescens* produce three chitinases (Suzuki et al., 2002), and *Streptomyces coelicolor* A3(2) possesses more than ten chitinase genes (some of which are putative) (Nazari et al., 2011). Bacterial endo- and exo-chitinases that cooperatively depolymerise chitin have potential as bio agents for crop protection (Neeraja et al., 2010b). Endo-chitinases randomly cleave glycosidic linkages, generating free ends and chitooligosaccharides (CHOS). The exo-chitinases release chitobiose from the reducing or non-reducing ends. The glycoside hydrolase family 18 (GH18) domain is the most common catalytic domain of microbial chitin depolymerases. Despite sharing a consensus sequence, and a conserved catalytic glutamic acid residue, GH18 domains differ in their activity towards polymeric chitin and CHOS (i.e., endo- versus exo-activity).

Many enzymes that hydrolyze insoluble crystalline polysaccharides such as cellulose and chitin guide detached single-polymer chains through long and deep active-site clefts, leading to processive (stepwise) degradation of the polysaccharide (Horn et al., 2006a). Processivity is thought to contribute to the degradation of crystalline polysaccharides because detached single-polymer chains are kept from reassociating with the solid material. Processivity reduces the number of times the enzyme has to carry out the energetically unfavorable process of gaining access to a single chain. Processive enzymes often have long and deep substrate binding cleft as illustrated by the first structures of processive cellulases (Divne et al., 1994).

The physicochemical properties of chitin and its derivatives (including oligomers) in linked forms are also suited for a wide range of applications in agricultural, food, cosmetic, pharmaceutical, and medical industries. Chitin that was chemically modified to obtain aminoethyl-chitin, showed antioxidant activity against free radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH),

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hydroxyl, superoxide, and peroxyl (Je and Kim, 2006). Chitin was an effective agent for homeostasis maintenance through aggregating platelets (Okamoto et al., 2003).

The key property of chitin-derived products for use in various biomedical applications is the immuno-modulating effect (Aam et al., 2010). NAG and chitobiose, the major end products from the chitinases, also have pharmacological applications. NAG possesses anti-inflammatory properties and was used to treat ulcerative colitis, gastrointestinal inflammations, and also as a nutritional substrate for pediatric chronic inflammatory bowel disease (Salvatore et al., 2000). The derivative of NAG, glucosamine, helps regeneration of joint cartilage and was used extensively to treat osteoarthritis (Huskisson, 2008). CHOS and NAG have been produced from chitin by acid hydrolysis. Alternatively, enzymatic hydrolysis of chitin has become more promising due to environmental compatibility, low cost, and reproducibility (Kadokura et al., 2007).

S. proteamaculans 568, a member of family *Enterobacteriaceae*, was isolated as a root endophyte from *Populus trichocarpa* (Taghavi et al., 2009). The Carbohydrate Active enZYme data base (CAZy-http://www.cazy.org/) (Henrissat and Davies, 1997) showed at least eight genes in the genome sequence of *S. proteamaculans* 568 could be potentially involved in chitin turnover, coding for four GH18 chitinases (*Sp* ChiA, *Sp* ChiB, *Sp* ChiC and *Sp* ChiD), three family 33 CBPs (*Sp* CBP21, *Sp* CBP28, and *Sp* CBP50), and a family 20 *N*-acetylhexosaminidase (*Sp* CHB). The present study, describes cloning, expression and characterization of *Sp* ChiA, *Sp* ChiB, and *Sp* ChiC from *S. proteamaculans* 568.

2. Methods

2.1. Bacterial strains, plasmids, culture conditions, bio chemicals and enzymes

S. proteamaculans 568 (gifted by Dr. Daniel van der Lelie, USA) was used as the source of genomic DNA for cloning the genes encoding for *Sp* ChiA, *Sp* ChiB, and *Sp* ChiC. The plasmid pET-28a (+) (Novagen, Darmstadt, Germany) and the host *E. coli* Rosetta-gami 2(DE3) (Novagen, Madison, USA) were used for heterologous expression. *S. proteamaculans* 568 was grown with shaking at 28 °C in LB medium (Difco, Detroit, USA) for 16 h. *E. coli* was grown in LB broth at 37 °C. Kanamycin (50 μg/mL) and chloramphenicol (25 μg/mL) were added to the LB broth as required. Oligonucleotide primers were purchased from Eurofins (Bangalore, India). Restriction enzymes, T4 DNA ligase and *Pfu* DNA polymerase were from MBI Fermentas (Ontario, Canada). Isopropyl-β-D-thiogalactoside (IPTG), ampicillin and all other chemicals were purchased from Calbiochem or Merck (Darmstadt, Germany), or Hi-media labs (Mumbai, India). The polymeric substrates α-chitin, β-chitin and chitosan were provided by Dr. Dominique Gillete, Mahtani Chitosan (Veraval, India). Colloidal chitin and glycol chitin were prepared as described by Neeraja et al. (2010a). CHOS with different degrees of polymerization (DP) were purchased from Seikagaku Corporation (Tokyo, Japan). Avicel was procured from Sigma–Aldrich (Missouri, USA).

2.2. Cloning of Sp ChiA, Sp ChiB, and Sp ChiC

The Oligonucleotide primers (Supplementary Table S1) for the cloning of *Sp* ChiA, *Sp* ChiB and *Sp* ChiC genes from *S. proteamaculans* 568 were designed based on the DNA sequence of respective genes (NCBI Accession Number ABV39247.1, ABV40327.1 and ABV42574.1) available in database. *S. proteamaculans* 568 gDNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, Düsseldorf, Germany) and used as the template for amplification of chitinase genes. The PCR thermal profile consisted of an initial denaturation at 95 °C for 2 min, followed by 30 cycles of 95 °C for 45 s, 56 °C for 55 s, and 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The genes were then cloned into the *Nco* I and *Xho* I sites of the pET- 28a (+) . These constructs were mobilized into *E. coli* Rosetta-gami 2(DE3) for expression of the genes. The transformants were selected by growing on LB broth with kanamycin (50 μg/mL) and chloramphenicol (25 μg/mL).

2.3. Expression and purification of Sp ChiA, Sp ChiB, and Sp ChiC

To express the recombinant *Sp* ChiA, *Sp* ChiB, and *Sp* ChiC, a single colony of *E. coli* Rosetta-gami 2(DE3) harboring recombinant plasmid was grown at 37 °C in LB medium containing kanamycin (50 μg/mL) and chloramphenicol (25 μg/mL). At 0.6 (OD_{600}) IPTG was added to a final concentration of 0.5 mM and incubated for 24 h at 18 °C and 200 rpm, followed by centrifugation at 9000*g* for 10 min at 4 °C for harvesting of cells. The cell pellets expressing *Sp* ChiA, *Sp* ChiB, and *Sp* ChiC were suspended in Ni–NTA equilibration buffer (50 mM NaH_2PO_4, 100 mM NaCl and 10 mM imidazole, pH 8.0). Cells were lysed by sonication at 20% amplitude with 30 × 15 s pulses (with 20 s delay between pulses) on ice, with a Vibra cell Ultrasonic Processor, converter model CV33, equipped with a 3 mm probe (Sonics, Newtown, CT, USA). The sonicated cell lysate was centrifuged at 15,200*g* for 10 min at 4 °C to pellet the insoluble cell debris. Ni–NTA affinity chromatography was used to purify C-terminal His-tag carrying *Sp* ChiA, *Sp* ChiB, and *Sp* ChiC according to the method described by Neeraja et al. (2010a).

2.4. Protein measurement

Purified *Sp* ChiA, *Sp* ChiB, and *Sp* ChiC were quantified by BCA protein assay kit (Novagen, USA) using a standard calibration curve constructed from BSA (bovine serum albumin). For the chitin binding assay, the protein concentration was measured from the absorbance at 280 nm using the molar extinction coefficients (ε) calculated from the amino acid composition of the protein as described by Pace et al. (1995).

2.5. Characterization of Sp ChiA, Sp ChiB, and Sp ChiC

2.5.1. Zymogram analysis

Dot blot assay was performed to detect the activity of purified recombinant *Sp* ChiA, *Sp* ChiB, and *Sp* ChiC. A composite gel supplemented with 0.1% glycol chitin was prepared. Five microgram each of *Sp* ChiA, *Sp* ChiB, and *Sp* ChiC was separately spotted onto the gel and placed in humid chamber at 37 °C for overnight. After incubation, the gel was stained with 0.01% calcofluor white M2R in 0.5 M Tris–HCl pH 8.9 for 10 min at 4 °C. Finally, the brightener solution was removed. The gel was washed with distilled water for 10 min at 4 °C. Lytic zones were visualized by placing the gels on a UV transilluminator.

2.5.2. Chitinase assay

Reducing end assay was performed for chitinase activity as described by Neeraja et al. (2010a), with slight modifications. Reaction mixture containing 5 μg of purified *Sp* ChiA/*Sp* ChiB/*Sp* ChiC and 4 mg/mL colloidal chitin, and 50 mM buffer (*Sp* ChiA: sodium phosphate pH 7.0, *Sp* ChiB: sodium acetate pH 6.0 and *Sp* ChiC: sodium phosphate pH 6.0), was incubated at 40 °C for 1 h with shaking at 190 rpm. One unit was defined as the amount of chitinase that liberated 1 μmol of reducing sugar per minute, against NAG standard.