Bioresource Technology 133 (2013) 213–220

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Chitinase A from *Stenotrophomonas maltophilia* shows transglycosylation and antifungal activities

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HIGHLIGHTS

- Multi domain chitinase A and a single catalytic domain chitinase B of *Stenotrophomonas maltophilia* were characterized.
- Chitinase A was an endo acting enzyme active on both oligomeric and polymeric substrates.
- Chitinase B was exo/endo acting enzyme, that can hydrolyze only oligomeric substrates.
- Chitinase A, and not chitinase B, exhibited transglycosylation on tetra- and hexameric chitooligosaccharides.
- Chitinase A also showed antifungal activity against *Fusarium oxysporum*.

ARTICLE INFO

Article history:
Received 2 May 2012
Received in revised form 13 January 2013
Accepted 17 January 2013
Available online 29 January 2013

Keywords:
Chitooligosaccharides
Stenotrophomonas maltophilia
Chitinase
Transglycosylation
Antifungal activity

ABSTRACT

Stenotrophomonas maltophilia chitinase (*Stm*ChiA and *Stm*ChiB) genes were cloned and expressed as soluble proteins of 70.5 and 41.6 kDa in *Escherichia coli*. Ni–NTA affinity purified *Stm*ChiA and *Stm*ChiB were optimally active at pH 5.0 and 7.0, respectively and exhibited broad range pH activity. *Stm*ChiA and *Stm*ChiB had an optimum temperature of 40 °C and are stable up to 50 and 40 °C, respectively. Hydrolytic activity on chitooligosaccharides indicated that *Stm*ChiA was an endo-acting enzyme releasing chitobiose and *Stm*ChiB was both exo/endo-acting enzyme with the release of GlcNAc as the final product. *Stm*ChiA showed higher preference to β-chitin and exhibited transglycosylation on even chain length tetra- and hexameric substrates. *Stm*ChiA, and not *Stm*ChiB, was active on chitinous polymers and showed antifungal activity against *Fusarium oxysporum*.

1. Introduction

Chitin is an abundant extracellular amino-sugar polymer that in association with other biomacromolecules forms an essential structural component of fungal cell walls, shells of crustaceans and insect cuticles. Chitin is one of the largest forms of renewable biomass on earth, second only to cellulose. Among the three different forms, α-chitin is stiffest, with higher intermolecular hydrogen bonding and exhibits highest tensile strength. The degradation of chitin is achieved by chitinases which belong to two families of glycoside hydrolases (GH), family 18 and family 19.

Enzymes acting on crystalline polysaccharides need to associate with the insoluble substrate, disrupt the polymer packing and guide a single polymer chain into the catalytic center. Association of accessory domains to the catalytic domain helps the enzyme to cleave polymeric substrates. A few chitinases have accessory domains like chitin-binding domain (ChBD), Fibronectin type III domain (FN3) and polycystic kidney disease domain (PKD) other than the catalytically active GH domain. The chitin-binding protein 21(CBP21) produced by *Serratia marcescens* potentiates chitinase action by disrupting the structure of the β-chitin substrate (Kolstad et al., 2010) strongly suggesting the synergistic effect of CBPs and chitinases. PKD domain of *Alteromonas* sp.O-7 ChiA participates in effective hydrolysis of powdered chitin through the interaction between two aromatic residues (W30 and W67) and chitin molecule (Orikoshi et al., 2005).

Some chitinases possess transglycosylation (TG) activity in addition to hydrolytic activity. TG is the ability to transfer the released oligosaccharide moiety to a suitable acceptor to form a new glycosidic bond. The TG activity of family 18 chitinases is of special interest because of the potential applications for chitooligosaccharides (CHOS) with specific degree of polymerization (DP). Family 18 chitinases are retaining GHs in which the TG reaction occurs through a double-displacement mechanism. GH18 chitinases like chitinase-1 from *Coccidioides immitis* (Fukamizo et al., 2001), chitinase A from *S. marcescens* (Aronson et al., 2006) and *Vibrio*

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http://dx.doi.org/10.1016/j.biortech.2013.01.103

harveyii (Suginta et al., 2005), chitinase A1 from *Bacillus circulans* (Sasaki et al., 2002) and chitinase D from *Serratia proteamaculans* (Purushotham and Podile, 2012; Madhuprakash et al., 2012) possess TG activity. Kobayashi et al. (1996) have successfully applied the chitinases from *Bacillus* sp. for the chemoenzymatic synthesis of artificial chitin and related polysaccharide derivatives, using N,N^0-diacetylchitobiose oxazoline and its modified forms as highly activated monomers for the chitinase-catalyzed polymerization.

Biocontrol of fungal diseases of plants by bacterial strains was achieved by destructive lysis of fungal cell walls through exploitation of their chitinolytic potential (Kishore et al., 2005). Gram-negative bacteria such as *Pseudomonas* sp., *Erwinia* sp., and *Agrobacterium* sp. represent the majority of bacteria investigated to control pathogenic fungi (Neeraja et al., 2010a). Chitinase from *S. proteamaculans* inhibited fungal spore germination and hyphal growth of *Fusarium oxysporum* and *Aspergillus niger* (Mehmood et al., 2009).

Stenotrophomonas maltophilia is a Gram negative bacterium coding for a cluster of chitin modifying enzymes. *S. maltophilia* has an extra range of activities that include beneficial effects for plant growth and degradation of xenobiotic compounds (Lee et al., 2002). The CAZy database indicating that nine genes in the genome sequence of *S. maltophilia* could be potentially involved in chitin turnover. This study, was aimed at cloning and characterization of two chitinases, ChiA and ChiB of *S. maltophilia*, and reports that Chi A shows TG and antifungal activity with a scope for wider applications.

2. Methods

2.1. Bacterial strains, plasmids, culture conditions and biochemicals

S. maltophilia (Strain No: ∗434 – Microbial Type Culture Collection, IMTECH, Chandigarh) was used as the source of genomic DNA for cloning the genes encoding chitinases A and chitinases B (referred to as *Stm*ChiA and *Stm*ChiB). The plasmid pET-22b (+) (Novagen, Darmstadt, Germany) and the host *Escherichia coli* Rosetta-gami 2(DE3) (Novagen, Madison, USA) were used for heterologous expression. *S. maltophilia* 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. Ampicillin (100 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- and β-chitin, and chitosan were provided by Dr. Dominique Gillete, Mahatani Chitosan (Veraval, India). Colloidal chitin and glycol chitin were prepared as described by Neeraja et al. (2010a). CHOS with different DP were purchased from Seikagaku Corporation (Tokyo, Japan) through CapeCod, USA. Laminarin and Avicel were procured from Sigma–Aldrich (Missouri, USA).

2.2. Cloning of StmChiA and StmChiB

The gene specific oligonucleotide primers were designed for the cloning of *StmchiA* and *StmchiB* genes from *S. maltophilia* based on the DNA sequence of respective genes (NCBI Accession Number CAQ44264 and CAQ46811). *S. maltophilia* gDNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, Düsseldorf, Germany) and used as the template. The genes were amplified using *Pfu* DNA polymerase using *StmChiA* Fp: 5′-CAT AAC CAT GGT GGC CAG CTG TGC CGG CGT G-3′ *StmChiA* Rp: 5′-ATC CGC TCG AGC TTC AGG CCA TCA CTG ACCGCC-3′ *StmChiB* Fp: 5′-CAT AACCAT GGT GGC GCC CGC GCA GCC ACC GATC-3′ *StmChiB* Rp: 5′-ATC CGC TCG AGA TCG CCG ATG CGC GCC TTC TG-3′ primers. The PCR thermal profile consisted of an initial denaturation at 96 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 40 s, and 72 °C for 5 min, and a final extension step at 72 °C for 7 min. The amplicons were cloned into the *Nco*I and *Xho*I sites of the pET-22b (+) and mobilized into *E. coli* Rosetta-gami 2(DE3) for expression. The transformants were selected by growing on LB broth with ampicillin (100 μg/mL) and chloramphenicol (25 μg/mL).

2.3. Expression and purification of StmChiA and StmChiB

To express the cloned *StmChiA* and *StmChiB*, a single colony of *E. coli* Rosetta-gami 2(DE3) harboring respective recombinant plasmid was grown at 37 °C in LB medium containing ampicillin (100 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 *StmChiA* and *StmChiB* 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 *Stm* ChiA and *Stm* ChiB as described by Neeraja et al. (2010b).

2.4. Protein measurement

Purified *Stm*ChiA and *Stm*ChiB were quantified by BCA protein assay kit (Novagen, USA) using a standard calibration curve constructed from BSA (bovine serum albumin).Molecular weights of *Stm*ChiA and *Stm*ChiB were estimated using ExPASy compute pI/MW software tool.

2.5. Characterization of StmChiA and StmChiB

2.5.1. Zymogram analysis

Dot blot assay was performed to detect the activity of purified *Stm*ChiA and *Stm*ChiB. An acrylamide gel supplemented with 0.1% glycol chitin was prepared. Five microgram each of *Stm*ChiA and *Stm*ChiB 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 and 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

Chitinase assay was performed in triplicates containing 1.79 μM of purified *Stm*ChiA or *Stm*ChiB and 300 μM chitin hexamer (DP6) in 50 mM buffer (*Stm*ChiA: sodium acetate pH 5.0 and *Stm*ChiB: sodium phosphate pH 7.0), was incubated at 40 °C for 1 h, generated reducing ends were estimated against *N*-acetyl glucosamine (NAG) standard. Specific activity in nkat/mg of protein was fitted to the Michaelis–Menten equation using GRAPHPAD PRISM (GraphPad Software Inc., USA) to determine V_{max}, K_m and k_{cat} of the chitinases.

2.5.3. Steady-state kinetics, temperature optima and pH

Kinetic parameters of the *Stm*ChiA and *Stm*ChiB were determined using DP6 as substrate. The reaction mixture containing 50–600 μM of DP6 and 5 μg *Stm*ChiA or *Stm*ChiB in 50 mM buffer