Biochimica et Biophysica Acta 1840 (2014) 2685–2694

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Mutagenesis and molecular dynamics simulations revealed the chitooligosaccharide entry and exit points for chitinase D from *Serratia proteamaculans*

Jogi Madhuprakash [a,1], Karunakar Tanneeru [b,1], Bhavana Karlapudi [a], Lalitha Guruprasad [b,*], Appa Rao Podile [a,**]

[a] Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Gachibowli, Hyderabad 500046, A.P., India
[b] School of Chemistry, University of Hyderabad, Gachibowli, Hyderabad 500046, A.P., India

ARTICLE INFO

Article history:
Received 19 March 2014
Received in revised form 27 May 2014
Accepted 18 June 2014
Available online 25 June 2014

Keywords:
Chitinases
Chitooligosaccharides
*Sp*ChiD
Transglycosylation
Mutagenesis
Molecular Dynamics Simulations

ABSTRACT

Background: Transglycosylation (TG) activity is a property of glycosyl hydrolases (GHs) with which new glycosidic bonds are introduced between donor and acceptor sugar molecules. This special property of the GHs has potential to generate longer chain chitooligosaccharides (CHOS) that show elicitor activity in plants. We hypothesize that TG activity could be improved by retaining the substrate for a longer duration in the catalytic site.
Methods: Four variants of chitinase D from *Serratia proteamaculans* (*Sp*ChiD) *i.e.* G119S, G119W, W120A and G201W were analyzed in detail for improved TG activity using high performance liquid chromatography (HPLC) and high resolution mass spectrometry (HRMS). The results were strongly supported by 50 ns molecular dynamics (MD) simulations and estimated solvated interaction energies (SIE).
Results: The mutant G119W lost much of both hydrolytic and TG activities, while the mutant G201W displayed increased TG. The trajectory of MD simulations of the mutant G119W showed that the indole rings of two adjacent Trp residues create a major hindrance for the DP4 movement towards the catalytic center. Increased van der Waals (vdW) and coulombic interactions between DP4 substrate and the Trp-201 resulted in enhanced TG activity with the mutant G201W. The average number of hydrogen bonds observed for the DP4 substrate was increased for the mutants G119W and G201W compared to *Sp*ChiD.
Conclusion: The increase in TG activity could be due to partial blocking of product exit of *Sp*ChiD.
General significance: This new approach can be used for generating mutants of GHs with improved TG activity to produce longer chain oligosaccharides.

1. Introduction

Chitinases are a class of glycosyl hydrolases (GHs) that catalyze hydrolysis of β-1, 4 glycosidic bonds present in the homopolymer chitin, made of *N*-acetylglucosamine (GlcNAc) repeating units. Based on sequence similarity, chitinases can be subdivided into two families, 18 and 19 that differ in structure and mode of action [1]. Family 18 chitinases are 'retaining glycoside hydrolases' often found in different organisms, from bacteria to humans [1,2]. Presence of $(\beta/\alpha)_8$ – TIM barrel fold along with several conserved sequence motifs [3] – in the catalytic domain is a characteristic feature of family 18 chitinases [4–8]. The most prominent of these motifs is the DXDXE motif that spans strand 4 of the TIM barrel and includes glutamate that acts as the catalytic acid. The concerted action of two acidic residues, one as general acid-base and the other as nucleophile, results in a reaction with retention of the configuration at the anomeric carbon atom, is known for family 18 chitinases [9]. The substrate-assisted mechanism of the retaining GHs proceeding *via* the formation of a potential oxazoline/oxazolinium ion intermediate was confirmed on the basis of kinetic measurements and crystallographic structures [10–13]. The active site grooves of the retaining chitinases are lined with aromatic amino acids that contribute to substrate binding [6,14].

The overall sequence similarity among family 18 chitinases is not very high. But, an average pairwise identity of 21% (http://www.sanger.ac.uk/Software/Pfam), and occurrence of highly conserved residues in the active site regions with catalytic functions are known [13]. In ChiB of *Serratia marcescens (SmChiB)*, apart from the catalytic twin aspartates Asp-140 & 142 and Glu-144, other amino acids like Tyr-10, Ser-93, Tyr-214, and Asp-215 are well studied. The mechanism of family 18 chitinases is conserved. But, with respect to processive and non-processive, including endo/exo-action, it is conceivable that the active

* Corresponding author. Tel.: +91 40 23134820.
** Corresponding author. Tel.: +91 40 23134503; fax: +91 40 2301 0120.
E-mail addresses: lgpsc@uohyd.ernet.in (L. Guruprasad), arpsl@uohyd.ernet.in (A.R. Podile).
[1] Contributed equally.

http://dx.doi.org/10.1016/j.bbagen.2014.06.014
0304-4165/

site residue flexibility and dynamics are adapted to the variations in the mode of action [10,13,15].

The degradation products of chitin *i.e.*, chitooligosaccharides (CHOS) are gaining special interest due to potential biological applications, especially in the food, medical, and agriculture fields [16,17]. A few of the very important biological activities of CHOS include antibacterial activities [18], antitumor properties [19], antifungal activities [20], and immuno-enhancing effects [21,22]. CHOS regulate the capsular architecture of *Cryptococcus neoformans* cells from both *in vitro* and infected tissues [23]. It was suggested that cellular pathways required for capsule formation and pathogenic mechanisms are affected by blocking chitin-derived structures at the cell surface of *C. neoformans* [23,24]. Thus, targeting CHOS with specific ligands could be a new therapeutic alternative to control cryptococcosis. Despite their biological interest and potential agronomical usefulness, CHOS with well-defined structures remain poorly accessible. Most biological activities require CHOS with a degree of polymerization (DP) ≥ 4 [25]. The synthesis of oligomers with DP ≥ 6 has been a daunting task [26]. Among several methods available for generating CHOS, enzymatic approaches are most promising. Few of the GHs show transglycosylation (TG) activity, forming new glycosidic bonds between donor and acceptor saccharides [26–28]. In retaining GHs, the TG occurs through a double-displacement mechanism [29]. This special property of the GHs can be exploited for the production of not only longer chain CHOS but also well-defined mixtures of CHOS with new or improved biological activity [30], by coupling smaller CHOS building blocks to each other or to other functional groups [27].

The TG activity was improved by chemical modification of residues Trp-62 and Asp-101, at -4 and -2 subsites of hen egg white lysozyme [31] or by mutating a Trp-167 located at subsite -3 of *S. marcescens* chitinase A (*Sm*ChiA) [32]. The Trp-167 was distantly located from the active site *i.e.*, in the chitin-binding cleft. The mutant *Sm*ChiA had improved TG activity with oligosaccharide substrates as well as *p*-nitrophenyl-di-*N*-acetyl-β-D-chitobiose co-incubated with chitotetraose [32]. The variants of Asp-140/311 and Asp-142/313, analogous residues in *Sm*ChiB and *Sm*ChiA, respectively, showed improved TG activity [27]. Alteration of amino acid residues at the catalytic center, catalytic groove, and solvent-accessible region substantially improved the TG activity in chitinase D from *Serratia proteamaculans* (*Sp*ChiD) [33], whereas, the aromatic side chains of Phe-166 and Trp-197, in class V chitinase from *Cycas revoluta* (*Cr*Chi-A), located in the acceptor binding site, were shown to control the TG activity [34] and the introduction of a tryptophan side chain into $+1$ subsite of family GH-18 (class V) chitinase from *Arabidopsis thaliana* (*At*ChiC) enhanced the TG activity [35].

To generate variants of chitinases with improved TG activity, a better understanding of the chitinase-catalyzed hydrolysis and/or TG of CHOS is essential. Introduction of a bulky residue like Trp at '119' and '201' in place of glycine, allowed us to elucidate the CHOS entry and exit points for *Sp*ChiD and unfolded a new possibility to increase the TG activity (Fig. 1). In the present study, the biochemical properties of *Sp*ChiD and its four mutants (G119S, G119W, W120A and G201W) were analyzed by HPLC, High Resolution Mass Spectrometry (HRMS), molecular dynamic (MD) simulations and estimated solvated interaction energies (SIE) free energy were compared to achieve better insights into the mutational effects on hydrolytic and TG activities.

2. Materials and methods

2.1. Bacterial strains, plasmids, culture conditions, biochemicals and enzymes

The plasmid pET-22b (+) and *Escherichia coli* BL21 (DE3) (Novagen, Madison, USA) were used for heterologous expression. *E. coli* was grown in LB broth (1% peptone, 0.5% yeast extract, 1% NaCl) at 37 °C. Ampicillin, at a working concentration of 100 μg/ml, was added to the LB broth as required. Oligonucleotide primers were purchased from Eurofins India (Bangalore, India). Restriction enzymes, T4 DNA ligase and *Pfu* DNA polymerase were obtained from MBI Fermentas (Ontario, Canada). Isopropyl-β-D-thiogalactoside (IPTG), ampicillin and all other chemicals were purchased from Calbiochem or Merck (Darmstadt, Germany). Ni–NTA His bind resin was procured from Novagen (Madison, USA) for protein purification. Different DP CHOS were obtained from Seikagaku Corporation (Tokyo, Japan), through Cape Cod (East Falmouth, USA).

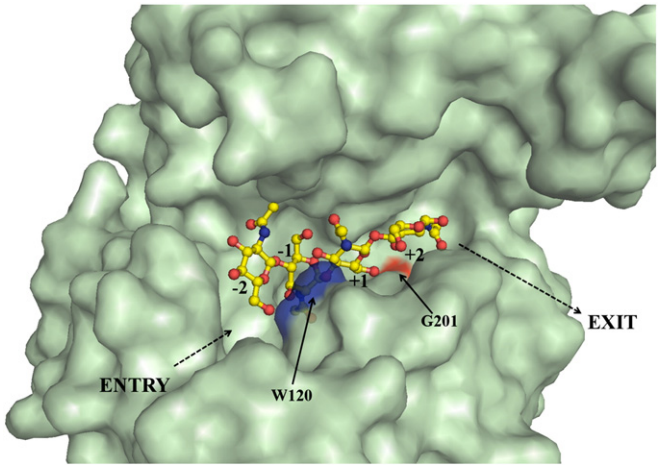

Fig. 1. Surface representation of *Sp*ChiD (PDB code: 4LGX) and the DP4 in the active site represented in the ball and stick (carbon—yellow, nitrogen—blue, oxygen—red). The residues at the probable entry (blue surface—W120) and exit (red surface—G201) sites for the protein *Sp*ChiD were indicated.

2.2. Construction of G119W and G201W mutants

*Sp*ChiD mutants were generated as described by Song-Hua and Madison [36] with pET-22b (+)-*Sp*ChiD as template [26]. Mutagenic primers were designed and followed similar conditions for PCR amplification as described earlier [33]. The primers used for site-directed mutagenesis were 5′-CTGTCCGTCGGT*TGG*TGGGGCGCTCGC-3′ for the Gly119 → Trp mutation (G119W) and 5′-ACCATCGCCGTC*TGG*GCCAAC GTGAAA-3′ for the Gly201 → Trp mutation (G201W) (the mutation sites are italicized and underlined). Each mutation was introduced into pET-22b (+)-*Sp*ChiD the inserted mutation was confirmed by automated DNA sequencing (Eurofins, Bangalore, India). The constructs, with desired mutations were transformed into *E. coli* BL21 (DE3) for protein over expression.

2.3. Protein expression, isolation and purification

Expression of *Sp*ChiD and the mutant proteins was done as described by Neeraja et al. [37]. The harvested culture pellet was processed for isolation of periplasmic fraction (PF) as described in pET manual (Novagen, Darmstadt, Germany). The PFs with desired proteins were purified as described in Qiagen (Duesseldorf, Germany) manual using Ni-NTA affinity chromatography under native conditions. The recombinant protein bound to the Ni-NTA matrix was eluted with different concentrations of imidazole containing buffers [33]. The purified fractions were electrophoresed on 12% SDS-PAGE and visualized using Coomassie brilliant blue. Fractions with high purity were selected for concentration and buffer exchanged with 20 mM sodium acetate, pH 5.6, using amicon filters of 10 kDa cut-off (Millipore, Billerica, MA).

2.4. Chromatography of CHOS generated by the mutants G119W and G201W

HPLC analysis was done to check the mutational effects on hydrolytic and TG activities of G119W and G201W mutants of *Sp*ChiD. Two mM chitotetraose (DP4) was taken as the starting substrate and incubated with 350 nM of the purified glycine variants. All the experimental parameters were kept unchanged to make a comparative study with