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Production of bioactive chitosan oligosaccharides using the hypertransglycosylating chitinase-D from *Serratia proteamaculans*

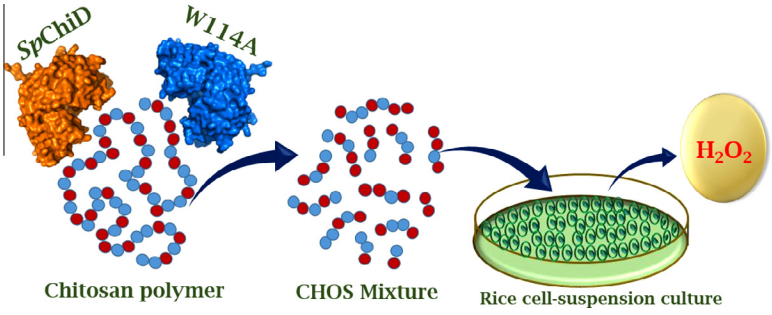
Jogi Madhuprakash [a], Nour Eddine El Gueddari [b], Bruno M. Moerschbacher [b], Appa Rao Podile [a,*]

[a] Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Gachibowli, Hyderabad, India
[b] Institute for Biology and Biotechnology of Plants, WWU, Münster University, Münster, Germany

HIGHLIGHTS

- Generated varied DP/DA CHOS from chitosans of DA35% and 61% by *Sp*ChiD/its mutant W114A.
- Analyzed the elicitor activity of crude/purified CHOS in suspension cultures of rice.
- CHOS crude mixtures produced by the mutant W114A were more active than *Sp*ChiD.
- Fully-deacetylated CHOS did not inhibit hydrolysis or the TG activity of *Sp*ChiD.
- TG activity of *Sp*ChiD on chitosans can be exploited to generate CHOS of defined DA/PA.

GRAPHICAL ABSTRACT



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ABSTRACT

The biological activities of chitosan and its oligosaccharides are greatly influenced by properties such as the degree of polymerization (DP), degree of acetylation (DA) and pattern of acetylation (PA). Here, structurally diverse chitosan oligosaccharides from chitosan polymers (DA = 35% or 61%) were generated using *Serratia proteamaculans* wild-type chitinase D (*Sp*ChiD) and the W114A mutant which lacks transglycosylase activity. The crude oligosaccharide mixtures and purified fractions with specific DP and DA ranges were tested for their ability to induce an oxidative burst in rice cell suspension cultures. The crude mixtures were more active when produced by the W114A mutant whereas the purified fractions were more active when produced by wild-type *Sp*ChiD. Neither hydrolysis nor transglycosylation by *Sp*ChiD was inhibited in the presence of fully-deacetylated oligosaccharides, suggesting that *Sp*ChiD could be exploited to generate oligosaccharides with defined DA and PA values.

1. Introduction

Chitin is a linear homopolysaccharide composed of *β*-(1,4)-linked *N*-acetylglucosamine residues (GlcNAc, A) and is the major structural component of fungal cell walls and arthropod exoskeletons. It can be deacetylated to form soluble cationic polysaccharides called chitosans, which are copolymers of GlcNAc and D-glucosamine (GlcN, D) residues (Aam et al., 2010). The chemical composition of chitosans depends on the number of residues (the degree of polymerization, DP), the relative proportion of acetylated and deacetylated residues (the degree of acetylation, DA) and their distribution along the chain (the pattern of acetylation, PA). These affect physicochemical properties such as

* Corresponding author at: Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Gachibowli, Hyderabad 500046, India. Tel.: +91 40 23134503; fax: +91 40 23010120.
E-mail address: arpsl@uohyd.ernet.in (A.R. Podile).

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pH-dependent solubility (Vårum et al., 1994) and biological properties such as gene and drug delivery (Koping-Hoggard et al., 2001; Schipper et al., 1996).

Plants defend themselves against fungal pathogens by recognizing and binding microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) by pattern-recognition receptors, and initiate various immune responses (Boller and Felix, 2009; Das et al., 2015). Oligomers of chitin/chitosan (CHOS) serve as PAMPs for the recognition of potential fungal plant pathogens. Oligomers with a DP range from 4 to 8 bind to the corresponding receptors but only those with DP values of 7 or 8 induce a significant defense response (Liu et al., 2012; Willmann and Nurnberger, 2012; Hamel and Beaudoin, 2010). The impact of DA and PA is not so well understood (Cabrera et al., 2006). In wheat leaves, stronger enzymatic defense responses were induced by chitosan oligomers with high DA values whereas lignin deposition and symptom development were induced more effectively by intermediate DA values (Vander et al., 1998). The importance of long-chain CHOS, in particular the fungal cell wall derived PAMPs, for induction of PTI suggests the need to generate a variety of CHOS with different DP, DA and if possible of different PA, to understand the subtle difference(s) in the interaction of CHOS ligands with their cognate receptors in plants.

Araucaria angustifolia cell suspension cultures were tested with chitosan oligomers with DA values, ranging from 1% to 69%, revealing that oligomers with the highest DA were the most potent inducers of an oxidative burst (dos Santos et al., 2008). Although most fungal cell walls contain chitin but no chitosan, a few species synthesize a mixture of chitin and chitosan, the latter produced by endogenous chitin deacetylases (Kafetzopoulos et al., 1993). These fungi may therefore produce MAMPs/PAMPs comprising chitosan oligomers with DA values ranging from 0% to 100% (Vander et al., 1998).

Chitinases are glycosyl hydrolases that catalyze the hydrolysis of β-(1,4) glycosidic bonds between GlcNAc units in chitin and chitosan. Some chitinases are also transglycosidases, allowing them to introduce new glycosidic bonds between donor and acceptor sugar molecules and thus generate longer-chain oligomers (Zakariassen et al., 2011; Purushotham and Podile, 2012) which may be useful for a range of biological applications (Das et al., 2015). Chitosan oligomers have been shown to induce defense reactions in wheat, barley, melon, and tomato, but rice cell suspension cultures are the best-characterized model system (Shibuya and Minami, 2001; Okada et al., 2002). It was shown that the CHOS with a DP $\geqslant$ 5 act as potent phytoalexin elicitors in suspension-cultured rice cells (Okada et al., 2002). Thus, the size and structure of CHOS can be crucial for induction of defense responses in plants.

A majority of bio-activity studies performed in different plant (s)/cell-suspension culture systems, employed CHOS that were prepared chemically by treating chitin with anhydrous hydrogen fluoride or by hydrolysis in hot concentrated HCl. For example, oligomers with a consistent DP but variable DA can be prepared by the partial re-acetylation of a mixture of GlcN oligomers using different quantities of acetic anhydride (Vander et al., 1998; dos Santos et al., 2008). Alternatively, crushed crab shell chitin can be powdered and passed through a 100-μm sieve to eliminate large particles before hydrolysis in 6 M HCl at 120 °C for 1 h (Nars et al., 2013). Longer oligomers can be prepared by treating the chitosan solution with commercially available Pectinex ultra SPL enzyme, followed by selective precipitation in 90% (v/v) methanol (Cabrera et al., 2006).

In the present study, a recombinant hypertransglycosylating chitinase D from *Serratia proteamaculans* (*Sp*ChiD) (Purushotham and Podile, 2012) and the W114A mutant lacking transglycosylase activity by with stronger hydrolyze activity (Madhuprakash et al., 2012) were used, for the first time, to generate oligomers from chitosans with DA values of 35% and 61%. A comparison of degradation products produced by the wild-type and mutant enzymes provided insight into the process of chitosan degradation and the activity of crude mixtures and oligomer fractions with DP values of 8–10 were tested in rice cell suspension cultures.

2. Methods

2.1. Bacterial strains, plasmids and biochemicals

The plasmid pET-22b (+) and *Escherichia coli* Rosetta-gami II (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. Construction of wild-type *Sp*ChiD and its mutant W114A were described earlier (Madhuprakash et al., 2012). Ampicillin, at a working concentration of 100 μg/mL, was added to the LB broth as required. Isopropyl-β-D-thiogalactoside, 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. CHOS with different DP were obtained from Seikagaku Corporation (Tokyo, Japan), through Cape Cod (East Falmouth, USA). Chitosans with DA35% and 61% were prepared as described previously by dos Santos et al. (2008).

2.2. Protein expression, isolation and purification

E. coli Rosetta-gami II (DE3) cells containing *Sp*ChiD and the mutant W114A were used for protein overexpression as previously described (Neeraja et al., 2010). Periplasmic fractions were prepared using the two-step osmotic shock procedure described in the pET expression system instructions (Novagen) with minor modifications. The supernatant was passed through a 0.2 μm sterile filter before purification. The periplasmic fraction was buffer exchanged against lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), which was also used as the equilibration buffer in further affinity purification steps and the elution of the bound recombinant protein was done as described earlier (Madhuprakash et al., 2015). The fractions were separated by 12% SDS–PAGE to check the purity of the protein and the activity was confirmed using zymogram analysis as described previously (Suma and Podile, 2013).

2.3. Preparation of elicitors

Chitosans with DA35% and 61% were hydrolyzed using *Sp*ChiD and its mutant W114A. To ensure that the hydrolysates contain substantial quantity of longer chain CHOS, time-dependent hydrolysis of chitosan polymers was performed. Hydrolysis was performed with 1 mg/mL of specific chitosan substrate prepared in 50 mM ammonium acetate pH 5.2, incubated with 5 μg of *Sp*ChiD or the mutant W114A at 40 °C. Fractions collected at regular intervals were analyzed using high pressure thin layer chromatography (HPTLC) as described below. To test the elicitor activity of specific CHOS, 100 mg of chitosan with DA61% was used as the substrate, followed by purification of CHOS using size exclusion chromatography (SEC) as described below. The SEC fractions were lyophilized overnight and dissolved in sterile MilliQ water to a final concentration of 1 mg/mL and used for MALDI-TOF-MS analysis and also to test elicitor activity.

2.4. High performance thin layer chromatography

Fractions collected at regular intervals were analyzed by high performance thin layer chromatography (HPTLC). The chitosan