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

ARTICLE *in* BIORESOURCE TECHNOLOGY · SEPTEMBER 2015

Impact Factor: 4.49 · DOI: 10.1016/j.biortech.2015.09.052

4 AUTHORS, INCLUDING:

Madhuprakash Jogi

Norwegian University of Life Sciences (NMBU)

8 PUBLICATIONS **25** CITATIONS

SEE PROFILE

Bioresource Technology 198 (2015) 503–509

Contents lists available at ScienceDirect

Bioresource Technology

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

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

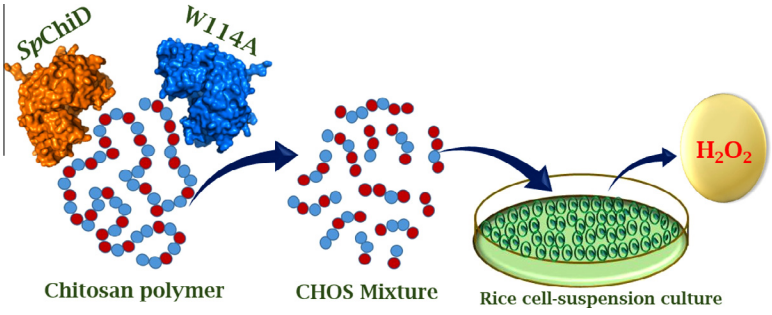
Jogi Madhuprakash [a], Nour Eddine El Gueddari [b], Bruno M. Moerschbacher [b], Appa Rao Podile [a,*]

[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



ARTICLE INFO

Article history:
Received 17 July 2015
Received in revised form 12 September 2015
Accepted 14 September 2015
Available online 25 September 2015

Keywords:
Chitinases
*Sp*ChiD
Hydrolysis
Transglycosylation
Chitosan oligosaccharides

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).

http://dx.doi.org/10.1016/j.biortech.2015.09.052
0960-8524/

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

hydrolysate generated by the wild-type or mutant enzyme was applied in 20-μL aliquots to HPTLC silica gel 60 F_{254} plates (Merck) using a Camag automatic TLC sampler 4 (Camag, Berlin, Germany). A solvent system comprising butanol/methanol/ammonia/water in the ratio 5:4:2:1 was used as the mobile phase. The solvent was evaporated and the products were visualized essentially as previously described by Purushotham et al. (2012), although the plate was sprayed with aniline diphenylamine reagent (400 μL aniline, 400 mg diphenylamine, 20 mL acetone, 3 mL 85% phosphoric acid) followed by baking for 8–10 min at 200 °C using a hot air gun (Black & Decker, Germany). As standard, 4 μg each of GlcN and GlcNAc monomers (Sigma Aldrich, München, Germany) and the oligomers $(GlcN)_{2-6}$ and $(GlcNAc)_{2-6}$ (Seikagaku Corporation, Tokyo, Japan) were prepared.

2.5. Purification of oligomers by size exclusion chromatography

Oligomers generated from chitosan with DA61% by *Sp*ChiD or its mutant W114A were separated as described previously (Hamer et al., 2015) using a SECcurity GPC System (PSS Polymer Standards Service, Mainz) with a refractive index detector (Agilent 1200 series RID®) and a set of three HiLoad™ 26/600 Superdex™ 30 preparatory grade columns equilibrated with filtered and degassed elution buffer (150 mM ammonium acetate, pH 4.5). Samples were passed through a 0.2-μm filter and loaded into a loop with a capacity of 5 mL. The elution flow rate was maintained at 0.6 mL/min for 22 h.

2.6. Analysis of CHOS by MALDI-TOF-MS

The SEC fractions described above were lyophilized overnight and dissolved in sterile MilliQ water to a final concentration of 1 mg/mL before MALDI-TOF-MS analysis as described by Purushotham and Podile (2012) with minor modifications. The crude chitosan hydrolysate or purified enzymatically-produced oligomers (1 μL of a 1 mg/mL solution) was mixed with 1 μL of matrix (10 mg/mL 2,5-dihydroxybenzoic acid in 1:1 water:acetonitrile) on a target plate. The samples were dried under a cold air stream and analyzed using an Autoflex Speed MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a SmartBeam™ NdYAG-laser (355 nm). The instrument was operated in positive acquisition mode and controlled using FlexControl v3.0. All spectra were obtained in reflectron mode with an acceleration voltage of 25 kV, a reflector voltage of 26 kV, and pulsed ion extraction of 40 ns in positive ion mode. The acquisition range was m/z 50–4000. The data were obtained by averaging 500 laser shots, with the lowest laser energy necessary to obtain sufficient signal-to-noise ratios. Peak lists were generated from the MS spectra using Bruker FlexAnalysis v3.0.

2.7. Maintenance of rice cell suspension cultures

Rice (*Oryza sativa* L.) cells were kindly provided by Dr. Burkhard Schmidt (RWTH Aachen University, Aachen, Germany). The cultures were grown in 50 mL Murashige & Skoog medium supplemented with 30 g/L sucrose and 1 mg/L 2,4-dichlorophenoxyacetic acid and were subcultured every 7 d in 15 mL medium (Ortmann et al., 2006). The cells were maintained at 26 °C in the dark with constant agitation at 120 rpm.

2.8. Oxidative burst measurements

The oxidative burst was characterized by measuring H_2O_2 levels using a modified luminol-dependent chemiluminescence method (Warm and Laties, 1982). The cells were gently separated from the medium through a sintered glass filter on the third day after subculturing. Aliquots of cells (50–60 mg) were then transferred to sterile 2-mL Eppendorf tubes containing 1 mL of fresh medium and were resuspended on a rotary shaker. The cells were pre-incubated at 26 °C in the dark with constant agitation at 120 rpm for 4 h, before adding oligomers derived from chitosan (DA = 61%). Rice cells in 20-μL aliquots were transferred to sterile microtiter plates at regular intervals and 50 μL of 0.1 mg/mL luminol was added to each well before measuring the chemiluminescence using a luminometer (Lumat LB 9501/16, Berthold, Germany). The chemiluminescence value was presented as relative light units, which are proportional to the amount of H_2O_2 released. The concentration of H_2O_2 was determined using a standard calibration curve.

2.9. Transglycosylase activity of SpChiD

The transglycosylase activity of *Sp*ChiD was analyzed with $(GlcNAc)_3$, $(GlcNAc)_4$ and the corresponding deacetylated oligomers in equimolar concentrations 2 mM $(GlcNAc)_3$ + 2 mM $(GlcN)_3$ or 2 mM $(GlcNAc)_4$ + 2 mM $(GlcN)_4$. All the reactions were carried out at 40 °C using 5 μg of the purified *Sp*ChiD in 50 mM sodium acetate buffer (pH 5.2). Fractions were collected at regular intervals and the reaction was stopped by adding an equal volume of 0.1 N NaOH. All the fractions were stored at −20 °C before 25-μL aliquots were analyzed by thin layer chromatography (TLC) as previously described (Purushotham et al., 2012).

3. Results and discussion

3.1. Generation and characterization of chitosan oligomers

The present study considered the ability of wild-type *Sp*ChiD and the mutant derivative W114A (lacking transglycosylase activity) to generate chitosan oligomers as elicitors of plant defense. This comparative analysis allowed the products of each enzyme to be determined, thus providing insight into the role of hydrolysis and transglycosylase activity in the generation of bioactive oligomers. Chitosan polymers with different DA values (35% and 61%) were hydrolyzed with each enzyme, and fractions collected at regular intervals were analyzed by HPTLC (Fig. 1). Time points for comparative analysis were chosen when substantial quantities of long-chain oligomers accumulated in each reaction. In the presence of the DA35% substrate, both enzymes generated oligomers with a DP $\geqslant$ 6 after 90 min. In the presence of the DA61% substrate, the wild-type enzyme took 90 min to generate substantial quantities of long-chain oligomers whereas the mutant took only 30 min. The crude hydrolysates representing each selected time point were lyophilized overnight and dissolved in sterile MilliQ water to a final concentration of 1 mg/mL. MALDI-TOF-MS analysis of the crude mixtures revealed minor differences in the oligomers produced by the wild-type and mutant enzymes (Figs. S1 and S2). One extra acetylated unit was detected in the oligomers produced by wild-type *Sp*ChiD presented with the chitosan DA61% substrate, whereas one extra deacetylated unit was detected in the oligomers produced by the mutant enzyme presented with the chitosan DA35% substrate (Table 1).

3.2. Elicitor activity of the crude hydrolysates

The rapid but transient release of H_2O_2 in an oxidative burst is a sensitive indicator for the induction of defense and common stress responses in plant cells and is thought to herald the hypersensitive reaction (Levine et al., 1994). The crude hydrolysates discussed above were tested for elicitor activity by measuring the oxidative burst response of rice cell suspension cultures. The cells were exposed to 50, 100 and 150 μg/mL of each crude hydrolysate.

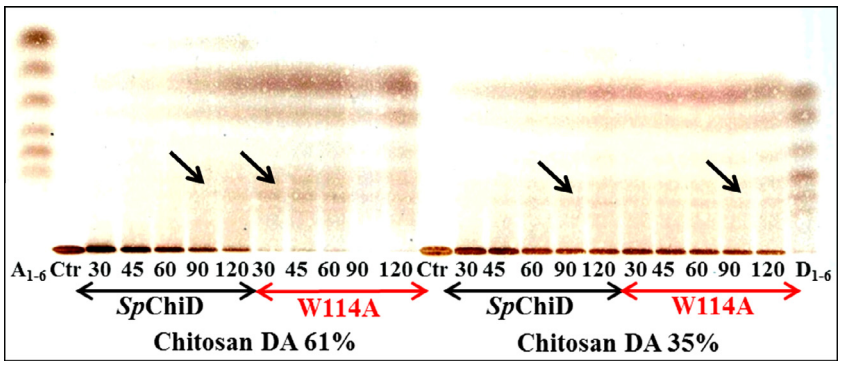


Fig. 1. HPTLC analysis of crude chitosan hydrolysates obtained using *Sp*ChiD and W114A. Fractions collected at different time intervals (arrows mark the selected sampling points) during the hydrolysis of chitosan (DA61% and DA35%) by *Sp*ChiD and W114A were analyzed by HPTLC. A_{1-6} was the standard mixture of $(GlcNAc)_1$–$(GlcNAc)_6$ and D_{1-6} represents the mixture of $(GlcN)_1$–$(GlcN)_6$. Ctr – indicates the substrate control without enzyme.

Table 1
MALDI-TOF-MS analysis of crude mixtures generated by *Sp*ChiD and W114A from chitosan oligomers with DA values of 61% and 35%. Unique products of each enzyme are shown in bold italics.

	*Sp*ChiD, substrate DA = 61%	W114A, substrate DA = 61%
DP2	AD, A2	AD, A2
DP3	AD2, A2D, ***A3***	AD2, A2D
DP4	A2D2, A3D	***AD3***, A2D2, A3D
DP5	A2D3, A3D2	A2D3, A3D2
DP6	A2D4, A3D3, A4D2	A2D4, A3D3, A4D2
DP7	A2D5, A3D4, A4D3, ***A5D2***	A2D5, A3D4, A4D3
DP8	A3D5, A4D4, ***A5D3***	***A2D6***, A3D5, A4D4
DP9	A4D5, ***A5D4***	***A3D6***, A4D5
DP10	A4D6, A5D5	A4D6, A5D5
DP11	A5D6	A5D6
DP12	***A2D10***	
	*Sp*ChiD, substrate DA = 35%	W114A, substrate DA = 35%
DP2	AD, A2	AD, A2
DP3	AD2, A2D	***D3***, AD2, A2D
DP4	D4, AD3, A2D2, A3D	D4, AD3, A2D2, A3D, ***A4***
DP5	A2D3, A3D2	***AD4***, A2D3, A3D2
DP6	A2D4, A3D3,	***D6***, ***AD5***, A2D4, A3D3
DP7	A2D5, A3D4	***AD6***, A2D5, A3D4
DP8	A2D6, A3D5	A2D6, A3D5
DP9	A2D7, A3D6	A2D7, A3D6
DP10		***A3D7***

The hydrolysates produced by the mutant enzyme were more potent than those produced by the wild-type enzyme despite the rather subtle differences in the DA of the components (Fig. 2). This may reflect the rapid hydrolytic activity of the mutant enzyme, which transformed much of the polymeric fraction into a highly active oligomeric fraction. The oxidative burst induced by the crude hydrolysate of the DA35% chitosan substrate produced a peak of H_2O_2 release 120–180 min after exposure and returned to the minimum level after 270 min (Fig. 2A). In contrast, the oxidative burst induced by the crude hydrolysate of the DA61% chitosan substrate produced a peak of H_2O_2 release 75–90 min (wild-type *Sp*ChiD) or 90 min (W114A) after exposure, and returned to the minimum level after 180 min (Fig. 2B).

Regardless of the enzyme used for degradation, the crude hydrolysate of the chitosan DA35% substrate showed elicitor activity only at doses of 100 and 150 μg/mL, whereas the crude hydrolysate of the DA61% chitosan substrate showed elicitor activity at all three different concentrations tested (Fig. 2). This indicates that the higher DA in the oligomeric fractions derived from chitosan DA61% may enhance the effect of the oligomers even at low concentrations. The rate of H_2O_2 production therefore depends on the substrate used to generate the oligomers as well as their concentration.

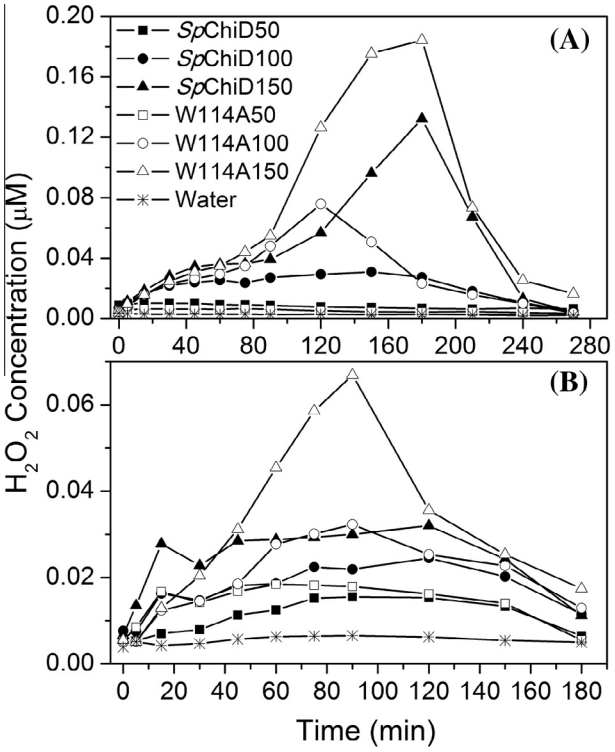


Fig. 2. Elicitor activity using crude chitosan hydrolysates generated by *Sp*ChiD and W114A. The dose-dependent elicitation of an oxidative burst was monitored in rice cell suspension cultures. Crude hydrolysates of chitosan DA35% (A) and DA61% (B) produced by *Sp*ChiD and W114A were used as elicitors at concentrations of 50, 100 and 150 μg/mL and sterilized MilliQ water was used as a negative control. Data given are from one representative of three independent experiments with similar results.

3.3. Elicitor activity of purified oligomers derived from chitosan DA61%

Chitosan substrates with high DA values may favor the transglycosylase activity of *Sp*ChiD and may generate oligomers with varying DA and PA values, which could in turn influence their elicitor activity. Therefore, 100 mg of chitosan DA61% was hydrolyzed with *Sp*ChiD or W114A, and the hydrolysates were fractionated by SEC. The size distribution of the oligomers in each hydrolysate and the corresponding HPTLC data are shown in Fig. 3. Fractions 12–17 from each experiment were investigated by MALDI-TOF-MS, revealing that each enzyme generated oligomers with the same range of DP values (Fig. S3). However, the oligomers generated by the wild-type enzyme carried an additional acetylated GlcN

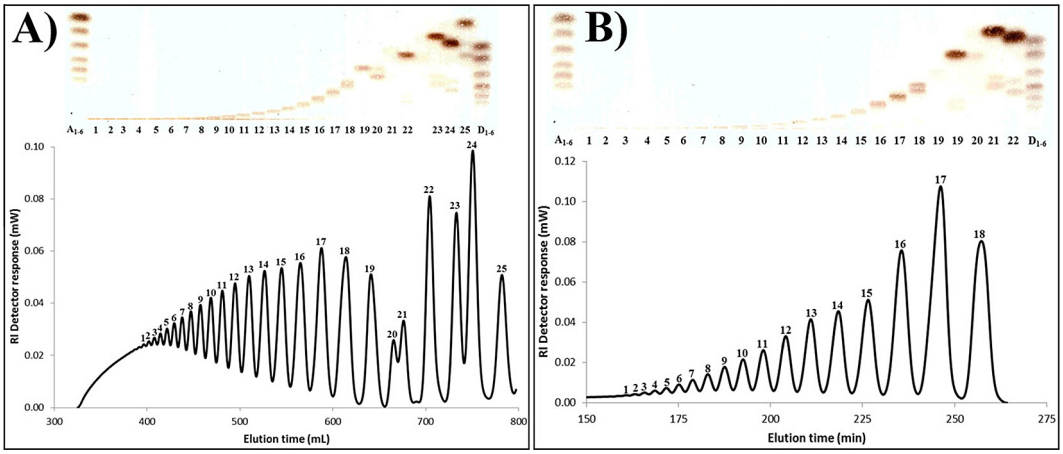


Fig. 3. Purification of chitosan oligomers derived from chitosan DA61% by *Sp*ChiD and W114A. Following the hydrolysis of 100 mg chitosan DA61% by each enzyme, the hydrolysates were purified by SEC. The lower panel represents the SEC purification profiles and includes the corresponding HPTLC images of oligomers in the upper panel generated by *Sp*ChiD (A) and W114A (B). A_{1-6} was the standard mixture of $(GlcNAc)_{1-6}$ and D_{1-6} represents the mixture of $(GlcN)_{1-6}$.

Table 2
MALDI-TOF-MS analysis of purified fractions generated by *Sp*ChiD and W114A from chitosan (DA = 61%). Unique products of each enzyme are shown in bold italics.

Fraction number	Oligomers obtained from chitosan DA = 61%		DP
	*Sp*ChiD	W114A	
12	A4D7, A5D6, A6D5, ***A7D4***	***A3D8***, A4D7, A5D6, A6D5	**11**
13	A4D6, A5D5, A6D4	***A3D7***, A4D6, A5D5, A6D4	**10**
14	A3D6, A4D5, A5D4, ***A6D3***	A3D6, A4D5, A5D4	**9**
15	A3D5, A4D4, A5D3, ***A6D2***	A3D5, A4D4, A5D3	**8**
16	A2D5, A3D4, A4D3, ***A5D2***	A2D5, A3D4, A4D3	**7**
17	A2D4, A3D3, A4D2, ***A5D***	A2D4, A3D3, A4D2	**6**

residue probably reflecting its transglycosylase activity (Table 2). Fractions with DP values of 8–10 produced by each enzyme were tested for their dose-dependent elicitor activity in rice cell suspension cultures (30, 50 and 70 μg/mL). The oxidative burst induced by DP8 and DP9 elicitors reached to peak at 60 and 15 min, after elicitation, respectively and then decreased to the initial stage by 120 min (Fig. 4A and B). The oxidative burst induced by DP10 elicitors was biphasic. An early response occurred between 5 and 15 min which was decreased and again reached to a maximum at 60 min (Fig. 4C).

There was no clear dose dependency, although the purified oligomeric fractions generated by wild-type *Sp*ChiD were more potent than those generated by the mutant enzyme (Fig. 4). Because both enzymes produced oligomers with equivalent DP values, the difference in potency could be caused by the DA or PA of the oligomers. Fully deacetylated oligomers retain their elicitor activity in *Arabidopsis thaliana* cell suspension cultures, and progressive re-acetylation impaired their ability to enhance H_2O_2 accumulation during both short and long exposures, e.g., even high concentrations of oligomers with a DA > 65% did not induce cells to accumulate H_2O_2 (Cabrera et al., 2006). A receptor for acetylated chitosan oligomers has been identified, but it is not clear how plants distinguish oligomers differing in DA or PA (Okada et al., 2002). The distinction may be non-specific, i.e., based on an interaction between a polycation and negatively-charged plasma membrane phospholipids that mimics and activates a common MAP kinase-dependent defense response (Shibuya and Minami, 2001). It is therefore difficult to determine whether the observed differences in elicitation potency reflect the transglycosylase activity of the wild-type enzyme and its influence on the DA and PA of the resulting oligomers.

3.4. $(GlcN)_3$ and $(GlcN)_4$ do not inhibit the activity of SpChiD

GH18-family chitinases use a substrate-assisted catalytic mechanism featuring a strong preference for an acetylated residue at the −1 subsite. If a partially deacetylated oligomer binds in such

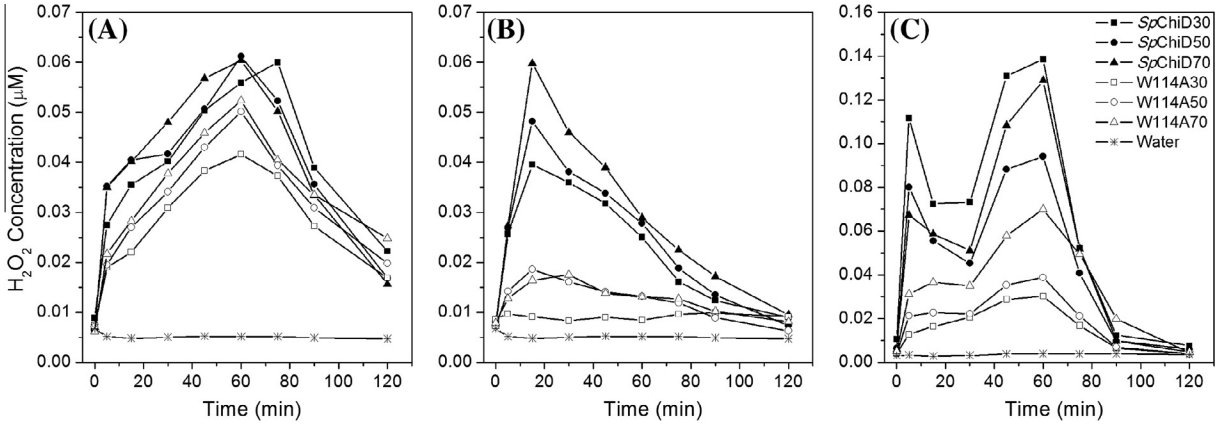


Fig. 4. Elicitor activity of purified chitosan oligomers (DP8–10) generated by *Sp*ChiD and W114A using chitosan DA61% as a substrate. The dose-dependent elicitation of the oxidative burst activity in rice cell suspension cultures was measured using 30, 50 and 70 μg of each of the purified oligomers with DP values of 8 (A), 9 (B) or 10 (C) and sterilized MilliQ water as a negative control. Data given are from one representative of three independent experiments with similar results.

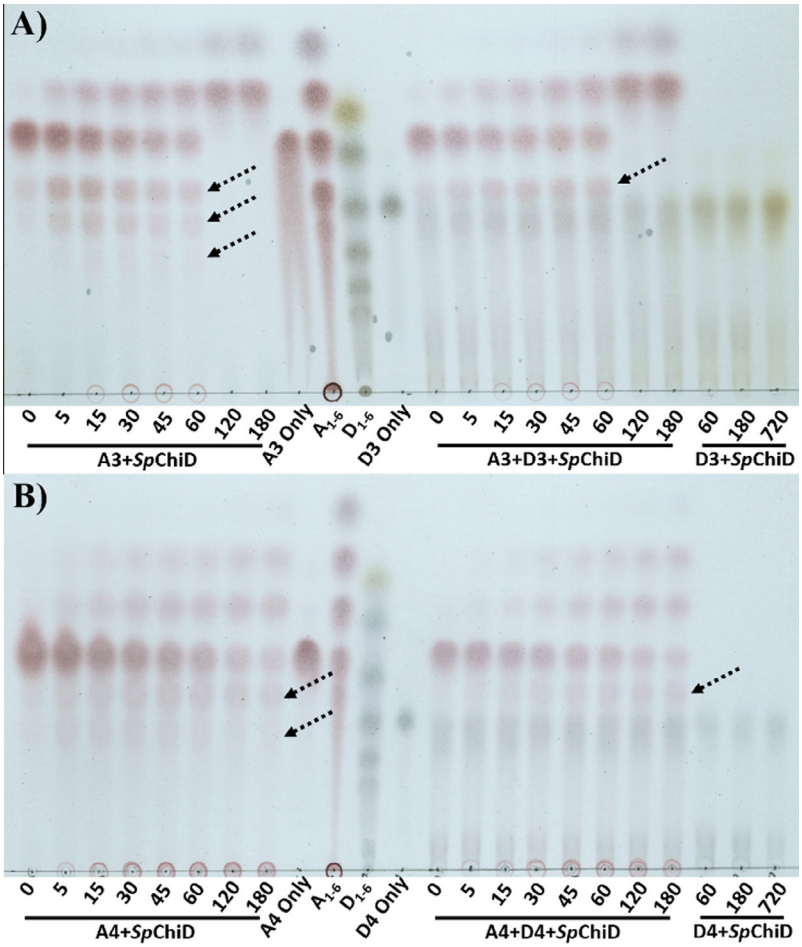


Fig. 5. TLC analysis of products generated by *Sp*ChiD using fully acetylated or deacetylated chitosan oligomer substrates. Each substrate was incubated with 5 μg purified *Sp*ChiD in 50 mM sodium acetate buffer (pH 5.2) at 40 °C. (A) and (B), represent the product profiles generated by *Sp*ChiD from oligomers with DP values of 3 and 4, respectively. TLC analysis was carried out using 25 μL of each fraction collected at different time points (indicated in min). A_{1-6} was the standard mixture of $(GlcNAc)_1$–$(GlcNAc)_6$ and D_{1-6} represents the mixture of $(GlcN)_1$–$(GlcN)_6$. The substrate control were A3 = $(GlcNAc)_3$, A4 = $(GlcNAc)_4$, D3 = $(GlcN)_3$ and D4 = $(GlcN)_4$. Dotted arrows indicate the formation of long-chain oligomers due to the transglycosylase activity of *Sp*ChiD.

a way that a deacetylated residue is loaded at the −1 subsite, catalysis is inhibited. The binding of deacetylated residues is non-productive but perhaps there is greater affinity compared to acetylated residues. Fully deacetylated oligomers with DP values of 2–7 thus served as efficient inhibitors of a chitinase from the insect *Ostrinia furnacalis*, human chitotriosidase, and two chitinases from *Serratia marcescens* (*Sm*ChiA and *Sm*ChiB), with IC_{50} values ranging from 10^1 to 10^3 μM (Chen et al., 2014). In contrast, the fully deacetylated oligomers $(GlcN)_3$ and $(GlcN)_4$ did not inhibit the hydrolysis and transglycosylase activities of *Sp*ChiD (Fig. 5A and B). This resistance to inhibition is unique to *Sp*ChiD within the GH18 family, supporting the hypothesis that the transglycosylase activity of *Sp*ChiD may promote the production of oligomers with high DA values, which act as strong elicitors. The biological activity of chitosan oligomers is thought to be dependent on PA as well as DP and DA, but this is difficult to demonstrate because there are few methods available for the control of PA in chitosan oligomers (dos Santos et al., 2008). Recently, this challenge has been addressed by the sequential use of two different chitin deacetylases, namely NodB from *Rhizobium* sp. GRH2 and chitin oligosaccharide deacetylase from *Vibrio cholerae*, which deacetylate the first and second units respectively, starting from the non-reducing end (Hamer et al., 2015). This method can control the PA but is inefficient because the enzymes can only deacetylate one residue at a time.

4. Conclusions

A chitinase with modest hydrolase activity combined with transglycosylase activity can generate long-chain chitosan oligomers from polymer substrates, whereas a chitinase with stronger hydrolase activity will yield more shorter-chain oligomers that are poor elicitors. A transglycosidase such as *Sp*ChiD, combined with chitin deacetylases, would therefore provide the means to create more diverse oligomers with defined DA and PA. In addition, engineering the subsite preferences of *Sp*ChiD may facilitate the production of chitosan oligomers with defined PA, which can be evaluated in detail for their biological activity.

Competing financial interests

The authors declare no competing financial interests.

Acknowledgements

We thank the Department of Science and Technology, Government of India, Funds for Infrastructure in Science and Technology, Level II support to the Department of Plant Sciences at the University of Hyderabad. We gratefully acknowledge support from the University Grants Commission (UGC) in the form of the Centre for Advanced Studies in Life Sciences. Thanks to the Department

of Biotechnology, Government of India, Centre for Research and Education in Biology and Biotechnology, and the School of Life Sciences. The authors also thank UGC-supported University with Potential for Excellence (Phase II) to the University of Hyderabad for the infrastructural support. This work was carried out in the context of an International Research and Training Group on "Molecular and Cellular Glycosciences" involving the University of Munster and the University of Hyderabad, supported by the Deutsche Forschungsgemeinschaft (DFG), Germany, and UGC, India. J.M. thanks the Council of Scientific and Industrial Research (CSIR), Government of India for a Senior Research Fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.09.052.

References

Aam, B.B., Heggset, E.B., Norberg, A.L., Sørlie, M., Vårum, K.M., Eijsink, V.G., 2010. Production of chitooligosaccharides and their potential applications in medicine. Mar. Drugs 8, 1482–1517.

Boller, T., Felix, G.A., 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu. Rev. Plant Biol. 60, 379–406.

Cabrera, J.C., Messiaen, J., Cambier, P., Van Cutsem, P., 2006. Size, acetylation, and concentration of chitooligosaccharide elicitors determine the switch from defence involving PAL activation to cell death and water peroxide production in *Arabidopsis* cell suspensions. Physiol. Plant 127, 44–56.

Chen, L., Zhou, Y., Qu, M., Zhao, Y., Yang, Q., 2014. Fully deacetylated chitooligosaccharides act as efficient glycoside hydrolase family 18 chitinase inhibitors. J. Biol. Chem. 289, 17932–17940.

Das, S.N., Madhuprakash, J., Sarma, P.V.S.R.N., Purushotham, P., Suma, K., Manjeet, K., Rambabu, S., El Gueddari, N.E., Moerschbacher, B.M., Podile, A.R., 2015. Biotechnological approaches for field applications of chitooligosaccharides (COS) to induce immunity in plants. Crit. Rev. Biotechnol. 35, 29–43.

dos Santos, A.L., El Gueddari, N.E., Trombotto, S., Moerschbacher, B.M., 2008. Partially acetylated chitosan oligo- and polymers induce an oxidative burst in suspension cultured cells of the gymnosperm *Araucaria angustifolia*. Biomacromolecules 9, 3411–3415.

Hamel, L.P., Beaudoin, N., 2010. Chitooligosaccharide sensing and downstream signaling: contrasted outcomes in pathogenic and beneficial plant–microbe interactions. Planta 232, 787–806.

Hamer, S.N., Cord-Landwehr, S., Biarnés, X., Planas, A., Waegeman, H., Moerschbacher, B.M., Kolkenbrock, S., 2015. Enzymatic production of defined chitosan oligomers with a specific pattern of acetylation using a combination of chitin oligosaccharide deacetylases. Sci. Rep. 5, 8716.

Kafetzopoulos, D., Martiniou, A., Bouriotis, V., 1993. Bioconversion of chitin to chitosan: purification and characterization of chitin deacetylase from *Mucor rouxii*. Proc. Natl. Acad. Sci. U.S.A. 90, 2564–2568.

Koping-Hoggard, M., Tubulekas, I., Guan, H., Edwards, K., Nilsson, M., Vårum, K.M., Artursson, P., 2001. Chitosan as a nonviral gene delivery system: structure–property relationships and characteristics compared with polyethylenimine *in vitro* and after lung administration in vivo. Gene Ther. 8, 1108–1121.

Levine, A., Tenhaken, R., Dixon, R., Lamb, C., 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79, 583–593.

Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., Fan, F., Wang, J., Jin, C., Chang, J., Zhou, J. M., Chai, J., 2012. Chitin-induced dimerization activates a plant immune receptor. Science 336, 1160–1164.

Madhuprakash, J., Tanneeru, K., Purushotham, P., Guru Prasad, L., Podile, A.R., 2012. Transglycosylation by chitinase D from *Serratia proteamaculans* improved through altered substrate interactions. J. Biol. Chem. 287, 44619–44627.

Madhuprakash, J., El Gueddari, N.E., Moerschbacher, B.M., Podile, A.R., 2015. Catalytic efficiency of chitinase-D on insoluble chitinous substrates was improved by fusing auxiliary domains. PLoS One 10, e0116823.

Nars, A., Rey, T., Lafitte, C., Vergnes, S., Amatya, S., Jacquet, C., Dumas, B., Thibaudeau, C., Heux, L., Bottin, A., Fliegmann, J., 2013. An experimental system to study responses of *Medicago truncatula* roots to chitin oligomers of high degree of polymerization and other microbial elicitors. Plant Cell Rep. 32, 489–502.

Neeraja, C., Moerschbacher, B.M., Podile, A.R., 2010. Fusion of cellulose binding domain to the catalytic domain improves the activity and conformational stability of chitinase in *Bacillus licheniformis* DSM13. Bioresour. Technol. 101, 3635–3641.

Okada, M., Matsumura, M., Ito, Y., Shibuya, N., 2002. High-affinity binding proteins for *N*-acetyl chitooligosaccharide elicitor in the plasma membranes from wheat, barley and carrot cells: conserved presence and correlation with the responsiveness to the elicitor. Plant Cell Physiol. 43, 505–512.

Ortmann, I., Conrath, U., Moerschbacher, B.M., 2006. Exopolysaccharides of *Pantoea agglomerans* have different priming and eliciting activities in suspension-cultured cells of monocots and dicots. FEBS Lett. 580, 4491–4494.

Purushotham, P., Podile, A.R., 2012. Synthesis of long-chain chitooligosaccharides by a hypertransglycosylating processive endochitinase of *Serratia proteamaculans* 568. J. Bacteriol. 194, 4260–4271.

Purushotham, P., Sarma, P.V.S.R.N., Podile, A.R., 2012. Multiple chitinases of an endophytic *Serratia proteamaculans* 568 generate chitin oligomers. Bioresour. Technol. 112, 261–269.

Schipper, N.G.M., Vårum, K.M., Artursson, P., 1996. Chitosans as absorption enhancers for poorly absorbable drugs. 1. Influence of molecular weight and degree of acetylation on drug transport across human intestinal epithelial (Caco-2) cells. Pharmaceut. Res. 13, 1686–1692.

Shibuya, N., Minami, E., 2001. Oligosaccharide signalling for defence responses in plant. Physiol. Mol. Plant Pathol. 59, 223–233.

Suma, K., Podile, A.R., 2013. Chitinase A from *Stenotrophomonas maltophilia* shows transglycosylation and antifungal activities. Bioresour. Technol. 133, 213–220.

Vander, P., Vårum, K.M., Domard, A., Eddine El Gueddari, N., Moerschbacher, B.M., 1998. Comparison of the ability of partially *N*-acetylated chitosans and chitooligosaccharides to elicit resistance reactions in wheat leaves. Plant Physiol. 118, 1353–1359.

Vårum, K.M., Ottøy, M.H., Smidsrød, O., 1994. Water solubility of partially N-acetylated chitosans as a function of pH – effect of chemical composition and depolymerization. Carbohydr. Polym. 25, 65–70.

Warm, E., Laties, G.G., 1982. Quantification of hydrogen peroxide in plant extracts by the chemiluminescence reaction with luminol. Phytochemistry 21, 827–831.

Willmann, R., Nurnberger, T., 2012. How plant lysin motif receptors get activated: lessons learned from structural biology. Sci. Signal. 5, 28.

Zakariassen, H., Hansen, M.C., Jøranli, M., Eijsink, V.G.H., Sørlie, M., 2011. Mutational effects on transglycosylating activity of family 18 chitinases and construction of a hypertransglycosylating mutant. Biochemistry 50, 5693–5703.