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Production and purification of a fungal chitosanase and chitooligomers from *Penicillium janthinellum* D4 and discovery of the enzyme activators

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ABSTRACT

Chitosanases have received much attention because of their wide range of applications. Although most fungal chitosanases use sugar as their major carbon source, in the present work, a chitosanase was induced from a squid pen powder (SPP)-containing *Penicillium janthinellum* D4 medium and purified by ammonium sulphate precipitation and combined column chromatography. The purified D4 chitosanase exhibited optimum activity at pH 7–9, 60 °C and was stable at pH 7–11, 25–50 °C. The D4 chitosanase that was used for chitooligomers preparation was studied. The enzyme products revealed various chitooligomers with different degrees of polymerisation (DP) from 3 to 9, as determined by a MALDI-TOF mass spectrometer, confirming the endo-type nature of the D4 chitosanase. D4 chitosanase activity was significantly inhibited by Cu^{2+}, Mn^{2+}, and EDTA. However, Fe^{2+} activated or inhibited D4 chitosanases at different concentrations. The D4 chitosanase was also activated by some small synthetic boron-containing molecules with boronate ester side chains.

1. Introduction

Chitosanases have been found in abundance in a variety of bacteria, fungi, cyanobacteria, and plants, and they are capable of catalysing the hydrolysis of chitosan into smaller chitooligomers (Muzzarelli et al., 2012; Thadathil & Velappan, 2013). The use of colloidal chitosan or chitosan as a major carbon source in chitosanase production from microorganisms is a common strategy, and in the majority of cases, its inductor effect has been established (Gao, Ju, Jung, & Park, 2008; Kurakake, Yo-u, Nakagawa, Sugihara, & Komaki, 2000). Currently, chitosan is produced commercially from shellfish waste by demineralisation, deproteinisation, and then *N*-deacetylation to different degrees using strong acids or bases (Liang, Chen, Yen, & Wang, 2007). Hence, direct utilisation of shellfish waste as the sole carbon/nitrogen source not only solves environmental problems but also decreases microbial chitosanase production cost (Wang, Liang, & Yen, 2011).

Recent studies on chitosan have attracted interest for oligosaccharide conversion because oligosaccharides are not only water-soluble but also possess versatile functional properties such as antitumor and antimicrobial activities (Busilacchi, Gigante, Mattioli-Belmonte, Manzotti, & Muzzarelli, 2013; Liang et al., 2007; Wang, Lin, Yen, Liao, & Chen, 2006; Wang et al., 2008a). Traditionally, chitooligomers were processed in industry using chemical methods. The chemical hydrolysis method for preparing chitooligomers is performed at high temperatures and under acidic conditions. Many problems exist in these chemical processes such as producing large amounts of monosaccharides, low oligosaccharide yields, the high cost of separation, and environmental pollution. Alternatively, with its advantages in environmental compatibility, low cost, reproducibility, and high oligosaccharide yields, enzyme hydrolysis has become more popular in recent years (Wang et al., 2008a). Among the microbial chitosanases, bacterial chitosanases have been studied extensively, especially from *Bacillus* spp. and *Streptomyces* spp. in terms of their catalytic features, enzymatic mechanisms, and protein structures (Adachi et al., 2004; Boucher et al., 1995; Fukamizo et al., 2005; Katsumi, Lacombe-Harvey, Tremblay, Brzezinski, & Fukamizo, 2005; Marcotte, Monzingo, Ernst, Brzezinski, & Robertas,

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1996). However, compared with bacterial chitosanases, fungal chitosanases have been poorly studied. Therefore, chitosanase characterisation from novel chitosanase-producing fungi is of value in understanding their catalytic mechanisms.

Penicillium janthinellum D4 is a novel chitosanase-producing fungus that is isolated from soil. In this study, the production, purification, and characterisation of SPP-induced D4 chitosanase are reported. The endo-type D4 chitosanase is potentially valuable for industrial applications that produce functional chitooligomers. We further discovered some small, synthetic boron-containing molecules that activated the D4 chitosanase, suggesting that they could be used as new activators in industrial processes for preparing chitooligomers by the enzymatic hydrolysis method.

2. Materials and methods

2.1. Materials

The squid pen powder (SPP) used in these experiments was prepared as described previously (Wang et al., 2008a). The squid pens were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). During the SPP preparation, the squid pens were washed thoroughly with tap water and then dried. The resulting dried materials were milled to powders for use as the carbon source for chitosanase production. DEAE-Sepharose CL-6B and Sephacryl S-100 were purchased from GE healthcare UK Ltd. (Little Chalfont, Buckinghamshire, England). The weak base anion exchange Macro-prep DEAE was obtained from Bio-Rad (Hercules, CA, USA). The test enzyme activators (compounds 001, 004, 016, 046, 037, 040, 039, and 045) were kindly supplied by Dr. P.S. Pan, Department of Chemistry, Tamkang University, New Taipei City, Taiwan. All of the other reagents used were of the highest grade available.

2.2. Sampling, isolation, and screening fungal strains with high chitosanase activities

Fungal strains were isolated from the soil in the Dak Lak province, Central Highland, Vietnam. One gram of soil was ground in a porcelain mortar, 10 mL sterile distilled water was then added, and the soil suspension was stirred. Chitosan-supplemented Czapek media was inoculated with 0.5 mL soil suspension and incubated at 28 °C for 3–4 days. Single fungal strain colonies that appeared were subcultured on PDA media.

Screening for fungal strains with high chitosanase activity was conducted in cotton-plugged 250-mL Erlenmeyer flasks containing 30 mL chitosan-supplemented Czapek media 0.25% (w/v). The medium was inoculated with 1 mL solution containing 5×10^7 spores and incubated at 28 °C for 14 days. The selected strains were based on the culture supernatant chitosanase activity. The best strains were selected for further process parameter optimisation. The fungal strains with high chitosanase activity were identified by morphology and by molecular biology based on 18S sequencing.

2.3. Optimal chitosanase production

Different incubation periods (3–15 days) were employed to study chitosanase production. Chitosan was replaced with various fishery wastes as carbon/nitrogen sources, and Czapek media was replaced with liquid media containing 0.1% (w/v) K_2HPO_4 and 0.05% (w/v) $MgSO_4{\cdot}7H_2O$ (pH 7) to assess optimal chitosanase production from *P. janthinellum* D4. The optimal culturing temperature was investigated (25–37 °C). Similarly, the optimum pH was determined to be in the range from 4 to 11. After incubation with the optimal process, the culture broth was centrifuged (4 °C at 12,000 × *g* for 20 min), and the supernatant was used for further purification via chromatography.

2.4. Chitosanase activity measurement

Enzyme chitosanase activity was measured by incubating 0.2 mL enzyme solution with 1 mL 0.3% (w/v) water-soluble chitosan (Kiotec Co., Hsinchu, Taiwan; with 60% deacetylation) in 50 mM phosphate buffer, pH 7, at 37 °C for 30 min. The reaction was stopped by heating the reaction mixture to 100 °C for 15 min. The amount of reducing sugar produced was measured using the method of Imoto and Yagishita (1971) with glucosamine as a reference compound; one unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol reducing sugars per min. The specific activity was expressed as units per mg protein (U/mg protein) of the enzyme extract.

2.5. Chitosanase purification

2.5.1. DEAE-Sepharose CL-6B chromatography

Ammonium sulphate (608 g/L) was added to the culture supernatant (660 mL). The resulting mixture was stored at 4 °C overnight, and the precipitate that formed was collected by centrifugation at 4 °C for 20 min at 12,000 × *g*. The precipitate was then dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7) and dialysed against the buffer. The resulting dialysate (20 mL) was loaded onto a DEAE-Sepharose CL-6B column (5 cm × 30 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7). The column was washed with the same buffer and eluted with a linear gradient of 0–1 M NaCl in the same buffer. The fractions containing the chitosanase activity peaks were independently pooled and concentrated using ammonium sulphate precipitation. The pooled enzyme solution fractions were purified further.

2.5.2. Macro-prep DEAE chromatography

The obtained enzyme solution (the adsorbed chitosanase fractions from the DEAE-Sepharose CL-6B column) was then chromatographed on a Macro-prep DEAE column (12.6 mm × 40 mm) that had been equilibrated with 50 mM sodium phosphate buffer (pH 7). The chitosanase was eluted using a linear 0–1 M NaCl gradient in the same buffer. The fractions containing chitosanase activity were pooled and concentrated using ammonium sulphate precipitation. The pooled enzyme solution fractions were used as a purified preparation.

2.6. Protein determination

Protein content was determined using the Bradford method with a Bio-Rad dye reagent concentrate and bovine serum albumin as the standard. After column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm (Wang et al., 2006).

2.7. Molecular mass determination

The purified chitosanase molecular mass was determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) with 12.5% acrylamide and 2.67% methylene bis acrylamide in a 0.375 M Tris–HCl buffer (pH 8.8) with 0.1% (w/v) SDS. Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7) containing β-mercaptoethanol. The electrode buffer was 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS (pH 8.3). Electrophoresis was performed at a constant current of 70 mA through the stacking gel and 110 mA through the resolving gel. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 in a

methanol–acetic acid–water (5:1:5, v/v) solution and decoloured in 7% acetic acid. The molecular mass of the purified chitosanase in its native form was determined using a gel filtration method. The sample and standard proteins were applied to a Sephacryl S-100 column (2.5 cm × 100 cm) that had been equilibrated with 50 mM phosphate buffer (pH 7). Bovine serum albumin (molecular mass: 67 kDa), *Bacillus* sp. α-amylase (50 kDa), and hen egg white lysozyme (14 kDa) were used as molecular mass markers (Wang et al., 2006).

2.8. Effect of pH and temperature on chitosanase activities

The optimum pH for D4 chitosanase activity was studied by assaying the samples at different pH values. D4 chitosanase stability was determined by measuring the residual activity at pH 7 as described above after the sample had been dialysed against a 50 mM buffer solution of various pH values (pH 4–11) in seamless cellulose tubing (Sankyo). The buffer systems used were acetate (50 mM, pH 4–5), phosphate (50 mM, pH 6–8), and Na_2CO_3-$NaHCO_3$ (50 mM, pH 9–11). To determine the optimum temperatures for D4 chitosanase activity, sample activity values were measured at various temperatures (25–90 °C). The thermal stability of D4 chitosanase was studied by incubating the samples at various temperatures for 60 min. The residual activity was measured as described above.

2.9. Effect of various chemicals on enzyme activities

The effects of metal ions (5 mM) were investigated using Mg^{2+}, Cu^{2+}, Fe^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, and Ba^{2+}. The effects of enzyme inhibitors were studied using phenylmethylsulfonyl fluoride (PMSF) and ethylenediamine tetraacetic acid (EDTA). The enzyme was pre-incubated with various chemicals for 30 min at 25 °C, and residual chitosanase activities were then assessed.

2.10. Assay system for chitosanase activation

D4 chitosanase activation was measured in response to different small synthetic boron-containing molecules. The enzyme solution was added to the activator solution, and this mixture was incubated at 37 °C for 5 min. The enzymatic reaction was initiated by adding the substrate. Activation of chitosanase activity was calculated as a percentage by the following equation:

$$\text{Activation ratio}(\%) = \frac{S}{C} \times 100,$$

where C is the enzyme activity of the control group and S is the enzyme activity of the experimental group.

3. Results and discussion

3.1. Isolation and identification of a chitosanase-producing strain

From the soil samples, 34 fungal strains with chitosanase activity were isolated, and five of these strains had high chitosanase activity (data not shown). The D4 strain produced the highest chitosanase activity. The potent D4 strain was selected to optimise processes and nutritional parameters.

Based on morphology, the D4 strain was preliminarily identified, and the result demonstrated that D4 belonged to the *Penicillium* species. Strain D4 was further identified as *P. janthinellum* by sequencing and BLASTing the 18S rRNA gene.

Table 1
Purification of the chitosanase from *P. janthinellum* D4[a]

Step	Total protein (mg)	Total activity (U)	Specific activity (mU/mg)	Purification fold	Yield (%)
Culture supernatant	2449.2	5.3	2.2	1	100
$(NH4)_2SO_4$ ppt	128.2	3.7	28.9	13.1	70
DEAE-Sepharose	40.7	1.5	36.9	16.8	28
Macro-prep DEAE	7.5	0.4	53.3	24.2	8

[a] *P. janthinellum* D4 was grown in 100 mL liquid medium in an Erlenmeyer flask (250 mL) containing 0.5% SPP, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ in a shaking incubator for 6 days at 30 °C.

3.2. D4 chitosanase production and purification

Chitosanase production by the D4 strain was investigated during 9 days of cultivation in the production media. Various fishery wastes as carbon/nitrogen sources were tested, and SPP gave the best chitosanase activity. Basal medium (100 mL, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$, pH 5) containing 0.5% SPP was the most suitable for chitosanase production by strain D4 at 30 °C for 6 days. Most of the other fungal chitosanases were produced using sugar as the major carbon source, for instance, two types of chitosanase (ChiA and ChiB) from *Aspergillus* sp. CJ22-326 (Chen, Xia, & Yu, 2005), a chitosanase from *Aspergillus* sp. Y2K (Cheng & Li, 2000), *Trichoderma koningii* (da Silva, Honorato, Franco, & Rodrigues, 2012), *P. islandicum* (Fenton & Eveleigh, 1981), *T. reesei* PC-3-7 (Nogawa et al., 1998), *Gongronella* sp. JG (Wang, Zhou, Yuan, & Wang, 2008b; Zhou, Yuan, Wang, & Yao, 2008), and *Aspergillus* sp. QD-2 (Zhang, Sang, & Zhang, 2012). *P. janthinellum* D4 produced chitosanase in the medium containing squid pen as the sole carbon/nitrogen source, but not in the medium containing sugar, which was different than the other reported chitosanase-producing fungi.

The D4 chitosanase was eluted in the DEAE-Sepharose CL-6B chromatography step with a linear gradient of 0–1 M NaCl in the same buffer. The eluted peak fractions containing the higher chitosanase activity were pooled for further purification. After the Macro-prep DEAE chromatography step (data not shown), approximately 7.5 mg D4 chitosanase was obtained (Table 1). A summary of the purification is presented in Table 1. The purification steps were combined to give an overall approximately 24.2-fold purification of D4 chitosanase. The overall D4 chitosanase activity yield was 8% with 53.3 mU/mg specific chitosanase activity. The purified chitosanase molecular mass was approximately 49 kDa as confirmed by SDS-PAGE (data not shown). The D4 chitosanase molecular mass (49 kDa) was markedly different from the other fungal chitosanase masses such as *Gongronella* JG (90 kDa and 28 kDa) (Wang et al., 2008b; Zhou et al., 2008), *A. fumigatus* Y2K (25 kDa) (Cheng & Li, 2000), *A. oryzae* IAM 2660 (40 kDa and 135 kDa) (Zhang et al., 2000), *Aspergillus* sp. CJ22-326 (29 kDa and 109 kDa) (Chen et al., 2005), *T. reesei* PC-3-7 (93 kDa) (Nogawa et al., 1998), *Mucor rouxii* (76 kDa and 58 kDa) (Alfonos, Martines, & Reyes, 1992), and *P. islandicum* (30 kDa) (Fenton & Eveleigh, 1981).

3.3. Effects of pH and temperature

The D4 chitosanase pH activity profiles revealed maximum activity at pH 7–9 (Fig. 1a). The optimum pH (pH 7–9) for D4 chitosanase activity was higher than that of most fungal chitosanases, which display optimum activities at acidic pH values in a range from 4.0 to 6.5. Furthermore, for most chitosanases, a sharp drop in activity was observed at pH values higher than 6.5 (Alfonos et al., 1992; Chen et al., 2005; Cheng & Li, 2000; Fenton & Eveleigh, 1981; Nogawa et al., 1998; Wang et al., 2008b; Zhang et al., 2000; Zhou et al., 2008), but D4 chitosanase was markedly different from most fungal chitosanases, with maximum activity at pH 7–9. The D4

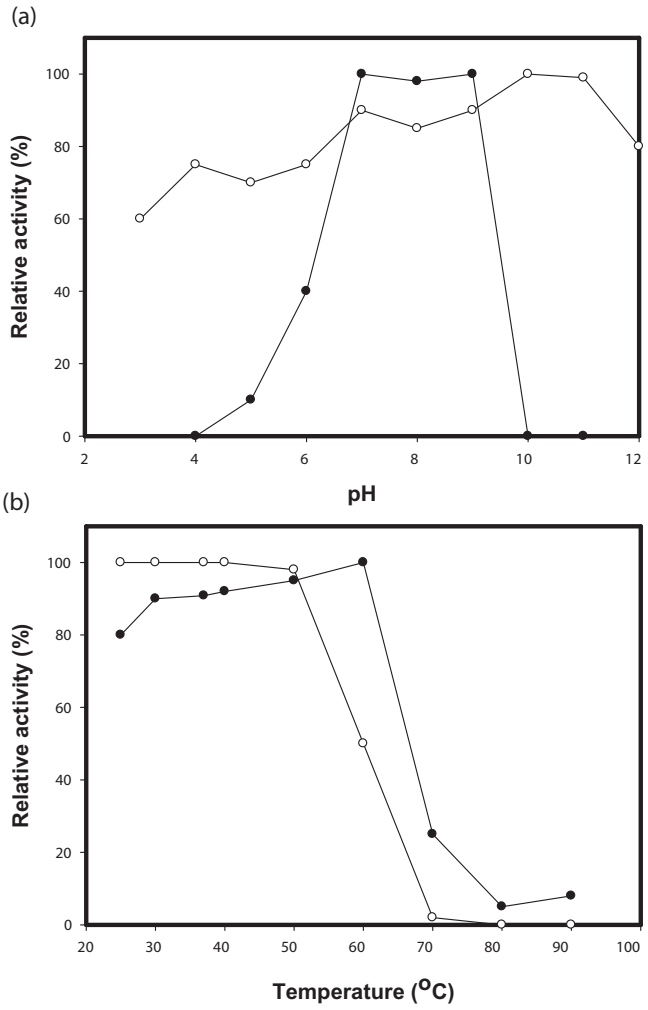


Fig. 1. Effect of pH (a) and temperature (b) on D4 chitosanase activity (●) and stability (○).

chitosanase pH stability profiles were determined by residual activity measurement at pH 7 after incubation at various pH values at 37 °C for 60 min. The D4 chitosanases were relatively stable at pH 7–11 and retained more than 85% of the initial activity (Fig. 1a). The D4 chitosanase became more sensitive to pH changes below pH 7 and above pH 11, especially acidic pH (pH 3–6) (Fig. 1a). The decreased activity at lower pH ranges may be because of protein instability rather than an acid-base catalytic mechanism, as reported in previous results (Jiang, Chen, Chen, Yang, & Zou, 2012; Katsumi et al., 2005). Previous investigations revealed pH stabilities of between 4 and 7.5 for fungal chitosanases from *Aspergillus* sp. Y2 K (Cheng & Li, 2000) and between 3.5 and 7.5 from *Aspergillus* sp. CJ22-326 (Chen et al., 2005), which fell between neutral and acidic. However, bacterial chitosanases exhibited broader pH stability such as between 3 and 10 for *Bacillus* sp. (Sakihama et al., 2004), between 5 and 8 for *Pseudomonas* sp. A-0 (Ando et al., 2008), and between 3 and 8 for *Streptomyces* sp. N17 (Lin Teng Shee, Arul, Brunet, & Bazinet, 2008). The chitosanase stability pH range differences might be explained by different chitosanase amino acid sequences in addition to the nature of the source (Gupta, Prasanna, Srivastava, & Sharma, 2012). In the present study, the D4 chitosanase exhibited pH stability and maximum activity in basic conditions. To our knowledge, this novel property of D4 chitosanase as an alkaline chitosanase has not been previously reported.

The effect of temperature on D4 chitosanase activity was also studied. The optimum temperature for D4 chitosanase activity was 60 °C (Fig. 1b). To examine the thermal stability of D4 chitosanase, the enzyme solution was incubated for 60 min at various temperatures in 50 mM phosphate buffer (pH 7), and the residual activity was then measured. The D4 chitosanase maintained its initial activity from 25 to 50 °C and had only 50% of its activity at 60 °C, but it was nearly completely inactivated at 70 °C (Fig. 1b).

Table 2
Effects of various chemicals on D4 chitosanase[a] activities.

Chemicals	Concentration (mM)	Relative activity (%)
None	0	100
Fe^{2+}	1	156
	2	123
	3	72
	4	13
	5	0
Cu^{2+}	1	16
	2	21
	3	18
	4	34
	5	5
Mn^{2+}	1	72
	2	65
	3	39
	4	35
	5	0
Mg^{2+}	5	76
Ca^{2+}	5	61
Zn^{2+}	5	68
Ba^{2+}	5	89
PMSF	5	100
EDTA	5	52

[a] Purified D4 chitosanase was preincubated with various reagents at 25 °C for 30 min, and residual chitosanase activities were determined as described in the text. A value of 100% was assigned to the enzymatic activity in the absence of a putative inhibitor.

3.4. Effects of various inhibitors and metal ions

To further characterise D4 chitosanase, we next examined the effects of some known enzyme inhibitors and divalent metals on its activity. The results are summarised in Table 2. D4 chitosanase activity that was measured in response to different metal ions demonstrated that preincubating the enzyme with 5 mM Fe^{2+}, Cu^{2+}, or Mn^{2+} resulted in 100, 95, and 100% inhibition, respectively (Table 2). In previous reports, Cu^{2+} and Mn^{2+} could activate or inhibit different chitosanases (Alfonos et al., 1992; Chen et al., 2005; Cheng & Li, 2000; Fenton & Eveleigh, 1981; Nogawa et al., 1998; Wang et al., 2008b; Zhang et al., 2000; Zhou et al., 2008); however, Cu^{2+} and Mn^{2+} both inhibited D4 chitosanase at 1–5 mM. Concentrations of 1–2 mM Fe^{2+} stimulated, while 3–5 mM Fe^{2+} negatively affected D4 chitosanase activity. The D4 chitosanase activity was markedly different from other fungal chitosanases; therefore, we hypothesised that different metal ions and metal ions at different concentrations might affect D4 chitosanase activity by influencing protein structure. D4 chitosanase activity was also lost after addition of EDTA. However, D4 chitosanase activity was not affected by phenylmethanesulphonyl fluoride (PMSF). Thus, PMSF could be used to control the crude extract serine protease activity and prevent exhaustive protein degradation during downstream processes.

3.5. Chitosan oligosaccharide preparation and product analysis

The chitosan oligosaccharides were produced from 60% DD chitosan with D4 chitosanase. The course of chitosan sample degradation was conveniently studied by measurement of total and reducing sugar. The results revealed the total sugar and reducing sugar of the sample as a function of reaction time. The chitosan

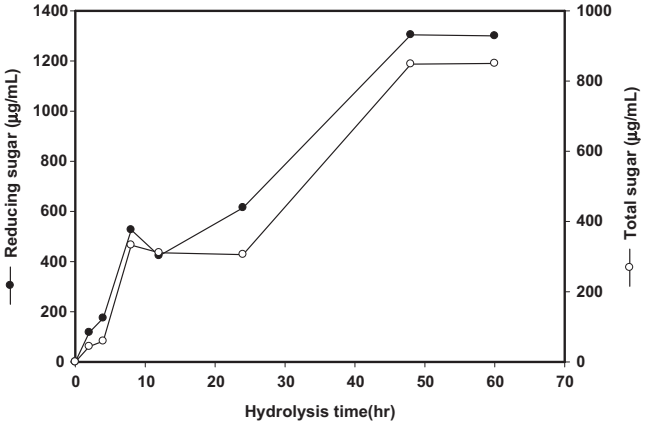


Fig. 2. Hydrolysis time course measurement of reducing sugar and total sugar with D4 chitosanase from *P. janthinellum* D4.

sample total and reducing sugar values produced a similar pattern (Fig. 2). The total and reducing sugar increased, and the chitosan sample recovery decreased dramatically in the early reaction stage, which can be attributed to an endo-type degradation process. The D4 chitosanase was added into a reaction solution after 48 h, but it did not improve the increase in total and reducing sugar levels (Fig. 2). Selective precipitation in 90% methanol and acetone solutions was performed to obtain low degrees of polymerisation (DP) oligomers as described earlier (Liang et al., 2007). Using MALDI-TOF analysis of the 90% acetone solution precipitation, in a mass spectrum, the *x* axis represents *m*/*z* – that is mass divided by charge and the *y* axis represents absolute intensity – that is the number of ions of each species that reach the detector. It appeared that the chitosan oligosaccharides had DP up to 9 (Fig. 3). The higher DP chitooligomers were precipitated as a light yellow powder in the methanol solution. The low DP oligomer fraction MALDI-TOF MS revealed pronounced differences among crude enzyme-generated chitooligomers, as demonstrated for chitosan depolymerisation in Fig. 3. The hydrolysate ions present in the mass spectra were identified as sodium adducts $[M+Na]^+$. MALDI-TOF analysis is limited to molecular weights higher than 500 Da because of matrix signal interference; therefore, DP < 2 oligomers could not be determined by this method. More information about the assigned structure of each signal at different hydrolysis times is given in Table 3. The hydrolysates contained chitooligomers (GlcN-oligomers) and several partial *N*-acetylated forms (Table 3). The D4 chitosanase reaction product is a mixture of DP 3-9 hetero-chitooligomers

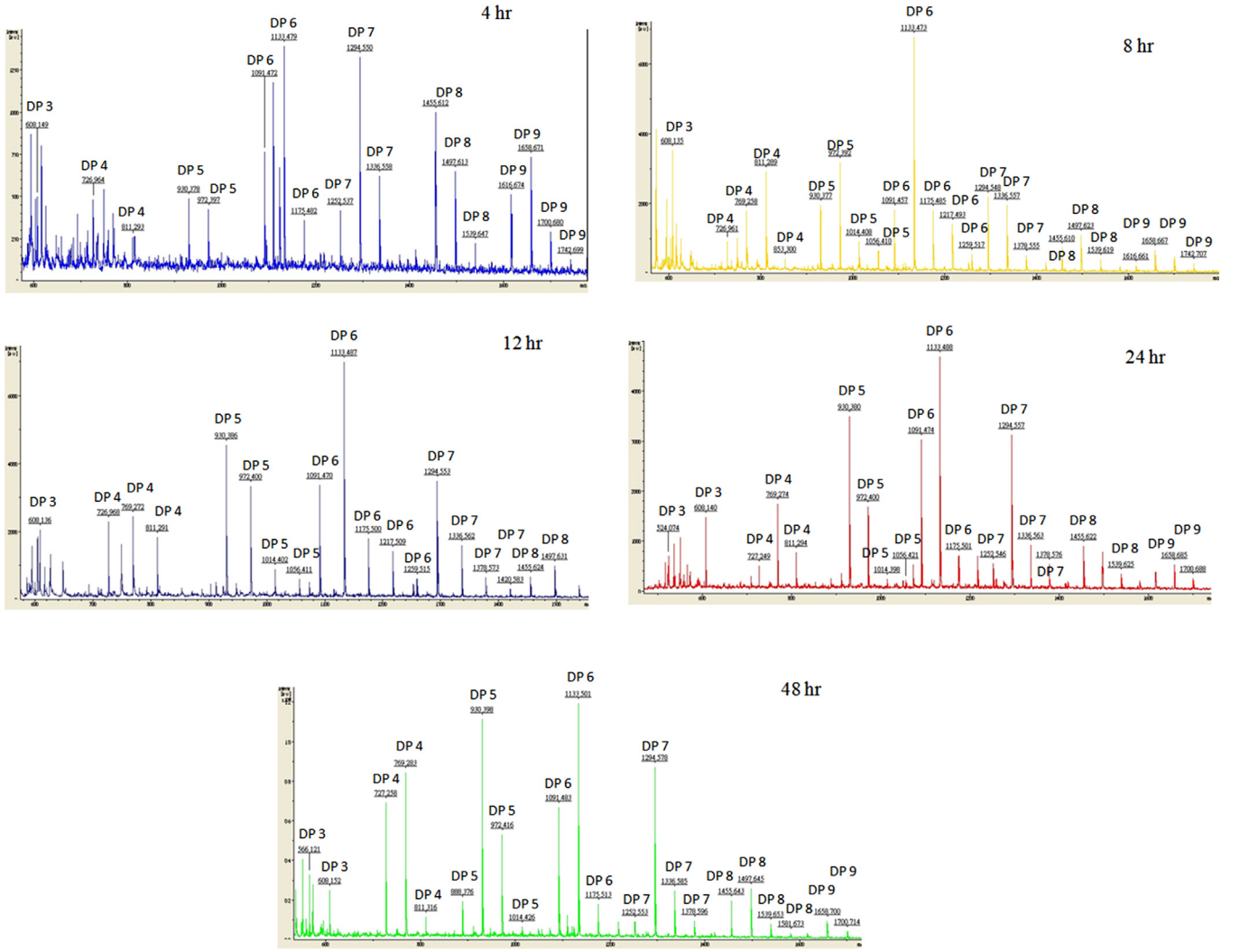


Fig. 3. MALDI-TOF-MS of chitosan oligosaccharide mixtures obtained during the 60% DD chitosan hydrolysis with D4 chitosanase. The proportion of low molecular weight oligomers was reduced by precipitation in 90% methanol soluble/90% acetone insoluble fraction. Identified peaks are labelled with DP, where DP indicates the degree of polymerisation. The hydrolysis time is labelled in each spectrum.

Table 3
Ion composition of chitosan oligosaccharides with DP below 9 as assigned by MALDI-TOF-MS spectra, prepared by enzymatic hydrolysis for the indicated times and fractionated using selective isolation with 90% methanol and acetone.

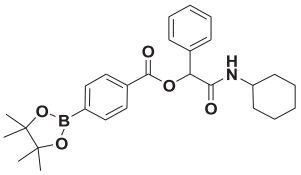
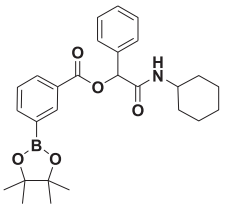
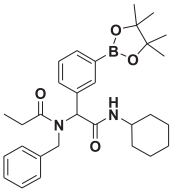
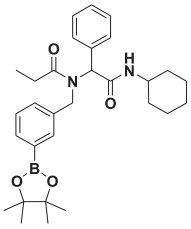
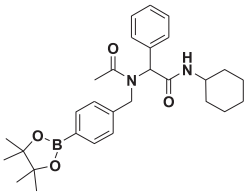
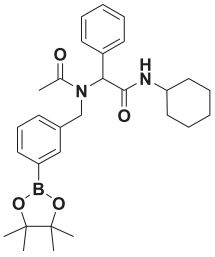
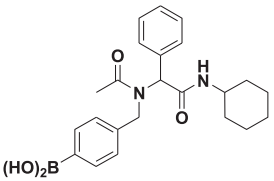
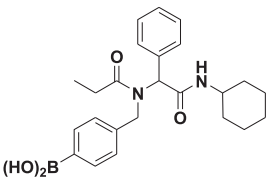
m/z	Ion composition	DP	Hydrolysis time (h)				
			4	8	12	24	48
566	GlcNAc-$(GlcN)_2$	3					+
608	$(GlcNAc)_2$-GlcN	3	+	+	+	+	+
727	GlcNAc-$(GlcN)_3$	4	+	+	+	+	+
769	$(GlcNAc)_2$-$(GlcN)_2$	4		+	+	+	+
811	$(GlcNAc)_3$-GlcN	4	+	+	+	+	+
853	$(GlcNAc)_4$	4		+			
888	GlcNAc-$(GlcN)_4$	5					+
930	$(GlcNAc)_2$-$(GlcN)_3$	5	+	+	+	+	+
972	$(GlcNAc)_3$-$(GlcN)_2$	5	+	+	+	+	+
1014	$(GlcNAc)_4$-GlcN	5		+	+	+	+
1056	$(GlcNAc)_5$	5		+	+	+	
1091	$(GlcNAc)_2$-$(GlcN)_4$	6	+	+	+	+	+
1133	$(GlcNAc)_3$-$(GlcN)_3$	6	+	+	+	+	+
1175	$(GlcNAc)_4$-$(GlcN)_2$	6	+	+	+	+	+
1217	$(GlcNAc)_5$-GlcN	6		+	+		
1259	$(GlcNAc)_6$	6		+	+		
1252	$(GlcNAc)_2$-$(GlcN)_5$	7	+			+	+
1294	$(GlcNAc)_3$-$(GlcN)_4$	7	+	+	+	+	+
1336	$(GlcNAc)_4$-$(GlcN)_3$	7	+	+	+	+	+
1378	$(GlcNAc)_5$-$(GlcN)_2$	7		+	+	+	+
1420	$(GlcNAc)_6$-GlcN	7			+		
1455	$(GlcNAc)_3$-$(GlcN)_5$	8	+	+	+	+	+
1497	$(GlcNAc)_4$-$(GlcN)_4$	8	+	+	+		+
1539	$(GlcNAc)_5$-$(GlcN)_3$	8	+	+		+	+
1581	$(GlcNAc)_6$-$(GlcN)_2$	8					+
1616	$(GlcNAc)_3$-$(GlcN)_6$	9	+	+			
1658	$(GlcNAc)_4$-$(GlcN)_5$	9	+	+		+	+
1700	$(GlcNAc)_5$-$(GlcN)_4$	9	+			+	+
1742	$(GlcNAc)_6$-$(GlcN)_3$	9	+	+			

containing $(GlcN)_2GlcNAc$ (short notation: D_2A_1), D_2A1, DA_2, D_3A, D_2A_2, DA_3, A_4, D_4A_1, D_3A_2, D_2A_3, D_1A_4, A_5, D_4A_2, D_3A_3, D_2A_4, D_1A_5, A_6, D_5A_2, D_4A_3, D_3A_4, D_2A_5, D_1A_6, D_5A_3, D_4A_4, D_3A_5, D_2A_6, D_6A_3, D_5A_4, D_4A_5, and D_3A_6 as the major components of DP 3-9 (Table 3). These products were generated by hydrolysis of 60% DD chitosan with D4 chitosanase and are not a series of fully deacetylated GlcN oligomers. During hydrolysis, the *O*-glycosidic and the *N*-acetyl linkages between residues can be hydrolysed. These results indicate that the D4 chitosanase might hydrolyse chitosan in an endo-type fashion. From these results, chitosan hydrolysis by the D4 chitosanase combined with a selective methanol precipitation is a quick and simple method to obtain good chitooligosaccharide yields with DPs up to 9 and low molecular weight oligomers.

3.6. Small synthetic boron-containing molecules as D4 chitosanase activators

Chitosanases have been extensively applied in industry and biotechnology. One of the most important chitosanase applications is preparation of chitooligomers from chitosan (Ming, Kuroiwa, Ichikawa, Sato, & Mukataka, 2006; Zhang et al., 2012). Enzymatic chitosan hydrolysis has some advantages for chitooligomers production; chitosanases can catalyse hydrolysis under mild conditions and do not produce monosaccharides (Liu et al., 2009). Chitooligomers possess higher biofunctional properties than chitosan because of their low molecular weight and water solubility. Therefore, it is important to activate chitosanase for larger scale chitooligomer production. In our previous study, we found that a small synthetic boron-containing molecule could enhance the growth of a chitosanase-producing strain in SPP-containing medium (Liang, Chen, Pan, & Wang, 2014). To investigate chitosanase activators, we synthesised several small boron-containing molecules and examined the effects of the small boron-containing molecules on D4 chitosanase activity. As shown in Table 4, the compounds interacted

Table 4
Effects of boron-containing molecules on D4 chitosanase activity.

Compound	Structure	Relative activity (%)	
		0.001 (concn.)	0.01 (concn.)
001		160	122
004		151	122
016		150	108
046		142	93
037		144	99
040		142	103
039		108	64
045		114	75

with D4 chitosanase for 5 min prior to substrate addition. The compound (001) exhibited an apparent activation (160%) at 0.001% (w/v), while the pre-treatment-elicited activation (at the same concentration) with compounds 004, 016, 037, 040, 046, and 039 was 151%, 150%, 144%, 142%, 142%, and 108%, respectively. However, D4 chitosanase was slightly inhibited by concentrations up to 0.01% (w/v) of compounds 046 and 037 but was significantly inhibited by compound 039 at the same concentration. Compounds 046, 037, and 039 could be either activators or inhibitors of D4 chitosanase. Comparing the structures of these small synthetic boron-containing molecules, differences in the side chain might be a key factor. The compound 039 side chain was $B(OH)_2$, which was different from compounds 001, 004, 016, 037, 040, and 046 that had a boronate ester side-chain. This observation indicated that much of the inhibition by compound 039 was likely associated with the $B(OH)_2$ side chain. To test this hypothesis, compound 045 was derived from compound 039, and the change in compound groups on activity was determined. The result was that compound 045 still inhibited D4 chitosanase activity at 0.01% (w/v). Compound 016 activated D4 chitosanase activity, but compound 045 was an inhibitor (0.01%, w/v). The only difference between the compound 045 and compound 016 structures was the side chain. The results confirmed that the synthetic compound with the boronate ester side chain could activate D4 chitosanase. These activating compounds with a boronate ester side chain were easily accessible at a low cost, and they have high efficiency towards D4 chitosanase at low concentrations. These activators can also be used in combination with D4 chitosanase to determine whether synergistic activation occurs.

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