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Short communication

Enzymatic production of *N*-acetyl chitooligosaccharides by crude enzyme derived from *Paenibacillus illioisensis* KJA-424

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Abstract

Production of *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl chitooligosaccharides is important to the food, agriculture and biotechnology sectors, where they can be employed as both plant growth promoters and agents of biocontrol for plant pathogens. *N*-Acetyl-D-glucosamine (GlcNAc) and *N*-acetyl chitooligosaccharides were produced from colloidal chitin by use of crude enzyme obtained from *Paenibacillus illinoisensis* KJA-424. The production rate of GlcNAc increased continuously during incubation, while that of GlcNAc oligomers declined. The maximum production of GlcNAc was 1.71 mg mL^{-1} (yield of 62.2%) after 24 h of incubation. At the same time chitobiose [$(GlcNAc)_2$], chitotriose [$(GlcNAc)_3$], chitoheptose [$(GlcNAc)_7$] and chitooctose [$(GlcNAc)_8$] were 0.13 (yield of 4.9%), 0.03 (yield of 1.2%), 0.01 (yield of 4.1%) and 0.24 mg mL^{-1} (yield of 9.6%), respectively. The rate of total *N*-acetyl chitooligosaccharide production decreased by over 44% and 87% after 2 and 24 h of incubation, respectively. The multi-chitinolytic enzyme complex produced by *Paenibacillus illinoisensis* KJA-424 is effective in the production of GlcNAc and *N*-acetyl chitooligosaccharides, facilitating its potential use in industrial applications.

Keywords: *N*-acetyl-D-glucosamine (GlcNAc); *N*-acetyl chitooligosaccharides; Crude enzyme; Chitin; *Paenibacillus illinoisensis* KJA-424

1. Introduction

Chitin, a linear polymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc), is synthesized in all the major groups of organisms (bacteria, fungi, plants and animals). In humans, GlcNAc monomers are precursors of the disaccharide units in glycosaminoglycans (such as hyaluronic acid, chondroitin sulfate and keratan sulfate), which are necessary to repair and maintain healthy cartilage and joint function. Chitin-oligosaccharides and *N*-acetylglucosamine can be produced by acid hydrolysis of chitin extracted from crustacean shells (Capon & Foster, 1970). In addition, GlcNAc and *N*-acetyl chitooligosaccharides are produced by chemical acetylation of glucosamine (GlcN) and its oligomers using acetic anhydride (Aiba, 1994). Recently, production of GlcNAc has been reported by enzymes derived from *Serratia marcescens* (Haynes, Aloise, & Creagh, 1999), *Bacillus thuringiensis* subsp. *pakistani* (Thamthiankul, Suan-Ngay, Tantimavanich, & Panbangred, 2001), *Aeromonas hydrophila* H2330 (Sashiwa et al., 2002), *Trichoderma viride* and *Acremonium cellulolyticus* (Sashiwa et al., 2003) and *Aeromonas* sp. (Kuk et al., 2005a, 2005b). Production of GlcNAc and *N*-acetyl chitooligosaccharides is of interest to the food, agriculture and biotechnology sectors. Their utilization is a way to improve biofertilizers, which can be comprised of microorganisms and elicitors of both plant pathogen defense responses and enhanced growth. This constitutes an interesting possibility for both plant growth promotion and biocontrol of plant pathogens. The potential to produce large

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amounts of these compounds makes them particularly promising for use as elicitors for disease control and growth promotion in sustainable agricultural systems. The work described here was performed to determine the potential for production of agricultural elicitors, through generation of GlcNAc and *N*-acetyl chitooligosaccharides from chitin using *Paenibacillus illinoisensis* KJA-424, which shows a strong chitinolytic activity.

2. Materials and methods

Chitin from crab shell was purchased from Sigma Chemical Co. (USA). Colloidal chitin was obtained according to the method of Monreal and Reese (1969). In our previous report, chitinase produced by *P. illinoisensis* KJA-424 showed a strong antifungal activity (Jung, Kuk, Kim, Kim, & Park, 2005). The KJA-424 was grown in 100 mL LB broth at 30 °C for 2 days with shaking. For enzyme production, 0.5 mL of this culture was added to 200 mL of medium, which contained 0.2% swollen chitin (Godoy, Rodriguez-Kabana, & Morgan-Jones, 1982). After 3 days of incubation the culture supernatant was centrifuged at 10,000*g* for 10 min and was precipitated overnight with 80% ammonium sulfate at 4 °C. The precipitant was dialyzed through Seamless-cellulose membrane (32 × 20.4 mm, Fisher Sci.), and the crude enzyme was concentrated with polyethylene glycol. Enzymatic activity was determined by measuring the amount of reducing sugar released from swollen chitin by the Schales' method (Imoto & Yamashita, 1971) using a standard curve for GlcNAc. One unit of chitinase activity was defined as the amount of enzyme which produces 1 μmol GlcNAc h^{-1}. To determine production of chitin-oligomers by enzymatic hydrolysis, reactions were carried out in a mixture containing 1 mL 0.5% (w/v) swollen chitin, 0.1 mL (0.66 U) crude enzyme in 0.9 mL 50 mM sodium acetate buffer (pH 5.0). Reaction mixtures were incubated at 37 °C. The reaction was terminated at 1, 2, 3, 6, 12 and 24 h by immersing the tube in boiling water for 3 min. The products were analyzed by HPLC, which was used for quantification of $(GlcNAc)_{1–8}$ under the following conditions: column, carbohydrate 4.6 × 250 mm (Waters, USA); mobile phase, acetonitrile/water (70:30, v/v); flow rate, 1 mL min^{-1}; detection at 214 nm. Quantification of chito-octomer [$(GlcNAc)_8$] was calculated as the same amount of standard [$(GlcNAc)_7$]. The yield of GlcNAc and other *N*-acetyl chitooligosaccharides were calculated by method of Sashiwa et al. (2003).

3. Results and discussion

Many bacteria, such as *S. marcescens* (Reid & Ogrydziak, 1981), *B. thuringiensis* (Barboza-Corona et al., 1999), and *Aeromonase* sp. (Kuk et al., 2005a, 2005b; Lien, Too, Wu, & Yu, 2005), secrete multi-chitinolytic enzyme complexes and these have potential utility in production of chitin monomer. In our work, the rate of total *N*-acetyl chitooligosaccharide production by crude enzyme decreased by over 44% during 2 h of incubation; thereafter the relative production rate decreased by 87% during the 24 h of incubation (Table 1).

HPLC chromatograms of the *N*-acetyl chitooligosaccharides obtained from reaction in enzyme are shown in Fig. 1. The production of GlcNAc increased over the 24 h incubation, while that of GlcNAc oligomers (dimer–octomer) was less. A similar result was reported by Sashiwa et al. (2002) who found that the selective production of GlcNAc from by chitinase, *A. hydrophila* H-2330, and chitin oligomers (dimer–heptamer) was negligible. They suggested that the selective production of GlcNAc through the combined action of the chitinolytic enzyme complex was the result of both endo- and exo-type enzymes.

In our work, HPLC analyses detected production of GlcNAc and *N*-acetyl chitooligosaccharides [except $(GlcNAc)_5$ and $(GlcNAc)_6$], as shown in Fig. 2. There was a maximum production of GlcNAc (1.71 mg mL^{-1}) at 24 h of incubation. Sukwattanasiniff, Zhu, Sashiwa, and Aiba (2002) reported that the yield of GlcNAc was 0.5 mg mL^{-1} at 24 h of incubation in 9:1 cellulase Ac:pectinase An. The concentration of chitobiose and chitotriose were 0.13 and 0.03 mg mL^{-1}, respectively, after 24 h of incubation. At the same time chitoheptose and chitooctose were 0.01 and 0.24 mg mL^{-1}, respectively. The concentrations of these four oligomers changed little during the incubation. Chitopentose and chitohexose were not detected until 24 h of incubation, which might be related to the rapid degradation of *N*-acetyl chitooligosaccharides to lower multimers during the early stages of incubation, and not to the smaller oligomers by 24 h. Similar results were reported by Lien et al. (2005) who found that chitin oligomers [$(GlcNAc)_5$ and $(GlcNAc)_6$] were not detected until 24 h during cultivation with *Aeromonase* sp. DYU-Too7. The existence of different chitin hydrolysates indicated that the chitinases produced from *P. illinoisensis* KJA-424 were both endo-chitinase and exo-chitinase.

The time course for production of *N*-acetyl chitooligosaccharides from chitin by crude enzyme is shown Fig. 3. The production of GlcNAc increased continuously, while *N*-acetyl chitooligosaccharides remained nearly constant during incubation. The yield of GlcNAc was 62.2% after

Table 1
Production of total *N*-acetyl chitooligosaccharides over time, as measured by HPLC

Reaction time (h)	Total yield (mg mL^{-1})	Relative production rate[a]
1	0.71 ± 0.03	100
2	0.79 ± 0.04	55.9
3	0.89 ± 0.09	41.8
6	1.32 ± 0.04	31.0
12	1.62 ± 0.10	19.1
24	2.21 ± 0.13	13.0

[a] The relative production rate on the *n* h was calculated from $Yield_n/(Yield_1 \times time) \times 100$. Total yield values are the means of three replicates and are given as ± SE.

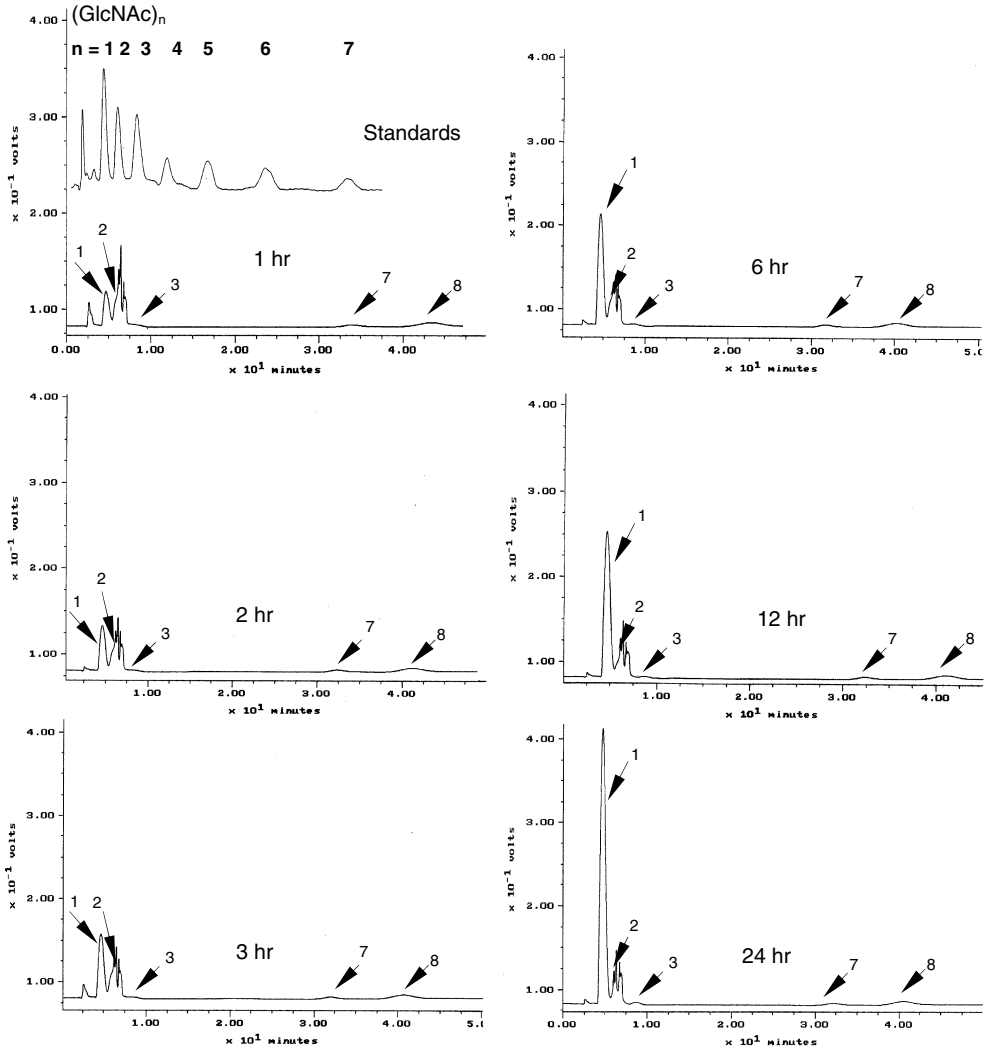

Fig. 1. Time course on HPLC chromatograms of the hydrolyzates of chitin formed by incubation of chitin with crude enzyme produced by *Paenibacillus illionisensis* KJA-424.

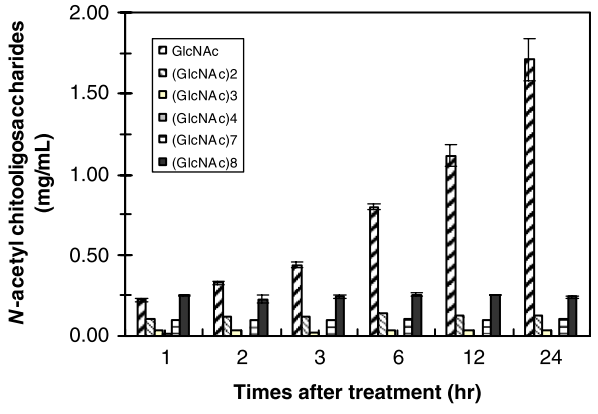

Fig. 2. Time course of chitin hydrolyzates by crude enzyme produced by *Paenibacillus illionisensis* KJA-424. Histogram bars represent the average of three replicates and are given as ± SE.

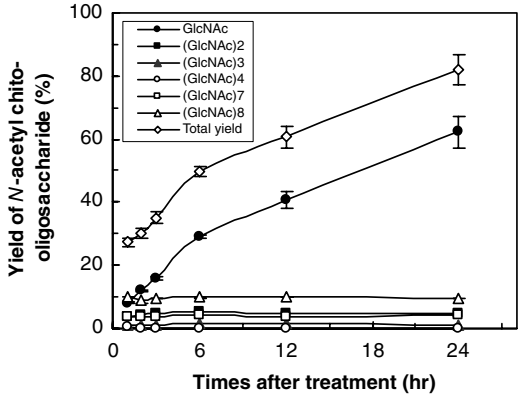

Fig. 3. Changes in yield percent of *N*-acetyl chitooligosaccharides from chitin by crude enzyme produced by *Paenibacillus illionisensis* KJA-424. Each data point represents the average of three replicates and is given as ± SE.

24 h of incubation. The yield of chitoheptose was below 10% during 24 h of incubation. During the same time period, the other oligomers [$(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$ and $(GlcNAc)_7$] remained below 5%. Kuk et al. (2005a, 2005b) reported that, after 5 days of incubation, GlcNAc and $(GlcNAc)_2$ were selectively produced by

crude enzyme of *Aeromonas* sp. GJ-18, with yields of 74% and 35% at 45 and 55 °C, respectively. Non-chitinolytic crude enzymes such as cellulose, hemicelluase, and pectinase also degraded chitin and partially degraded *N*-acetylated chitiosan. Sashiwa et al. (2003) reported that the production of GlcNAc was 74% of β-chitin and 16% of α-chitin by cellulases derived from *T. viride* (T) at day 19 after the onset of treatment. GlcNAc was produced at 66–77% (mol mol^{-1}) yield from α-chitin at day 10 after treatment (Sashiwa et al., 2002). To prepare agricultural elicitor we are currently developing a simple purification process for production of GlcNAc and *N*-acetyl chitooligosaccharides using enzymes obtained from chitinase producing bacteria.

In conclusion, the multi-chitinolytic enzyme complex produced by *P. illinoisensis* KJA-424 is effective in the production of GlcNAc and *N*-acetyl chitooligosaccharides, facilitating its potential use in industrial applications.

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