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Selective preparation of *N*-acetyl-D-glucosamine and *N*,*N*′-diacetylchitobiose from chitin using a crude enzyme preparation from *Aeromonas* sp.

Ju Hee Kuk, Woo Jin Jung, Gyung Hyun Jo, Joon Seob Ahn, Kil Yong Kim & Ro Dong Park*

Glucosamine Saccharide Materials-National Research Laboratory (GSM-NRL), Division of Applied Bioscience and Biotechnology, Institute of Agricultural Science and Technology, Chonnam National University, Gwangju 500-757, Korea

**Author for correspondence (Fax: +82-62-530-0876; E-mail: rdpark@chonnam.ac.kr)*

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Abstract

A bacterium, *Aeromonas* sp. GJ-18, having strong chitinolytic activity was isolated from coastal soil and used for crude enzyme preparations. This enzyme preparation contained *N*-acetyl-D-glucosaminidase and *N*,*N*′-diacetylchitobiohydrolase. *N*-Acetyl-D-glucosaminidase was inactive above 50 °C, but *N*,*N*′-diacetylchitobiohydrolase was stable at this temperature. Utilizing the temperature sensitivities of the chitin degradation enzymes in crude enzyme preparation, *N*-acetyl-D-glucosamine (GlcNAc) and *N*,*N*′-diacetylchitobiose [$(GlcNAc)_2$] were selectively produced from chitin. At 45 °C, GlcNAc was produced as a major hydrolytic product (94% composition) with a yield of 74% in 5 d, meanwhile at 55 °C $(GlcNAc)_2$ was the major product (86%) with a yield of 35% within 5 d.

Introduction

Chitin, a linear polymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc), is the second most abundant biopolymer next to cellulose (Roberts 1992). Chitin degradation products, GlcNAc and chitin oligosaccharides, are widely used in medicine, agriculture (Jung *et al.* 2003), and food (Agulló *et al.* 2003). Traditionally, GlcNAc and chitin oligomers have been produced by acid hydrolysis of chitin (Capon & Foster 1970). This chemical process is costly and produces undesirable effluents which are of environmental concern. Therefore, much attention has been focused on the enzymatic hydrolysis of chitin (Sashiwa *et al.* 2002), which could minimize pollution. This study was performed to develop biological methods for preparing *N*-acetyl-D-glucosamine (GlcNAc) and *N*,*N*′-diacetylchitobiose ($GlcNAc_2$) from chitin as an alternative to the chemical treatment. We describe a selective and efficient production of GlcNAc and $(GlcNAc)_2$ using the crude enzyme preparation from an isolated bacterium, *Aeromonas* sp. GJ-18, which showed a strong chitinolytic activity.

Materials and methods

Materials

Chitin from crab shell was purchased from Shinyong Chitosan Ltd. (Korea). Swollen chitin was obtained according to the method of Monreal & Reese (1988).

Isolation of chitinolytic bacteria

Soils from the west coast of Korea were washed with 1% NaCl. Five ml of the resulting suspension were incubated in Erlenmeyer flasks containing 50 ml medium supplemented with 1% swollen chitin as the sole carbon source. After the samples were incubated with shaking at 37 °C for 5 d, 2 ml portions of each were transferred into fresh chitin medium, and incubated under the same conditions for 5 d. Subsequently, the samples were spread onto the agar medium containing 1% swollen chitin and incubated at 30 °C for 5 d. The pure culture showed large clear zone obtained by successively isolating single colonies.

Preparation of crude enzyme

GJ-18 was grown in 20 ml LB broth at 30 °C for 3 d with shaking. For enzyme production, 1 ml of this culture was added to 500 ml medium, which contained 1% swollen chitin, 1% tryptone, and 1% NaCl. After incubation for 5 d, the culture supernatant was centrifuged at $5000 \times g$ for 20 min, and dialyzed through cellulose membrane.

Enzymatic activity

The 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 per h.

Enzymatic hydrolysis of chitin

To determine the optimum temperatures for production of *N*-acetyl-D-glucosamine and *N,N′*-diacetylchitobiose by enzymatic hydrolysis, reactions were carried out in a mixture containing 1 ml 3% (w/v) swollen chitin, 1 ml (1.44 U) crude enzyme in 100 mM sodium acetate buffer (pH 5). Reaction mixtures were incubated at 20 °C to 60 °C. The reaction was terminated by immersing the tube in boiling water for 3 min.

Analytical methods

Enzymatic hydrolysis of chitin was performed as given above. Products were analyzed by TLC and HPLC. TLC was done on silica gel $60F_{254}$ plates (Merck, Germany) using *n*-propanol/water/NH_4OH (70:30:1, by vol.) as solvent. Amino sugars were detected by spraying the plate with aniline/diphenylamine reagent and holding at 180 °C for 3 min (Tanaka *et al.* 1999). HPLC was used for quantification of $(GlcNAc)_{1-3}$ under the following conditions: column, NH2P-50 4E (Shodex, Japan); mobile phase, acetonitrile/water (70:30, v/v); flow rate, 1 ml min^{-1}; detection at 210 nm.

Results

Isolation and identification of Aeromonas *sp. GJ-18*

During the screening experiments, various types of microbial colonies formed halos on chitin agar medium. Several bacterial strains were able to use swollen chitin as a sole carbon source, and these cultures were tested for chitinolytic activity after having been multiplied in chitin-containing liquid medium. Bacterial strain GJ-18 showed the highest chitinolytic activity and was selected for further study. On the basis of the nucleotide sequence of 16S rRNA gene, GJ-18 was identified as on *Aeromonas* sp. Phylogenetic analysis showed that the sequence of GJ-18 was the most similar to *Aeromonas* sp. Tf 234 with more than 99% similarity.

Chitinase production of Aeromonas *sp. GJ-18*

Aeromonas sp. GJ-18 produced an extracellular chitinase when grown in a medium containing swollen chitin. The chitinase activity was the highest when culture was maintained at 30 °C. Enzyme activity of the culture supernatants was maximal at 5 d after inoculation (1.44 U ml^{-1}), and decreased thereafter (Figure 1). Therefore, culture supernatants from 5 d-old cultures were used in preparation of crude enzyme for hydrolysis of swollen chitin.

Selective production of N-*acetyl-D-glucosamine and* N,N′-*diacetylchitobiose*

To investigate the efficiency of hydrolysis of swollen chitin by crude enzyme, reaction mixtures were incubated at various temperatures ranging from 20 to 60 °C. During investigation of the enzymatic properties of the crude enzyme prepa-

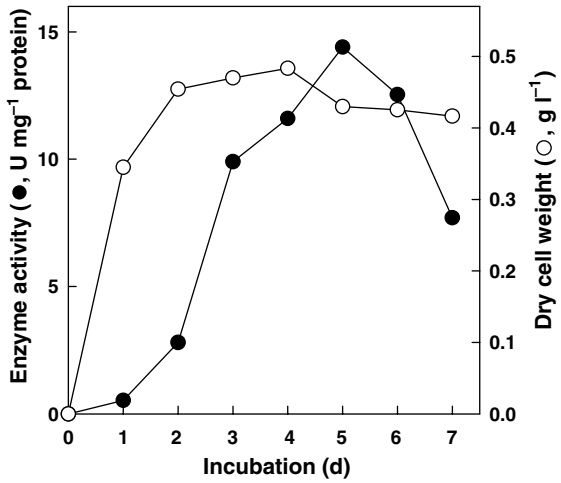

Fig. 1. Time course of cell growth (○) and enzyme production (●) of *Aeromonas* sp. GJ-18.

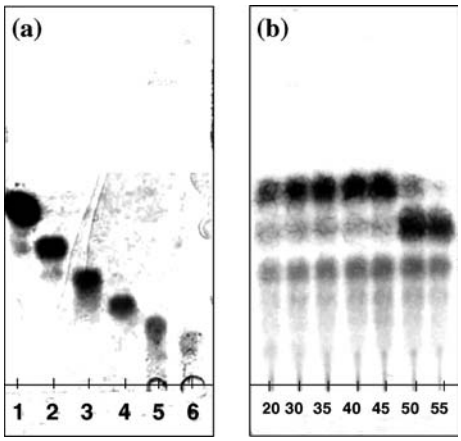

Fig. 2. Effect of temperature on the degree of depolymerization of chitin by crude enzyme of *Aeromonas* sp. GJ-18. (a) Standards of $(GlcNAc)_{1-6}$; (b) products after 2 d of incubation at various temperatures.

ration, we found that the composition of chitinolytic products by the enzyme preparation was dependent on the incubation temperature as shown in Figure 2. Interestingly, when incubated below 45 °C, the major reaction product was GlcNAc, with a small amount of $(GlcNAc)_2$ and $(GlcNAc)_3$, whereas $(GlcNAc)_2$ was a main product above 50 °C.

HPLC indicated that a selective production of the monomer (GlcNAc) was achieved at 45 °C. At this temperature, a small quantity of $(GlcNAc)_2$ and negligible quantity of $(GlcNAc)_3$ were produced. At 50 °C, the ratio of $(GlcNAc)_2$ to GlcNAc suddenly increased and $(GlcNAc)_2$ was the major product at 55 °C (data not shown). Table 1 shows the % yield of chitinolytic products at 45 and 55 °C. At 45 °C, the major product was GlcNAc with less than 5% $(GlcNAc)_2$, and the yield of GlcNAc gradually increased with incubation time. Swollen chitin was hydrolyzed with a yield of 74% GlcNAc and 4.8% $(GlcNAc)_2$ within 5 d, and the relative composition of GlcNAc in total hydrolyzate produced was 93.7%. At 55 °C $(GlcNAc)_2$ was the major product. The yield of GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$ was 3.9%, 34.7% and 1.6%, respectively, and the relative composition of $(GlcNAc)_2$ was 86.3% after 5 d.

Discussion

Selective production of *N*-acetyl-D-glucosamine (GlcNAc) or *N*,*N*′-diacetylchitobiose [$(GlcNAc)_2$] was achieved by the crude enzyme preparation of *Aeromonas* sp. GJ-18. Although the purification of chitinase and *β*-*N*-acetylglucosaminidase from

Table 1. Yield of chitin hydrolyzates at 45 °C and 55 °C.

Reaction time (h)	45 °C			55 °C		
	GlcNAc (%)	$(GlcNAc)_2$ (%)	$(GlcNAc)_3$ (%)	GlcNAc (%)	$(GlcNAc)_2$ (%)	$(GlcNAc)_3$ (%)
0.25	9.7	1.8	0	0.3	9.1	0.7
0.5	15.1	2.4	0	0.7	11.7	0.7
1	24.3	2.5	0.1	0.9	15.2	0.8
2	41.2	3.5	0	1.4	21	1.1
3	55.5	3.5	0.2	2	26.9	1.3
4	68.3	4.7	0.2	2.5	32.1	1.8
5	74	4.8	0.2	3.9	34.7	1.6

Aeromonas species has been intensively studied in several cases (Yabuki *et al*. 1986, Lee *et al*. 1993, Ueda *et al*. 1995, 2003, Hiraga *et al*. 1997, Sugita *et al*. 1999, Lan *et al*. 2004), only limited studies on the enzymatic production of chitin hydrolyzates by those strains have been reported. Sashiwa *et al*. (2002) reported that GlcNAc was produced from chitin by crude enzymes of *Aeromonas hydrophila* H-2330 to yield of 64–77% toward various particle sizes of chitin after incubation for 10 d. In this study, swollen chitin was hydrolyzed with a yield of 74% GlcNAc and 4.8% $(GlcNAc)_2$ at 45 °C, and a yield of 3.9% GlcNAc, 34.7% $(GlcNAc)_2$ and 1.6% $(GlcNAc)_3$ at 55 °C within 5 d.

Previously elucidated biodegradation pathways of chitin are as follows: chitin is degraded into dimer unit of GlcNAc by the combination of endo- and exo-type chitinases, and then *N*-acetyl-D-glucosaminidase further hydrolyzes $(GlcNAc)_2$ to GlcNAc (Tanaka *et al*. 2003). Our results indicated that at least two types of exo-chitinase (*β*-*N*-acetylglucosaminidase and *N*,*N*′-diacetylchitobiohydrolase) are associated in enzymatic preparation of GJ-18. The crude enzyme preparation from *Aeromonas* sp. GJ-18 showed higher activity of *β*-*N*-acetylglucosaminidase compared to that of *N*,*N*′-diacetylchitobiohydrolase and likely resulted in selective production of GlcNAc below 45 °C. But *β*-*N*-acetylglucosaminidase was inactivated above 50 °C while *N*,*N*′-diacetylchitobiohydrolase remained active at this temperature which resulted in production of $(GlcNAc)_2$ as a major component.

Up to date, there are not many reports about *N*,*N*′-diacetylchitobiohydrolase and enzymatic production of $(GlcNAc)_2$. An enzyme which hydrolyzed chitin to *N*,*N*′-diacetylchitobiose has been purified from an *Aeromonas* sp. but there are no data about production of $(GlcNAc)_2$ from chitin by this bacterium (Huang *et al*. 1996). The present work is, to our knowledge, the first example of selective production of GlcNAc and $(GlcNAc)_2$ from chitin by controlling reaction temperature with crude enzyme preparation. Thus, this typical protocol using *Aeromonas* sp. GJ-18 crude enzyme preparation should be a valuable biological process for a selective and efficient production of GlcNAc and $(GlcNAc)_2$ from chitin by simply controlling the enzymatic reaction temperature. Further study of enzyme purification and characterization is being undertaken for more understanding chitinolytic system in *Aeromonas* sp. GJ-18.

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