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Induction of the Enzymes that Degrade Chitin and Chitosan from *Enterobacter* sp. G-1

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Abstract *Enterobacter* sp. G-1 produced both chitinolytic and chitosanolytic enzymes when the carbon source was slightly deacetylated chitin or its oligomer, but not when the carbon source in a medium was highly deacetylated chitosan or its oligomer. Synthesis of these enzymes was not inhibited by chitosan, but was promoted in proportion to the amount of added chitin. The synthesis was induced by insoluble chitin or by its oligomers, but not by its monomer. These results indicate that *Enterobacter* sp. G-1 produces both chitinolytic and chitosanolytic enzymes in response to an N-acetyl group in chitin, and that when there are more N-acetyl groups the production of the enzyme is higher, which led to more cell divisions. The results of cultures in which $GlcNAc_2$ is the only carbon source led us to conclude that $GlcNAc_2$ does not directly enter the cell. Instead, GlcNAc derived from $GlcNAc_2$ is metabolized. However, GlcNAc did not induce production of both enzymes. These results imply that *Enterobacter* sp. G-1 secretes the enzymes after recognizing insoluble chitin or its oligomers. Then the monomer enters the cell and is metabolized by the bacteria.

Key words: *Enterobacter* sp. G-1; chitinolytic and chitosanolytic enzymes; chitin; chitosan.

Introduction

Enzymes hydrolyzing chitin or chitosan are widely distributed in animals, plants, fungi, and bacteria. Plants produce chitinase in response to microbial infections or other injury.[1−3] Since plants do not contain chitin, it has been postulated that they produce chitinase to protect themselves from chitin-containing pathogens.[4),5] ROBERT *et al.*[6] reported that chitinase help limit and define the fungal species that can parasitize plants by acting directly on growing hyphal tips or in concert with other hydrolytic enzymes. Insects produce a chitinolytic enzyme during each moulting cycle.[7] In general, there are two types of chitinolytic enzymes, an endo-type and an exo-type. Living organisms such as bacteria, molds, and fungi produce chitinolytic enzymes or chitosanolytic enzymes to obtain energy. Thus, many kinds of organisms produce chitinolytic and chitosanolytic enzymes for various purposes. There are many reports on the inducers of chitinolytic enzyme production. Ulhoa and Peberdy.[8] suggested that inducers of chitinase in *Trichoderma harzianum* 39.1 are soluble oligomers of N-acetyl-glucosamine derived from chitin. Monreal and Reese.[9] demonstrated that the chitinase in *Serratia marcescens* is induced by soluble oligomers of N

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-acetyl- glucosamine, but not by the monomer. However, there are no reports on the induction of both chitinolytic and chitosanolytic enzymes produced by a sole bacterium. In a previous study, we isolated bacteria that degraded chitin and chitosan in a medium containing chitin, and called it *Enterobacter* sp. G-1.[10] The chitosanolytic enzyme precipitated by salting with ammonium sulfate was immobilized on chitosan beads (Chitopearl) and continuously produced chitosan hydrolyzates.[11] Moreover, the chitosanolytic enzyme precipitated by salting with 30% ammonium sulfate was purified, and its mode of action was studied.[12] In this paper, we describe the physiological conditions and mechanisms that induce production of chitinolytic and chitosanolytic enzymes by *Enterobacter* sp. G-1.

Methods

Reagents and chemicals: Chitin and chitosan, the latter deacetylated to various degrees, were purchased from Funakoshi Chemical Co.(Tokyo). Colloidal chitin was prepared by the method of Jeuniaux.[13], and colloidal chitosan by the method of YABUKI *et al.*[14] Other reagents were all of analytical grade.

Culture conditions: *Enterobacter* sp. G-1 was incubated in 2 ml of basic medium (0.07% K_2HPO_4, 0.03% KH_2PO_4, 0.05% $MgSO_4$, 0.03% polypepton, and 0.03% yeast extract) supplemented with carbon substrates (2% chitin or 2% chito -san) and LB medium.

Chitinolytic and chitosanolytic activities: The assay was based on the estimation of reducing sugars released during the hydrolysis of colloidal chitosan or colloidal chitin. The reaction mixture, containing McIlvaine's buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 7.0) and 0.25% colloidal chitosan or colloidal chitin in a final volume of 2.0 ml, was incubated and shaken for 30 min at 30°C, and then centrifuged at 3000 r.p.m. for 5 min. The amount of reducing sugar released in the supernatant was determined by a modified version of the method of Scharles.[15] One unit (U) of activity was defined as the amount of enzyme that catalyzed the release of 1μmol of reducing sugar (D-glucosamine for chitosanolytic activity and N-acetyl-D - glucosamine for chitinolytic activity) for 1 min.

N-acetyl-glucosaminidase activity was assayed as follows: 10μl of culture supernatant was added to 100μl of $GlcNAc_2$ (1 mM) in McIlvaine's buffer and incubated at 30°C. The reaction was stopped by boiling for 5 min. A 20μl sample of the reaction mixture was analyzed by HPLC. One unit (U) of activity was defined as the amount of enzyme that catalyzed the release of 1μmol of GlcNAc for 1 min.

Chitinolytic and chitosanolytic activities of the culture solution precipitate: The culture solution was centrifuged at 8,000×g for 20 min and its precipitate was washed with McIlvaine's buffer. The enzyme activities in the supernatant and the precipitate were assayed. Total activities are the enzymes activities of supernatant plus precipitate.

Vial cell number: Dilute culture supernatant was spread on a medium containing the agar and incubated at 30°C, and the number of colonies that grew on the medium was counted.

Activity staining:[16] SDS-slab polyacrylamide gel electrophoresis was done with the culture supernatant concentrated with ammonium sulfate as the sample. Then the protein band in the gel was transblotted into a gel with 0.01% glycol chitin as the substrate, with semi-dry transblotter (Sartblot II-S, Sartorius. Co. Ltd). The transblotted gel was shaken to remove the SDS and the enzyme was allowed to react in the tris-HCl buffer (pH 7) for 2 hours at room temperature. The gel was soaked in 0.01% Fluostain I for 5 minutes, and incubated in water for 1 hour. Under ultraviolet light, the degraded glycol chitin was observed as a dark band.

Analytical methods: $GlcNAc_n$ and GlcNAc in the culture supernatant were analyzed by HPLC with a Tosoh TSK gel NH2-60 column, eluted with an acetonitril -water mixture (65: 35) at 1.0 ml per minute. They were measured by monitoring the absorbance at 210 nm.

Results

Effects of various carbon sources on chitinolytic and chitosanolytic enzyme induction during cultivation

The chitinolytic and chitosanolytic enzymes were induced only when *Enterobacter* sp. G-1 grew in medium containing chitin. They were not induced when the bacteria were cultured in LB medium or in media without chitin or with chitosan (Fig. 1).

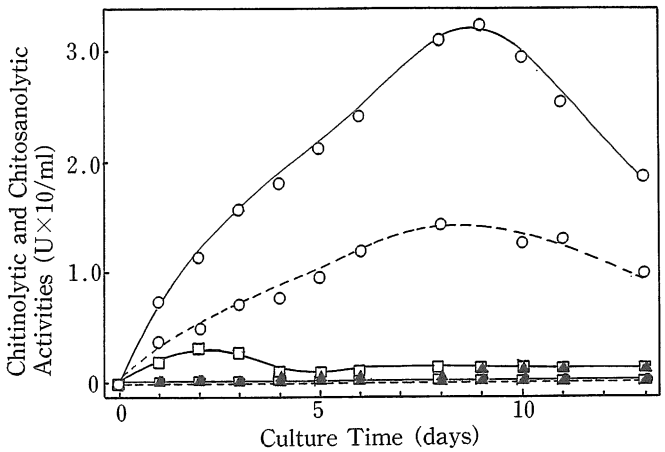

Fig. 1. Changes in Enzyme Activities during Cultivation in Various Substrates.
○ : medium containing 2% chitin, ● : Basic medium (described in the text), □ : medium containing 2% chitosan, ▲ : LB medium,
----- : chitinolytic activity, —— : chitosanolytic activity.

Effects of degree of deacetylation of chitosan

Almost no chitinolytic and chitosanolytic enzymes were secreted when the bacteria were incubated in the media with over 70% deacetylated chitosan, but both enzymes were induced when with incubation in media containing less than 20% deacetylated chitosan (Fig. 2). There were over 10^{10} cells per ml (maximum) during cultivation in the medium containing less than 20% deacetylated chitosan, but this

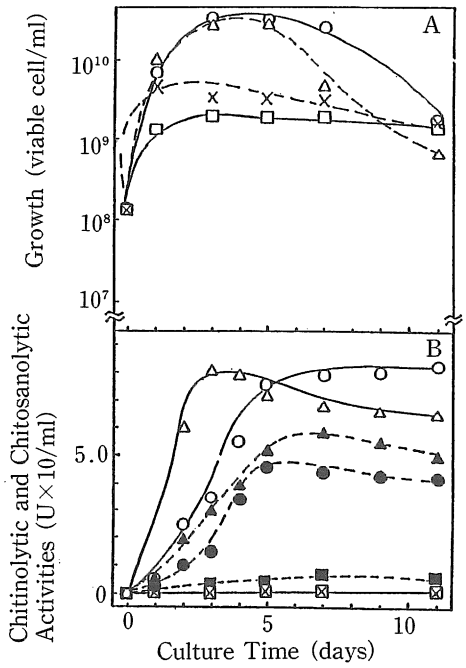

Fig. 2. Effects of Degree of Deacetylation of Chitin. (A) Number of viable cells per ml: less than 20% deacetylated chitosan (○), 20-30% (△), 75% (□), and 90% (×). (B) Chitinolytic activities: less than 20% deacetylated chitosan (●), 20-30% (▲), 75% (■), and 90% (×). Chitosanolytic activities: less than 20% deacetylated chitosan (○), 20-30% (△), 75% (□), and 90% (×).

number was lower by an order of magnitude during cultivation in the medium containing over 70% deacetylated chitosan. During 1 day of cultivation in the LB medium, the viable cells multiplied into over 10^{10}, and thereafter gradually decreased.

Effects of chitin: chitosan ratios during cultivation

As shown in Fig 3, both chitinolytic and chitosanolytic enzyme activities were higher when the ratio of chitosan to chitin was high, and were also higher when more chitin was added. These results indicate that enzyme production depended on the amount of chitin added, but was not inhibited by chitosan.

Effect of particle size of chitin

The chitosanolytic enzyme in culture with 2% ground chitin was secreted earlier than with 2% chitin flakes, and showed the same activity as 2% ground chitin washed with distillied water to remove soluble oligomers (Fig. 4). N-acetyl-glucosaminidase activity was higher than the chitosanolytic activity, but was the same time course. These results supported that the enzyme production increased with increasing of surface area of chitin. The soluble oligomers contained in the ground chitin did not affect induction of enzymes.

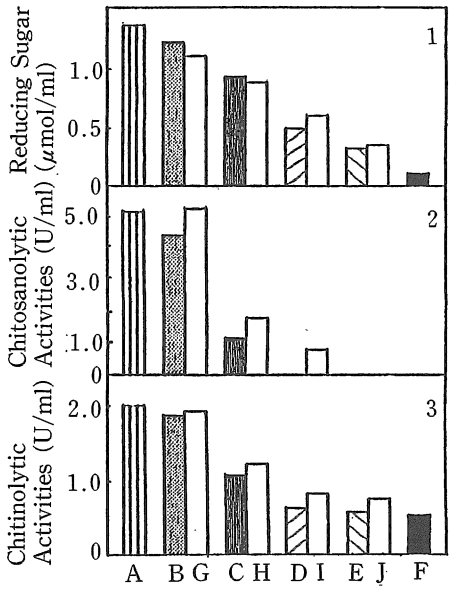

Fig. 3. Enzyme Induction during Cultivation with Various Mixtures of Chitin and Chitosan. After *Enterobacter* sp. G-1 was cultured for 3 days in media containing chitin and chitosan in various ratios, or various amounts of added chitin, the culture supernatant was assayed. (1): reducing sugar, (2): chitosanolytic activities, (3): chitinolytic activities. Ratios of chitin to chitosan were 10:0 (A), 8:2 (B), 6:4 (C), 4:6 (D), 2:8 (E), and 0:10 (F). Amount of added chitin was 1.6% (G), 1.2% (H), 0.8% (I), and 0.4% (J).

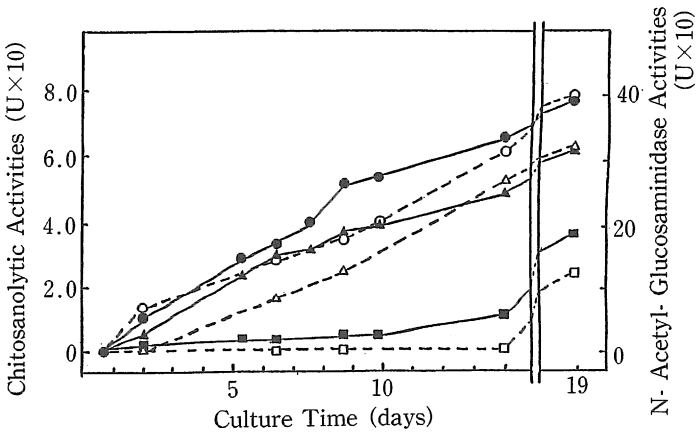

Fig. 4. Effect of Particle Size of Chitin. *Enterobacter* sp. G-1 was incubated in 2 ml of basic medium supplemented with carbon substrates (2% ground chitin, 2% ground chitin washed with distillied water to remove soluble oligomer, chitin flakes), and the culture supernatant was assayed. 2% ground chitin (circle), 2% ground chitin washed with distillied water (triangle), Chitin flakes (square). Chitosanolytic activity (black mark), N-acetyl- glucosaminidase activity (white mark).

Effects of $GlcNAc_n$ (dimer-hexamer), $GlcN_n$ (dimer-pentamer) and their monomer

$GlcNAc_n$ induced production of both enzymes, but $GlcN_n$, GlcNAc, and GlcN did not induce production of either enzyme (Table. 1). The reducing sugar in the culture was about 80% consumed when the bacteria were incubated in the medium containing $GlcNAc_n$ and GlcNAc, but only trace quantities were consumed when they were incubated in the medium containing GlcN, and none at all was consumed from the medium containing $GlcN_n$. There were about 10^{10} cells per ml in the culture with $GlcNAc_n$ and GlcNAc, but about 10^9 per ml in those with $GlcN_n$ and GlcN.

Table 1. Enzyme induction during cultivation with $GlcNAc_n$ (n=1-5) and GlcN as carbon sources. *Enterobacter* sp. G-1 was incubated in 2 ml of basic medium supplemented with 0.5% $GlcNAc_n$ or GlcN. After 3 days in culture, the supernatant of the culture was analyzed.

Carbon source	Chitinolytic activity (U/ml)	Chitosanolytic activity (U/ml)	Consumed rate of reducing sugar (%)	Viable cell (number/ml)
GlcNAc	0.01	0.01	79.6	1.47×10^{10}
$GlcNAc_2$	1.80	1.90	60.7	5.16×10^9
$GlcNAc_3$	2.20	2.21	76.2	6.26×10^9
$GlcNAc_4$	2.33	2.14	69.7	7.00×10^9
$GlcNAc_5$	2.41	2.33	69.0	5.12×10^9
GlcN	0.00	0.00	13.5	1.10×10^9

Effects of glucose, GlcNAc, and GlcN on chitinolytic and chitosanolytic activities

The basic medium containing 0.5% chitin was supplemented with 1.0% GlcNAc, 1.0% GlcN, or 0.1, 0.5, or 1.0% glucose after 4 days of incubation. Activities of both enzymes were lower one day later, but thereafter the activities recovered, and inceased to a level greater than control in the culture with GlcN (Fig. 5). This phenomenon is known as catabolite repression in general.

Enzyme activities of precipitates in the culture solution

The activities (1.4 U) in the precipitate comprised 30% of the total activities (3.5 U). This shows that the enzymes secreted from the bacteria were absorbed on the chitin substrate.

Activity staining

As shown in Fig. 6, the three chitinolytic isozymes were observed as dark bands, and their molecular weights were estimated to be 50,000, 44,000, and 40,000, respectivery, by SDS-PAGE.

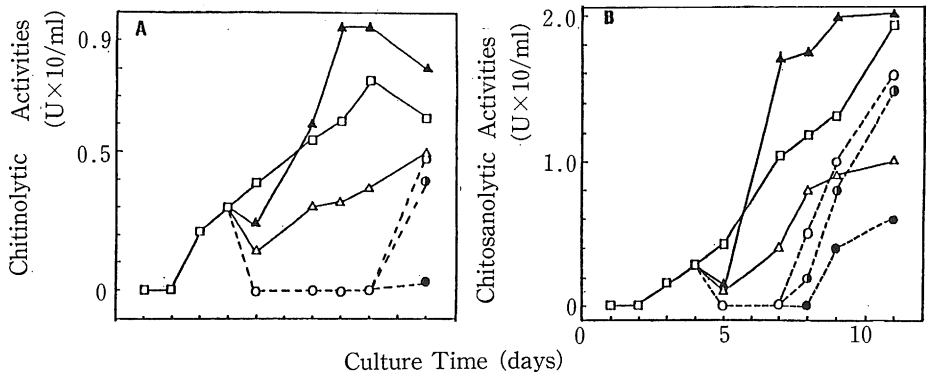

Fig. 5. Effects of Addition of Glucose, GlcNAc, and GlcN to the Chitin Medium. After *Enterobacter* sp. G-1 was inoculated into the basic medium containing 0.5% chitin and incubated at 30°C for 4 days, 1.0% GlcNAc, 1.0% GlcN, or 0.1, 0.5, 1.0% glucose was added to the culture solution. (A) Chitinolytic activities, (B) Chitosanolytic activities□ : basic medium containing 0.5% chitin (control), ▲ : GlcN, △ : GlcNAc, ○ : 0.1% glucose, ○ : 0.5% glucose, ● : 1.0% glucose.

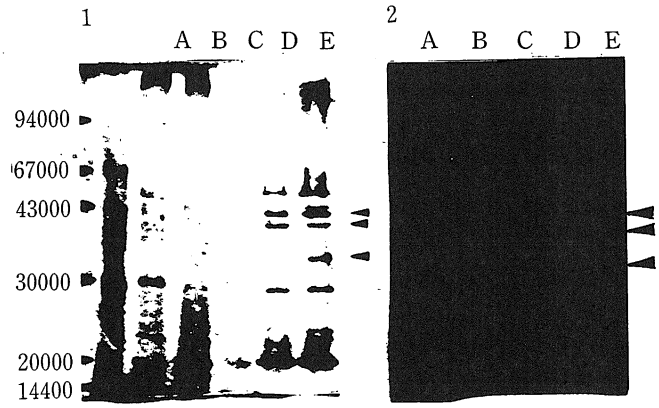

Fig. 6. Activity Staining of Glycol Chitin Degrading Activities. (1) SDS-PAGE, (2) activity staining. Lane A: supernatant in LB medium, Lane B: in LB medium containing 1 % chitin, Lane C: in basic medium, Lane D: in basic medium containing 1 % chitin, Lane E: in basic medium containing 1 % chitin cultured under oxygen-rich conditions. The allows show enzyme bands.

Time course of reducing sugar components during incubation in media containing $GlcNAc_2$, GlcNAc, $GlcN_2$, and GlcN

When *Enterobacter* sp. G-1 were incubated in the medium containing $GlcNAc_2$ as the carbon source, the time courses of the amounts of $GlcNAc_2$ and GlcNAc in the culture supernatant were as shown in Fig. 7. $GlcNAc_2$ decreased at 0.2 mM/hr after 3 hr of cultivation. GlcNAc increased at 0.12 mM/hr after 5 hr of cultivation, and decreased at 0.3 mM/hr after 10 hr. When the bacteria were incubated in the medium containing GlcNAc as the carbon source, GlcNAc began to decrease after 2 hr of cultivation and had almost disappeared by 10 hr. GlcN in the medium containing GlcN

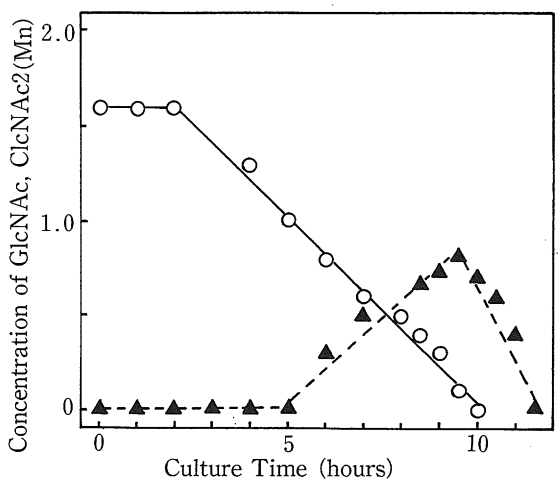

Fig. 7. *Time Course of Cultivation with $GlcNAc_2$.* After *Enterobacter* sp. G-1 was incubated in the medium containing $GlcNAc_2$ as the carbon source, $GlcNAc_2$ and GlcNAc in the culture supernatant were analyzed by HPLC. ○ : $GlcNAc_2$, ● : GlcNAc.

as the carbon source decreased very slowly (0.08 mM/hr). $GlcN_2$ in the medium containing $GlcN_2$ did not decrease for 20 hr.

Discussion

Chitinolytic and chitosanolytic enzymes are produced by a wide range of living organisms, including, plants, insects, molds, and microorganisms. Higher plants that do not contain chitin in their cell walls secrete chitinase to defend themselves against parasites containing chitin. Insects secrete both endo-and exo-type chitinolytic enzymes to shed their old cuticle when they molt. Some microorganisms produce chitinolytic enzymes and then use chitin as an energy source. There are many reports of inducers of chitinolytic enzyme production in microorganisms. Monreal and Reese[9] suggested that *Serratia marcescens* induces chitinase production by soluble chitodextrins capable of entering the cell, but not by GlcNAc, and that chitin is partially hydrolyzed to yield soluble inducers. Berg and Petterson[17] studied cellulase and reported that *Trichoderma viride* was induced to produce it by physical contact between the cell surface and insoluble substrates, and that the enzyme is bound to the outside of the cell wall and is released by lysis. we describe our findings regarding induction of chitinolytic and chitosanolytic enzymes produced by *Enterobacter* sp. G-1.

These bacteria produced both chitinolytic and chitosanolytic enzymes when they were cultured in media containing slightly deacetylated chitin or their oligomers (dimer-hexamer) as carbon sources, but not in media with highly deacetylated chitosan or their oligomers. Moreover, GlcNAc (monomer of chitin) and GlcN (monomer of chitosan) did not induce synthesis of either enzyme. The number of cells in the culture solution that induced production of both enzymes was an order of magnitude greater than the number of cells in the culture solution that did not induce

enzyme production. These results indicate that this strain of bacteria secretes both enzymes when it recognizes an N-acetyl group of carbon substrates. When the bacteria were cultured in media with various ratios of chitin to chitosan, the activities of both enzymes in the culture supernatant were higher when the proportion of chitin was greater. When only various amounts of chitin were added to the medium, activities of both enzymes in the culture supernatant were similarly higher in proportion to the amount of chitin. From these results, we conclude that the synthesis of these enzymes was not inhibited by chitosan, but was promoted in proportion to the amount of added chitin. Thus, we suspect that *Enterobacter* sp. G-1 produces both chitinolytic and chitosanolytic enzymes in response to an N-acetyl group in the insoluble chitin, and that when there were more N-acetyl groups, enzyme production was higher, and this led to more cell division.

Synthesis of both enzymes was induced both by insoluble chitin and by its oligomers, but not by its monomer GlcNAc. Chitosanolytic and glucosaminidase activity were higher when the probability of contact with bacteria and insoluble chitin were higher. These results mean that *Enterobacter* sp. G-1 may recognize insoluble chitin or its oligomers by physical contact with the cell surface, and then the enzymes are synthesized and secreted. They hydrolyze insoluble chitin or its oligomers to produce the monomer, which is metabolized by the bacterium. To test this hypothesis, *Enterobacter* sp. G-1 were cultured in a medium containing $GlcNAc_2$ as the sole carbon source and the time courses of $GlcNAc_2$ and GlcNAc (the hydrolyzates) were studied by HPLC. $GlcNAc_2$ decreased at 0.2 mM/hr after 3 hours. GlcNAc increased at 0.12 mM/hr after 5 hours and then decreased at 0.3 mM/hr after 10 hours. The total amount of $GlcNAc_2$ consumed was 1.0 mM. These data indicate that GlcNAc degraded from $GlcNAc_2$ was metabolized, and that $GlcNAc_2$ does not enter the cell.

Ulhoa and Peberdy(1991)[8] suggested that *Trichoderma harzianum* 39.1 produces chitinase in response to soluble oligomers derived from the chitin preparation, or by the action of constitutive chitinase, because the constitutive level of chitinase activity may be sufficient to initiate chitin degradation and release soluble oligomers. St Leger *et al.*[18] reported that GlcNAc, not $GlcNAc_2$, was the major inducer of chitinase production in *Metarhizium anisopliae*. They argued that extracellular and cell-bound activities of the constitutive chitobiase would probably degrade chitobiose to GlcNAc before it enters the cell, and the major product of both chitobiase and chitinase is GlcNAc.

For the following two reasons, we speculate that *Enterobacter* sp. G-1 produce chitinolytic and chitosanolytic enzymes in response to insoluble chitin or oligomers on the cell surface, GlcNAc is produced with enzymes, enters the cell, and is metabolized. First, in the experiments chitinolytic and chitosanolytic enzymes were induced, not constitutive. Second, GlcNAc did not induce enzyme synthesis and $GlcNAc_2$ did not enter the cell.

The induction of these enzymes was strongly affected by glucose. Synthesis of enzymes can be repressed by glucose or by metabolizable compounds. This phenome-

non has been called catabolic repression, and it occurs in many microorganisms. The production of chitinase by *Serratia marcescens* is repressed by various metabolites (mannose, ribose, sucrose, GlcNAc, glucose).[9] Glucose inhibited enzyme synthesis more than GlcNAc at the same concentration, and catabolic repression disappeared eventually. GlcN had almost no effect on catabolic repression. This is consistent with the finding that the presence of an amino group in the chitosan molecule did not affect enzyme synthesis. Figure 8 shows proposed mode of enzyme induction from *Enterobacter* sp. G-1.

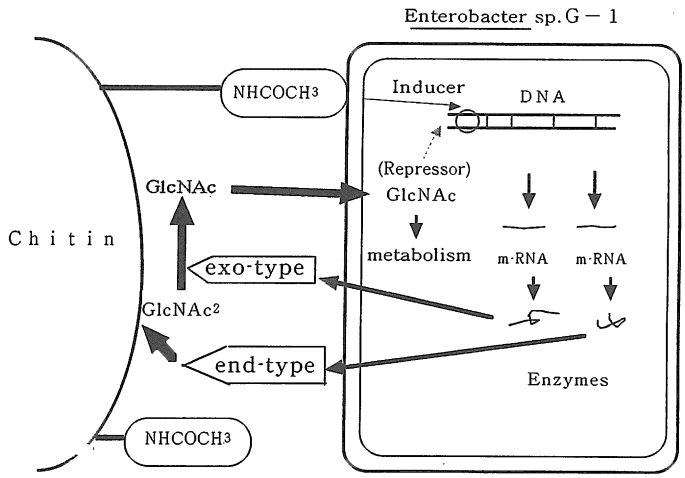

Fig 8. Proposed Mode of Chitinolytic and Chitosanolytic Enzyme from *Enterobacter* sp. G-1. *Enterobacter* sp. G-1 recognize N-acetylated groups in insoluble chitin or its oligomers by physical contact with the cell surface. The mRNAs from activated gene with signal of N-acetylated groups may produce proteins containing chitinolytic and chitosanolytic enzyme. The enzyme produced are secreted from cell, degrade insoluble chitin or chitosan to GlcNAc, and metabolize in the cell as energy sources.

Acknowledgments

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