A chitosanolytic enzyme was purified from Enterobacter sp. G-1 by fractionation of 30% saturation with ammonium sulfate, isoelectric focusing, and Sephadex G-100 gel chromatography. The purified enzyme showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the molecular mass was estimated to be 50 kDa. The enzyme degraded N-acetyl-chitooligosaccharides, glycol chitin, colloidal chitin, and colloidal chitosan (about 80% deacetylated), but did not degrade chitooligosaccharides, colloidal chitosan (100% deacetylated), or Micrococcus lysodeikticus cell walls. It hydrolyzed GlcNAc\textsubscript{4-6} and colloidal chitin to GlcNAc\textsubscript{3-6} finally. The main cleavage site with GlcNAc\textsubscript{3-6} was the second linkage from the non-reducing end, based on the pattern of pNp-GlcNAc\textsubscript{3-6}. Colloidal chitosan was hydrolyzed to GlcNAc\textsubscript{2} and to similar partially N-acetylated chitooligosaccharides.

Chitin is an important constituent of cell walls in many fungi.\textsuperscript{1} It is a β-1,4 linked polymer of N-acetyl-d-glucosamine. When microorganisms degrade chitin, the first step is hydrolysis by chitinase (EC 3.2.1.14). This produces oligomers, mainly dimers, which are then degraded to free N-acetyl-d-glucosamine by chitobiase (EC 3.2.1.30). Monosaccharides are released, which can be metabolized by a variety of organisms.\textsuperscript{2} Chitosan is generally a partially N-acetylated compound. It can be hydrolyzed to chitosan oligosaccharides by enzymes called chitosanases.\textsuperscript{3} Some of these enzymes hydrolyze only chitosan,\textsuperscript{4-6} and others hydrolyze both chitosan and carboxymethyl cellulose.\textsuperscript{7-10} Chitinase from Aeromonas hydrophila,\textsuperscript{11} Streptomyces griseus,\textsuperscript{12,13} and Pseudomonas cinnabarinus\textsuperscript{14} hydrolyzed both chitin and the N-acetyl-β-d-glucosaminic bonds in partially N-acetylated chitosan. This produced hetero-chitooligosaccharides, with GlcNAc at the reducing end. Chitosanases from Bacillus sp. No. 7-M\textsuperscript{14} and Streptomyces griseus HUT 6037\textsuperscript{15} specifically hydrolyzed the GlcN-GlcN bonds in chitosan molecules, and could produce chitooligosaccharides and hetero-chitooligosaccharides. Thus, there was a difference between chitinase and chitosanase in their mode of action on chitosan, and this difference remained to be studied. We have reported that Enterobacter sp. G-1\textsuperscript{16} produced both chitin degrading activity and chitosan degrading activity when cultivated in a chitin medium. This paper deals with the purification and mode of action of a chitosan degrading enzyme from Enterobacter sp. G-1.

Materials and Methods

Materials. Chitin, N-acetyl-chitooligosaccharides (GlcNAc\textsubscript{n}), chitooligosaccharides (GlcNAc\textsubscript{n}), and p-nitrophenyl N-acetyl-chitooligosaccharides (pNP-GlcNAc\textsubscript{n}) (n=1-6) were purchased from Sekagaku Kogyo (Tokyo), and chitosan was purchased from Funakoshi Co., Ltd. Colloidal chitin was prepared from chitin by the method of Jeuniaux.\textsuperscript{17} Colloidal chitosan was prepared by the method of Yakbi et al.\textsuperscript{18} All other chemicals were purchased from Commercial suppliers.

Microorganism. The microorganism used in this study was isolated from a culture containing chitin, and was identified as Enterobacter sp. G-1, as described previously.\textsuperscript{19} The strain was cultured in a chitin medium (0.07% K\textsubscript{2}HPO\textsubscript{4}, 0.03% KH\textsubscript{2}PO\textsubscript{4}, 0.05% MgSO\textsubscript{4}, 0.03% polypropylene, 0.03% yeast extract, 2% chitin) for 5 days at 30°C, and the culture was centrifuged at 8000 × g for 20 min to obtain the supernatant.

Purification of chitosanolytic enzyme. Ammonium sulfate was added to the supernatant to give 30% saturation. The supernatant was left to stand overnight, and the precipitate that formed was collected by centrifugation. Then it was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) and treated by isoelectric focusing with Ampholine (LKB Co.). The chitosanolytic enzyme, which was obtained by isoelectric focusing, was put on a Sephadex G-100 column equilibrated with 0.01 M citric acid-0.02 M sodium phosphate buffer (pH 7.0), and then the active enzyme was eluted with the same buffer solution. Fractions with chitosanolytic activity were pooled and lyophilized.

Chitosanolytic and chitinolytic activity. The assay is 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\textsubscript{2}HPO\textsubscript{4} and 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 for 30 min at 30°C with shaking, and then centrifuged at 3000 rpm for 5 min. The amount of reducing sugar released in the supernatant was measured with a modified version of the method of Scharies.\textsuperscript{19} One unit (U) of activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of reducing sugar (d-glucosamine for chitosanolytic activity, and N-acetyl-d-glucosamine for chitinolytic activity) for 1 min.

Enzymatic reaction. When GlcN\textsubscript{p}, GlcNAc\textsubscript{p}, pNP-GlcNAc\textsubscript{p}, colloidal chitosan, and colloidal chitosan were used as the substrates, the enzymatic reaction was done in a 10 ml test tube as follows: 10 μl of the enzyme solution (0.1 mg) was added to 100 μl of GlcN\textsubscript{p} (1 mg) or GlcNAc\textsubscript{p} (1 mg), pNP-GlcNAc\textsubscript{p} (0.1 mg), 0.5% colloidal chitin, or 0.5% colloidal chitosan in McIlvaine’s buffer, and incubated at 35°C. The reaction was stopped by boiling for 5 min. A 0.2 μl-sample of the reaction mixture was analyzed by HPLC with a Tosos TSK gel NH2-60 column, and eluted with an acetonitrile-water mixture (65:35) at 1.0 ml per min. The GlcNAc\textsubscript{p} and pNP-GlcNAc\textsubscript{p} eluted were detected by monitoring the absorbance at 210 nm, and GlcN\textsubscript{p} was detected by the refractive index (RI). The products of the enzymatic hydrolysis of colloidal chitosan were analyzed by thin-layer chromatography on Merk Silica Gel 60 aluminum plates, with propanol, 30% ammonium (2:1) as the developing solvent. GlcN\textsubscript{p} was stained by a ninhydrin spray.\textsuperscript{20}

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Ammonium sulfate was added to the supernatant of culture solution to 70% saturation; the precipitate that formed was dialyzed against 10 mM Tris-HCl buffer (pH 8.0), and was used as the 70% saturation fraction. On the other hand, the fraction precipitated at various degrees of saturation of ammonium sulfate were also used. The 40% saturation fraction was prepared with supernatant from which the 30% saturation fraction had been removed, and 50 and 60% saturation fractions were similarly prepared. SDS-PAGE
was done in 12.5% polyacrylamide gel containing 0.1% SDS, by the method of Laemmli.\(^2\)

**Results**

**Purification of chitosanolytic enzyme**

The culture supernatant was fractioned by ammonium sulfate. The protein precipitated by addition of various amounts of ammonium sulfate were analyzed by SDS–PAGE. A fraction that precipitated at 30% saturation contained a main protein of 50 kDa (Fig. 1). This fraction was dialyzed and analyzed by isoelectric focusing. The isoelectric point of the chitosanolytic enzyme was 5.5 (Fig. 2). The active fractions were concentrated with polyethylene glycol 20,000, put on a Sephadex G-100 column and eluted with McIlvaine’s buffer (pH 7.0). A symmetrical protein peak was recorded. It was entirely associated with chitosanolytic activity (Fig. 3) and the active fractions were lyophilized. The purified enzyme was confirmed to be homogeneous by SDS–PAGE (Fig. 4) and the molecular masses were estimated to be 50 kDa. The results of the purification are summarized in Table I. The chitosanolytic enzyme was purified about 11-fold, with recovery of 26% from the culture supernatant.

**Substrate specificity**

The substrate specificity of the purified enzyme was analyzed with 0.5% glycol chitin, glycol chitosan, *Micrococcus lysodeikticus* cell walls, pNp-GlcNAc, colloidal

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Fig. 1. SDS–PAGE at Various Degrees of Saturation with Ammonium Sulfate.

SDS polyacrylamide slab gel electrophoresis was done as described in Materials and Methods. Protein bands were stained with Coomassie Brilliant Blue R-250. Lane M, standard proteins; phosphorylase b (M, 94,000), BSA (M, 67,000), ovalbumin (M, 43,000), carbonic anhydrase (M, 30,000), soybean trypsin inhibitor (M, 20,000), a-lactalbumin (M, 14,400). Lane 1, 70% saturation. Lane 2, culture supernatant. Lane 3, 60% saturation. Lane 4, 50% saturation. Lane 5, 40% saturation. Lane 6, 30% saturation.

Fig. 2. Elution Pattern of Sephadex G-100 Chromatography.

The chitosanolytic fraction obtained by isoelectric focusing chromatography was put on Sephadex G-100 chromatography columns equilibrated with 0.01 M citric acid and 0.02 M sodium phosphate buffer (pH 7.0). Chitosanolytic activity (\(\bullet\)), absorbance at 280 nm (\(\circ\)).

Fig. 3. Elution Pattern of Sephadex G-100 Chromatography.

The chitosanolytic fraction obtained by isoelectric focusing chromatography was put on Sephadex G-100 chromatography columns equilibrated with 0.01 M citric acid and 0.02 M sodium phosphate buffer (pH 7.0). Chitosanolytic activity (\(\circ\)), absorbance at 280 nm (\(\circ\)).

Fig. 4. SDS–PAGE at Each Step of Chitosanolytic Enzyme Purification.

Lane M, standard protein; Lane A, fraction from Sephadex G-100 Chromatography; Lane B, fraction from isoelectric focusing chromatography; Lane C, 30% saturated with ammonium sulfate.
chitin, and colloidal chitosans (about 80% and 100% deacetylated) as substrates. The enzyme degraded glycol chitin, colloidal chitin, and colloidal chitosan (about 80% deacetylated), but did not degrade the other substrates. For degrading colloidal chitosan (about 80% deacetylated), the enzyme was about twice as active as it was for degrading colloidal chitin. Therefore, this enzyme was chitosanolytic, and was not a lysozyme or a β-N-acetyl glucosaminidase.

Effects of temperature on activity and stability
The optimum temperature of chitosanolytic activity was about 50°C, and that of chitinolytic activity was about 60°C (Fig. 5A). Chitosanolytic activity was stable between 20°C and 50°C, but chitinolytic activity was less stable between 20°C and 40°C, and by 50°C it had decreased to about 40% of its original value.

Hydrolysis products of GlcNAc₆
GlcNAc₄ was hydrolyzed mainly to two molecules of GlcNAc₂ or faintly to GlcNAc₃ and GlcNAc (Fig. 6A). GlcNAc₃ was hydrolyzed to GlcNAc₃ and GlcNAc₂, and then GlcNAc₃ was gradually hydrolyzed to GlcNAc₂ and GlcNAc (Fig. 6B). Each molecule of GlcNAc₆ was hydrolyzed to GlcNAc₆ and GlcNAc₅ or two molecules of GlcNAc₃, and then GlcNAc₄ was hydrolyzed mainly to two molecules of GlcNAc₄.

Kinetic analysis
The effects of substrate concentration on the velocity of hydrolysis of GlcNAc₆ were investigated. The Michaelis constant ($K_m$) and the maximum velocity were calculated from Lineweaver-Burk plots (Fig. 7, Table II). The $K_m$ decreased when the substrate chain was longer. However, substrate inhibition was observed in the reaction of GlcNAc₆ at the higher concentration of 1 mM.

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Table I. Purification of Chitosanolytic Enzyme

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>796</td>
<td>958.0</td>
<td>510.8</td>
<td>0.53</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>Ammonium Sulfate</td>
<td>140</td>
<td>257.2</td>
<td>443.7</td>
<td>1.73</td>
<td>86.9</td>
<td>3.24</td>
</tr>
<tr>
<td>Sulfite Precipitation</td>
<td>562</td>
<td>86.4</td>
<td>182.1</td>
<td>2.11</td>
<td>35.7</td>
<td>3.95</td>
</tr>
<tr>
<td>Isoelectric Focusing</td>
<td>447</td>
<td>22.6</td>
<td>131.8</td>
<td>5.84</td>
<td>25.8</td>
<td>11.00</td>
</tr>
<tr>
<td>Chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Cleavage pattern

GlcNAc₂ was not cleaved, and the order of the velocities of the cleavage reactions was GlcNAc₅ ≥ GlcNAc₆ > GlcNAc₄ > GlcNAc₃, the last of which was about 10% that of GlcNAcₑ. To locate the cleavage site, five forms of pNP-GlcNAcₖ (n = 1−5) were used as the substrates. The enzyme released GlcNAc₂ from the non-reducing end of each substrate except pNP-GlcNAc₃ (Table III). Neither GlcNAc₂ nor pNP-GlcNAc was cleaved, but a p-nitrophenol was liberated from pNP-GlcNAc₂, and this could be seen as a change in the color of the reaction solution. These results suggest that the enzyme releases p-nitrophenol by recognizing more than two N-acetyl-D-glucosamine residues bonded to p-nitrophenol. GlcNAc₃ was cleaved to GlcNAc and GlcNAc₂, and pNP-GlcNAc₂ was cleaved to p-nitrophenol and GlcNAc₂. Therefore, we deduce that GlcNAc₃ was cleaved at the second linkage from the non-reducing end. GlcNAc₄ was cleaved to two molecules of GlcNAc₂ (73%) or to GlcNAc and GlcNAc₂ (27%). pNP-GlcNAc₃ was cleaved to GlcNAc₂ and pNP-GlcNAc (27%), or to GlcNAc₃ and p-nitrophenol (73%). From these results, we deduce that GlcNAc₄ was cleaved at the second or third linkage from the non-reducing end, but the main cleavage sites of GlcNAc₄ and pNP-GlcNAc₃ differed. GlcNAc₅ was cleaved at the second or fourth linkage from the non-reducing end, and that p-nitrophenol was probably not liberated from pNP-GlcNAc₄, but from pNP-GlcNAc₂. GlcNAc₆ was cleaved at the second or third linkage from the non-reducing end. p-Nitrophenol was probably not released from pNP-GlcNAc₅ but from pNP-GlcNAc₃ and pNP-GlcNAc₂.

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Table II. Kinetic Parameters

The reaction were done as described in Fig. 7. Molecular activity, kₒ, is expressed as V_max/e₀ where e₀ is the molar concentration of the enzyme.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kₙ (mm)</th>
<th>kₒ (1/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc₄</td>
<td>1.25</td>
<td>0.025</td>
</tr>
<tr>
<td>GlcNAc₅</td>
<td>0.80</td>
<td>0.030</td>
</tr>
<tr>
<td>GlcNAc₆</td>
<td>0.77</td>
<td>0.037</td>
</tr>
</tbody>
</table>

---

Table III. Cleavage Patterns of N-Acetyl-chitooligosaccharides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction</th>
<th>Initial velocity (nmol/min)</th>
<th>Cleavage pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc₂</td>
<td>Not detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcNAc₃</td>
<td>G₃⇒ G₂ + G₁</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>GlcNAc₄</td>
<td>P-G₂⇒ G₂ + P</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>GlcNAc₅</td>
<td>G₄⇒ G₂ + G₂</td>
<td>18.0 (73%)</td>
<td></td>
</tr>
<tr>
<td>P-G₃⇒ G₂ + P</td>
<td>G₁ + G₃</td>
<td>5.0 (27%)</td>
<td></td>
</tr>
<tr>
<td>G₃ + P</td>
<td>22.0</td>
<td></td>
<td>G-G-G*</td>
</tr>
<tr>
<td>GlcNAc₆</td>
<td>G₅⇒ G₂ + G₃</td>
<td>31.0 (82%)</td>
<td></td>
</tr>
<tr>
<td>P-G₄⇒ G₂ + P</td>
<td>G₁ + G₄</td>
<td>7.0 (18%)</td>
<td></td>
</tr>
<tr>
<td>G₃ + P</td>
<td>23.0</td>
<td></td>
<td>G-G-G-G*</td>
</tr>
<tr>
<td>GlcNAc₇</td>
<td>G₆⇒ G₂ + G₄</td>
<td>31.0 (83%)</td>
<td></td>
</tr>
<tr>
<td>P-G₅⇒ G₂ + P</td>
<td>G₃ + G₃</td>
<td>6.0 (17%)</td>
<td></td>
</tr>
<tr>
<td>G₃ + P₂</td>
<td>33.0</td>
<td></td>
<td>G-G-G-G-G*</td>
</tr>
</tbody>
</table>

Hydrolysis products of GlcN$_2$-5, colloidal chitin and colloidal chitosan

Hydrolysis products of GlcN$_2$-5 were not detected even after a reaction for 3 h. In the hydrolysis products of colloidal chitin, GlcNAC$_2$ and a small amount of GlcNAC$_3$ were detected after reactions that lasted for 30 h when the purified enzyme was used, and GlcNAC was detected when the crude enzyme (70% saturation fraction with ammonium sulfate) was used (data not shown). In the hydrolysis products of colloidal chitosan, a small amount of GlcNAC$_2$ along with unknown peaks, were detected when the purified enzyme was used (Fig. 8A). GlcNAC, GlcNAC$_2$, and unidentified peaks were observed when the crude enzyme was used (Fig. 8B). Peak b corresponded to authentic GlcNAC$_2$, and peak c was between authentic GlcNAC$_2$ and GlcNAC$_3$. However, the hydrolysis products of colloidal chitosan were not detected by thin layer chromatography and RI, which can detect chitoooligosaccharides.

Discussion

The chitosanolytic enzyme of Enterobacter sp. G-1 was purified by a series of purifying steps: 30% saturation with ammonium sulfate, isoelectric focusing and Sephadex G-100, from the culture filtrate of a medium with chitin as a sole carbon source. The purified enzyme manifested as a single band on SDS–PAGE and seemed to be one of the three isozymes observed by activity staining after SDS–PAGE of crude enzyme of 70% saturation fraction with ammonium sulfate (data not shown). The optimum pH differed between chitinolytic and chitosanolytic activity. It is thought that the physical and chemical properties of chitin or chitosan vary with temperature. This will have to be studied. The enzyme hydrolyzed colloidal chitin, colloidal chitosan (about 80% deacetylated) and N-acetyl-chitoooligosaccharides (dimer–hexamer), but it did not hydrolyze chitoooligosaccharides (dimer–hexamer) or colloidal chitosan (100% deacetylated). These results suggest that the cleavage site of the enzyme recognize N-acetyl groups.

To study the cleavage pattern of N-acetyl-chitoooligosaccharides, the degradation of GlcNAC$_2$-6 was examined. The reaction was faster with long chains of N-acetyl-D-glucosamine residues than with short chains. The longer-chain substrates may be preferentially cleaved because they occupy more subsites than short-chain substrates. However, the velocities with GlcNAC$_2$ and GlcNAC$_3$ were almost the same, because of the substrate inhibition at high concentration of GlcNAC$_6$. Koga et al. reported on substrate inhibition in yam chitinase, $\beta$-N-acetyl-D-glucosaminidase from the silkworm Bombyx mori, and pupal alimentary canal enzymes. Subsite structures in relation to substrate inhibition in chitinases have to be studied.

Koga et al. described the mode of action of insect, yam, and prawn chitinase against N-acetyl-chitoooligosaccharides, and Roberts et al. reported on the differences between plant and bacterial chitinases. However, there are almost no previous reports regarding the mode of action of microorganism-derived chitosanolytic enzyme against N-acetyl-chitoooligosaccharides. Yam chitinase degrades GlcNAC faster than it degrades GlcNAC$_2$ or GlcNAC$_3$, and it cleaves GlcNAC$_6$ in three ways. In contrast, insect and prawn chitinases degrade GlcNAC$_5$ and GlcNAC$_6$ faster than they degrade GlcNAC$_4$, and they cleave GlcNAC$_6$ in only two ways. The wide substrate specificity of yam chitinase is consistent with the hypothesis that it is used in self-defense against plant pathogens. In contrast, insects have a very substrate-specific chitinase, which is consistent with the hypothesis that they use it only in ecdysis. Chitinase from Streptomyces griseus mainly splits GlcNAC$_2$ from the non-reducing end of p-nitrophenyl-chitoooligosaccharides, and releases p-nitrophenol from pNP-GlcNAC$_2$. The chitosanolytic enzyme from Enterobacter sp. G-1 that we isolated also mainly released GlcNAC$_2$ from the non-reducing end of N-acetyl-chitoooligosaccharides and p-nitrophenyl-chitoooligosaccharides. The enzyme reactions that continued for many hours produced mainly GlcNAC$_2$, but there was some GlcNAC$_3$ and GlcNAC. Colloidal chitin was hydrolyzed to the same products as N-acetyl-chitoooligosaccharides, and other N-acetyl-chitoooligosaccharides were not detected. These results indicate that the chitosanolytic enzyme degrades a high polymer (colloidal chitin) to GlcNAC$_2$ or GlcNAC$_3$ in order from the non-reducing end, but not to GlcNAC$_5$ or GlcNAC$_6$.

Ohtakara et al. reported that Aromonas hydrophila

Fig. 8. Hydrolyzate of Colloidal Chitosan.

The purified enzyme (0.1 mg) or crude enzyme (70% saturated with ammonium sulfate, 0.58 U) was incubated with 0.5% colloidal chitosan at 35°C and the hydrolyzates were analyzed by HPLC. A, hydrolyzates of purified enzyme; B, hydrolyzates of crude enzyme; C, standard. Peak 1, GlcNAC; peak 2, GlcNAC$_2$; peak 3, GlcNAC$_3$; Peak 4, GlcNAC$_4$; peak 5, GlcNAC$_5$; peak 6, GlcNAC$_6$. Peak a corresponds to GlcNAC, peak b corresponds to GlcNAC$_2$, peak c is between GlcNAC$_3$ and GlcNAC$_4$, and peaks d and e are unknown.
chitinase and *Bacillus* sp. No. 7-M chitosanase produced partially N-acetylated-chitooligosaccharides that are not authentic N-acetylglucosamine residues. Therefore, the chitosanolytic enzyme from *Enterobacter* sp. G-1 probably splits colloidal chitosan (partially acetylated chitosan) at N-acetylated-D-glucosamine residues by recognizing N-acetyl groups, produces GlcNAc (Fig. 8, peak b) from a block of that residue, and also produces a partially N-acetylated-D-glucosamine oligomer (Fig. 8, peak c) from random N-acetylated-D-glucosamine residues. This enzyme may be classified as chitinolytic rather than chitosanolytic, but it is a new type of enzyme, that is, its chitosanolytic activity is stronger than its chitinolytic activity. Moreover, because the crude enzyme of 70% saturated ammonium sulfate produced GlcNAc and the previously mentioned four products of the purified enzyme (Fig. 8B), we deduce that *Enterobacter* sp. G-1 secretes N-acetyl glucosaminidase. We are now going to investigate the chitosanolytic enzyme fractions except the 30% saturation fraction of ammonium sulfate.

References
9) T. Horiuchi, Abstracts of Papers, the Annual Meeting of the Japan Society for Bioscience, Biotechnology, and Agrochemistry, Tokyo, April, 1984, p. 550.