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Chitosanase-Catalyzed Hydrolysis of 4-Methylumbelliferyl β-Chitotrioside

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4-Methylumbelliferyl β-chitotrioside [(GlcN)$_3$-UMB] was prepared from 4-methylumbelliferyl tri-*N*-acetyl-β-chitotrioside [(GlcNAc)$_3$-UMB] using chitin deacetylase from *Colletotrichum lindemuthianum*, and hydrolyzed by chitosanase from *Streptomyces* sp. N174. The enzymatic deacetylation of (GlcNAc)$_3$-UMB was confirmed by ^{1}H-NMR spectroscopy and mass spectrometry. When the (GlcN)$_3$-UMB obtained was incubated with chitosanase, the fluorescence intensity at 450 nm obtained by excitation at 360 nm was found to increase with proportion to the reaction time. The rate of increase in the fluorescence intensity was proportional to the enzyme concentration. This indicates that chitosanase hydrolyzes the glycosidic linkage between a GlcN residue and UMB moiety releasing the fluorescent UMB molecule. Since (GlcN)$_3$ itself cannot be hydrolyzed by the chitosanase, (GlcN)$_3$-UMB is considered to be a useful low molecular weight substrate for the assay of chitosanase. The k_{cat} and K_m values obtained for the substrate (GlcN)$_3$-UMB were determined to be 8.1×10^{-5} s^{-1} and 201 μM, respectively. From TLC analysis of the reaction products, the chitosanase was found to hydrolyze not only the linkages between a GlcN residue and UMB moiety, but also the linkages between GlcN residues. Nevertheless, the high sensitivity of the fluorescence detection of the UMB molecule would enable a more accurate determination of kinetic constants for chitosanases.

Key words: chitin deacetylase, chitooligosaccharide, chitosanase, fluorescent substrate, steady state kinetics.

Chitosanase [EC 3.2.1.132] hydrolyzes the β-1,4 glycosidic bond of chitosan producing glucosamine oligosaccharides [(GlcN)$_n$], which have attracted public attention because of their antibacterial and antitumor activities (*1*). In order to obtain the oligosaccharides efficiently, it is essential to determine the optimal conditions for the enzymatic degradation of the polysaccharides. Therefore, we have evaluated the kinetic constants of chitosanases from various sources. Chitosanase activity has been evaluated in terms of the increase in the reducing sugar concentration during chitosan hydrolysis (*2-5*). The degrees of acetylation of most chitosans, however, is in the intermediate range, with the *N*-acetylglucosamine residues (GlcNAc) randomly localized in the chitosan chain, resulting in chemical diversity of the polysaccharides. Thus, the kinetic constants obtained for the chitosan substrate contain some uncertainty, making it highly desirable to establish a strategy for obtaining definitive values for the kinetic constants of chitosanases.

Recently, we tried to determine the kinetic constants of chitosanases using (GlcN)$_n$ as a substrate. The time-course for oligosaccharide degradation was successfully obtained by separating and quantifying (GlcN)$_n$ with a gel-filtration HPLC system (*6*). Although information concerning the mode of enzymatic hydrolysis could be obtained by HPLC determination, the sensitivity of oligosaccharide detection using the refractive index was insufficient to allow an accurate determination of kinetic constants. On the other hand, synthetic chromophoric substrates have been used for the kinetic analysis of various hydrolases. In particular, 4-methylumbelliferyl (UMB) glycoside substrate enables the most sensitive assay of glycosyl hydrolases such as 1,3-1,4-β-D-glucanohydrolases, lysozymes, chitinases, and cellulases (*7-10*), because the enzymes release a fluorescent UMB moiety from the corresponding synthetic substrate. However, there is no previous report on the synthesis of fluorescent substrates for chitosanases. In order to obtain such a fluorescence substrate, we at first synthesized (GlcNAc)$_3$-UMB, and then tried to deacetylate it chemically. However, it was quite difficult to eliminate *N*-acetyl groups chemically without affecting the UMB glycosidic

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Abbreviations: FAB-MS, fast atom bombardment mass spectrometry; GlcN, 2-amino-2-deoxy-D-glucopyranose; (GlcN)$_n$, β-1,4-linked oligosaccharide of GlcN with a polymerization degree of *n*; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; (GlcNAc)$_n$, β-1,4-linked oligosaccharide of GlcNAc with a polymerization degree of *n*; MALDI-TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; UMB, 4-methylumbelliferone.

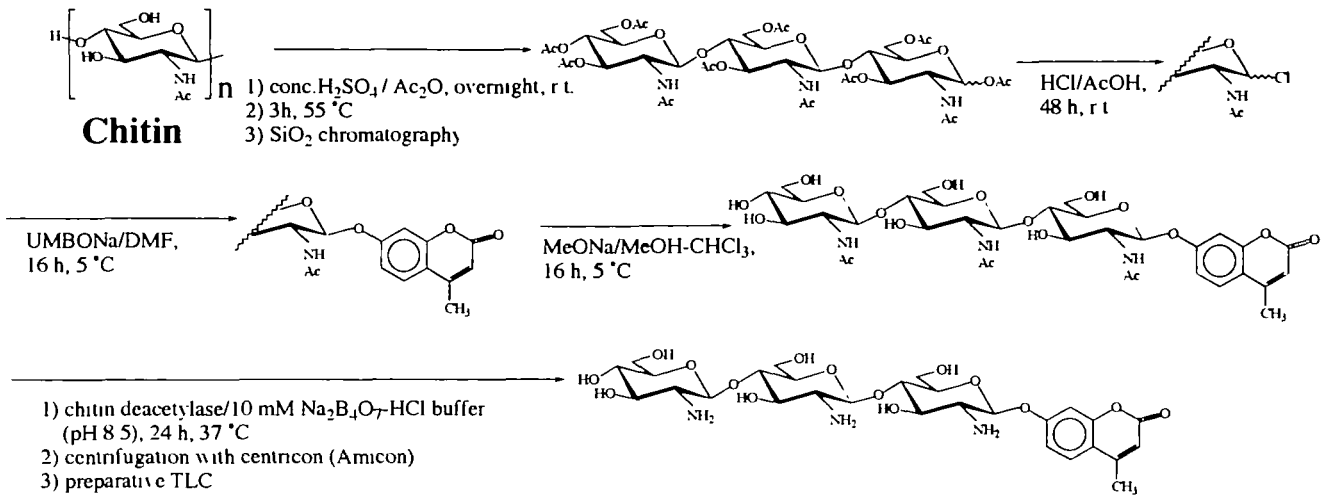

Fig. 1. **Strategy for the chemo-enzymatic synthesis of (GlcN)$_3$-UMB from chitin using chitin deacetylase from *Colletotrichum lindemuthianum*.**

bond. Recently, Tokuyasu *et al.* reported that chitin deacetylase from *Colletorichum lindemuthianum* fully catalyzes the deacetylation of chitooligosaccharides with chain lengths greater than three (*11, 12*). Thus this enzyme may be the best for obtaining the UMB glycoside of GlcN oligosaccharide from (GlcNAc)$_3$-UMB.

In the present study, we tried to prepare (GlcN)$_3$-UMB from (GlcNAc)$_3$-UMB using chitin deacetylase as shown in Fig. 1, and conducted a kinetic investigation of the chitosanase-catalyzed hydrolysis of (GlcN)$_3$-UMB in comparison with the kinetic data for (GlcN)$_n$ (n=4, 5, and 6).

MATERIALS AND METHODS

Materials—*Streptomyces* sp. N174 chitosanase was produced by an expression system in *Streptomyces lividans* TK24 and purified according to the method of Boucher *et al.* (*13*). Chitin deacetylase from *C. lindemuthianum* was obtained by the method previously reported (*11*). (GlcNAc)$_3$-UMB was prepared as described by Inaba *et al.* (*14*). UMB was obtained from SIGMA. Other reagents were commercially available and of analytical grade.

Enzymatic Deacetylation of (GlcNAc)$_3$-UMB—The deacetylation of (GlcNAc)$_3$-UMB was carried out with chitin deacetylase according to the method of Tokuyasu *et al.* (*11, 12*) with a slight modification. (GlcNAc)$_3$-UMB was dissolved in 0.8 ml of 10 mM sodium tetraborate-HCl buffer (pH 8.5) and mixed with chitin deacetylase (0.46 U). One unit of enzymatic activity was defined as described by Tokuyasu *et al.* (*11, 12*). The reaction mixture was incubated for 24 h at 37°C, and then the enzyme was removed from the reaction mixture using a Centricon YM-3 (amicon) membrane. The resultant solution was evaporated, and the residues were separated by preparative TLC on PSC-Fertigplatten Kieselgel 60 F_{254} plates (MERCK). The solvent system used was *n*-butanol:methanol:28% ammonium hydroxide (4:3:3) (*15*). The purified product was lyophilized.

Instrumental Analysis—The deacetylation of (GlcNAc)$_3$-UMB was confirmed by measuring the ^{1}H-NMR spectrum in D_2O using a JEOL JNM-GX-270 NMR spectrometer. For further identification of the product, the FAB-MS spectrum was obtained using a JEOL JMS-AX500 mass spectrometer using 3-nitrobenzylalcohol was used as the matrix. The MALDI-TOF-MS spectrum was also obtained on a Shimadzu KOMPACT MALDI using 10 mg/ml 2,5-dihydroxybenzoic acid in acetonitrile:0.1% trifluoroacetic acid (3:7) as the matrix.

Hydrolysis of (GlcN)$_3$-UMB by Streptomyces sp. N174 Chitosanase—(GlcN)$_3$-UMB was dissolved in 0.6 ml of 50 mM sodium acetate buffer (pH 5.5) to obtain a 28.0 μM solution. Enzyme solution was added to the substrate solution and the mixture was incubated at 40°C. A portion (0.1 ml) of the reaction mixture was withdrawn at an appropriate reaction time and mixed with 0.5 ml of 0.4 M Na_2HPO_4-NaOH buffer, pH 11.9, to terminate the enzymatic reaction. The fluorescence intensity of the resultant solution was measured at 450 nm with excitation at 360 nm using a Shimadzu-RF1500 spectrofluorometer. The concentration of 4-methylumbelliferone released from (GlcN)$_3$-UMB was calculated from the calibration curve obtained with an authentic solution of 4-methylumbelliferone.

For steady state kinetic analysis, (GlcN)$_3$-UMB solutions from 43 to 630 μM were prepared, and each solution was mixed with enzyme solution to obtain a final enzyme concentration of 0.353 μM. The initial velocity was determined from the increase in fluorescence intensity. The kinetic constants were calculated from double reciprocal plots of initial velocity *versus* substrate concentration.

Kinetics for Substrate (GlcN)$_n$ (n=4, 5, and 6)—The substrate, (GlcN)$_n$ (n=4, 5, or 6), was dissolved in 50 mM sodium acetate buffer, pH 5.5, to obtain 1-16.6 mM substrate solutions. The enzyme solution was added to the substrate solution, and the reaction mixture was incubated at 40°C. The final enzyme concentrations were 0.37 μM for (GlcN)$_4$ and 0.053 μM for (GlcN)$_5$ and (GlcN)$_6$. After an appropriate reaction time, a portion of the reaction mixture was withdrawn and mixed with an equal volume of 0.1 N NaOH to terminate the enzymatic reaction. The reaction products obtained were analyzed by HPLC on a TSK-GEL G2000PW (TOSOH, 0.5×600 mm) gel-filtration column.

The column was eluted with 0.5 M NaCl at a flow rate of 0.3 ml/min at room temperature using a Hitachi L-6200. The product oligosaccharides were detected with a Hitachi L3350 RI monitor and the oligosaccharide concentrations were calculated from peak areas using standard curves obtained from pure oligosaccharide solutions. Initial velocity was determined from the decrease in the substrate concentration. The kinetic constants were calculated from double reciprocal plots of initial velocity *versus* the substrate concentration.

Chitosanase Concentration—The protein concentration was determined from the UV absorbance at 280 nm using molar absorption coefficients; 30,300 $M^{-1} \cdot cm^{-1}$ (*13*).

TLC Analysis of the Enzymatic Products from $(GlcN)_3$-UMB—To determine the mode of the hydrolysis of $(GlcN)_3$-UMB by chitosanase, the hydrolytic products were analyzed by TLC. One milligram of $(GlcN)_3$-UMB was dissolved in 200 μl of 50 mM sodium acetate buffer, pH 5.5. The enzyme solution was added to the substrate solution and the mixture was incubated for 18 h at 40°C. The final concentration of the enzyme was 4.9 μM. The reaction mixture was concentrated to 30 μl, and a small portion of the mixture was spotted on a TLC plate, DC-Fertigplatten SIL G-25 UV_{254} (MACHEREY-NAGEL). The plate was developed with *n*-butanol:methanol:28% ammonium hydroxide (4:3:3). UMB and $(GlcN)_3$-UMB were developed on the same plate as controls. After development, the plate was dried and exposed to ultraviolet light (312 nm) for detection of the chromophore.

RESULTS

Enzymatic Deacetylation of $(GlcNAc)_3$-UMB—Figure 2A shows the upfield region of the ^{1}H-NMR spectrum of $(GlcNAc)_3$-UMB. The resonances at 1.8-1.9 ppm are derived from the acetyl methyl proton of the $(GlcNAc)_3$ moiety. After enzymatic deacetylation of the compound, the acetyl methyl resonances disappeared (Fig. 2B). A new resonance appearing at 1.72 ppm might be due to an acetate methyl resonance. FAB-MS and MALDI TOF-MS analyses indicated the enzymatic product to have a molecular weight of 659 (data not shown). By these results, we have confirmed that chitin deacetylase from *C. lindemuthianum* produces $(GlcN)_3$-UMB from the substrate $(GlcNAc)_3$-UMB. The $(GlcN)_3$-UMB obtained was successfully purified by the procedure described in "MATERIALS AND METHODS."

Hydrolysis of $(GlcN)_3$-UMB by Streptomyces sp. N174 Chitosanase—The fluorescence spectrum of $(GlcN)_3$-UMB obtained by excitation at 360 nm changed when chitosanase was added to the solution, as shown in the inset in Fig. 3. Before the addition of chitosanase, no fluorescence maximum was found (spectrum B). After incubation with chitosanase at 40°C for 80 min, however, a clear fluorescence maximum at 450 nm emerged (spectrum A). The main part of Fig. 3 shows time courses of the fluorescence intensity change in the presence or absence of chitosanase. Fluorescence intensity increased linearly with reaction time in the presence of chitosanase, but not in its absence. Furthermore, the rate of increase in fluorescence intensity was found to be proportional to the concentration of chitosanase added, as shown in Fig. 4. Thus, the increase in fluorescence intensity indicates that the chitosanase hydrolyzes the glycosidic linkage between the GlcN residue and UMB moiety of the substrate releasing the fluorescent UMB molecule.

Steady State Kinetics—Figure 5 shows a double reciprocal plot for $(GlcN)_3$-UMB hydrolysis by chitosanase. The kinetic constants of the enzyme were calculated to be $K_m = 201\ \mu M$, $k_{cat} = 8.1 \times 10^{-5}\ s^{-1}$, $k_{cat}/K_m = 0.40\ s^{-1} \cdot M^{-1}$.

For comparison, we attempted to determine the steady state kinetic constants using $(GlcN)_n$ ($n = 4$, 5, or 6) as the substrate. Figure 6 shows double reciprocal plots for $(GlcN)_n$ hydrolysis catalyzed by *Streptomyces* sp. chitosanase. In the case of substrate $(GlcN)_6$, the relationship between the initial velocity and the substrate concentration exhibited a normal Michaelis-Menten type profile, resulting in a straight line in the double reciprocal plot. When $(GlcN)_5$ was used instead of $(GlcN)_6$, however, substrate inhibition was observed in the substrate concentration

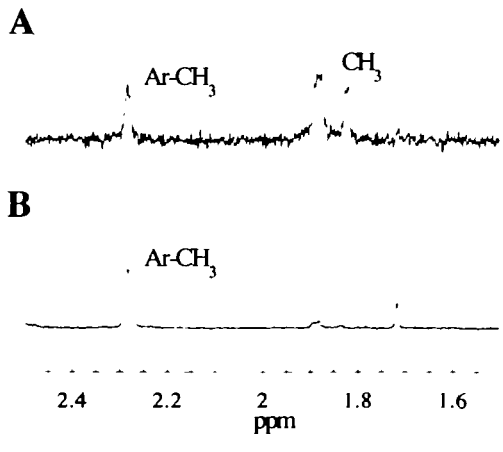

Fig. 2. **Upfield region of the ^{1}H-NMR spectra of $(GlcNAc)_3$-UMB (A) and the product obtained by enzymatic deacetylation of $(GlcNAc)_3$-UMB (B).** Ar-CH_3 indicates the signal derived from the UMB group. CH_3 indicates the signals derived from the *N*-acetyl groups of the $(GlcNAc)_3$ moiety.

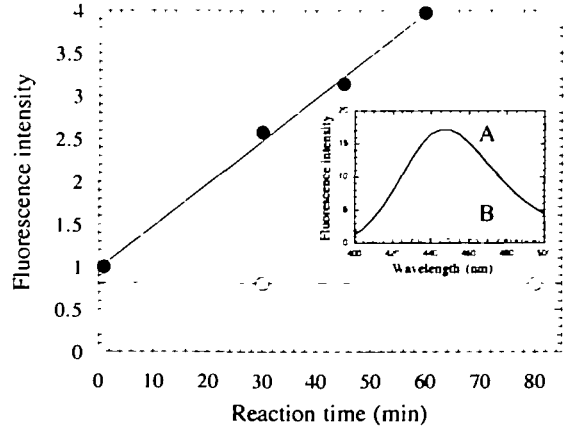

Fig. 3. **Time course of the increase in fluorescence intensity in the presence or absence *Streptomyces* sp. N174 chitosanase.** $(GlcN)_3$-UMB concentration was 28.0 μM. Enzyme concentration was 1.98 μM. The other reaction conditions are described in the text. Inset: Fluorescence spectrum of $(GlcN)_3$-UMB exited at 360 nm. A, after incubation with *Streptomyces* sp. N174 chitosanase (3.95 μM) for 80 min at 40°C; B, intact $(GlcN)_3$-UMB.

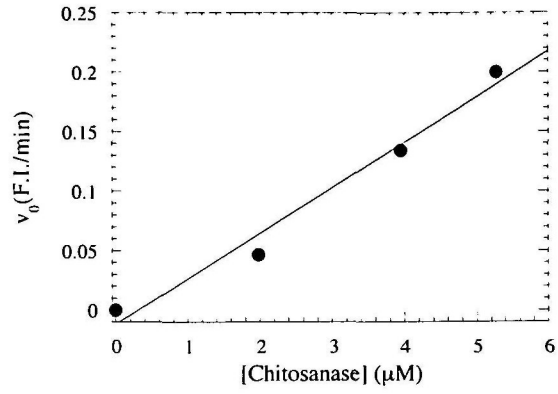

Fig. 4. **Effect of *Streptomyces* sp. N174 chitosanase concentration on the rate of increase in fluorescence intensity.** $(GlcN)_3$-UMB concentration was 28 μM. The other reaction conditions are described in the text.

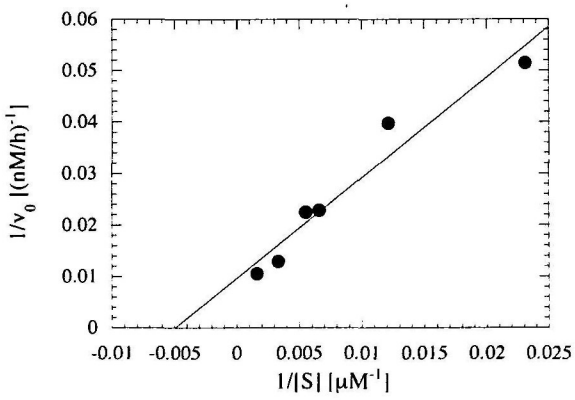

Fig. 5. **Double reciprocal plot for $(GlcN)_3$-UMB hydrolysis catalyzed by *Streptomyces* sp. N174 chitosanase.** Experimental conditions and kinetic parameters are described in the text.

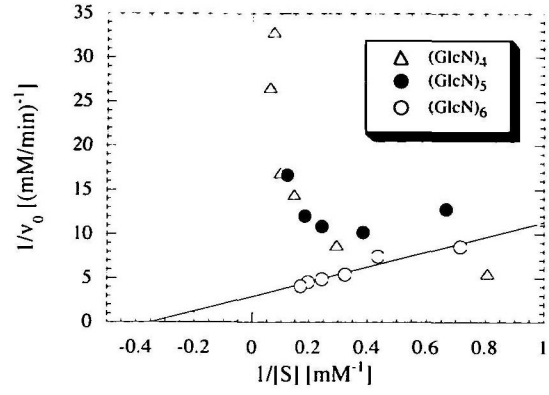

Fig. 6. **Double reciprocal plots for $(GlcN)_n$ (n=4, 5, and 6) hydrolysis catalyzed by *Streptomyces* sp. N174 chitosanase.** Experimental conditions and the kinetic constants obtained are described in the text.

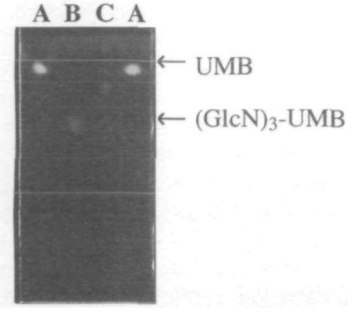

Fig. 7. **TLC profile of the chitosanase products from $(GlcN)_3$-UMB.** A, UMB; B, $(GlcN)_3$-UMB; C, enzymatic products. Details are described in the text.

range above 3 mM. This substrate inhibition was more intense in the case of substrate $(GlcN)_4$. Thus we determined only the kinetic constants for $(GlcN)_6$. By fitting line as shown in Fig. 6, the kinetic constants for $(GlcN)_6$ were calculated to be $K_m = 2.9$ mM, $k_{cat} = 107\ s^{-1}$, $k_{cat}/K_m = 3.7 \times 10^4\ s^{-1} \cdot M^{-1}$.

Mode of the Chitosanase Hydrolysis of (GlcN)$_3$-UMB— Chitosanase might cleave not only the glycosidic linkage between the GlcN residue and UMB moiety but also the linkage between GlcN residues. Thus we tried to obtain a product distribution profile for the enzymatic hydrolysis of $(GlcN)_3$-UMB. Figure 7 shows the TLC profile of the enzymatic products from $(GlcN)_3$-UMB. Two fluorescent spots were observed (lane C). One of the spots showed the same R_f value as that of the UMB control (lane A), but the other migrated to a position between the UMB and $(GlcN)_3$-UMB spots. Considering the R_f-value and the splitting specificity of the chitosanase, we identified the latter spot as GlcN-UMB. Thus, the enzymatic hydrolysis of $(GlcN)_3$-UMB occurs in two positions, producing the products $(GlcN)_3$ + UMB and $(GlcN)_2$ + GlcN-UMB.

DISCUSSION

In order for the fluorescent aglycon to be released from the synthetic substrate by the action of chitosanase, the design of the substrate should be based on the minimum structural requirements of the natural substrates. Marcotte *et al.* reported that the substrate binding cleft of *Streptomyces* sp. N174 chitosanase consists of six subsites, so called A, B, C, D, E, and F, from the non-reducing end residue (*16*). Bond cleavage takes place between sites D and E through the concerted action of the acidic amino residues Glu22 and Asp40 (*13*). We previously reported that the enzyme produces mainly $(GlcN)_3$ from the substrate $(GlcN)_6$, and that the $(GlcN)_3$ produced is not further decomposed by the enzyme. The substrate $(GlcN)_5$ is hydrolyzed to equimolar concentrations of $(GlcN)_3$ and $(GlcN)_2$, and $(GlcN)_4$ is hydrolyzed to two molecules of $(GlcN)_2$ (*6*). From the structural and functional information, we decided to synthesize $(GlcN)_3$-UMB because this should be the minimum structural requirement for the release of the UMB molecule by chitosanase action. Furthermore, the splitting mode of the chitosanase is similar to that of chitinase from barley seeds, that is, the chitinase produces mainly $(GlcNAc)_3$ from the substrate $(GlcNAc)_6$ (*9*). Comparative X-ray crystallography showed both enzymes to have an identical archetypal catalytic core in their crystal structures (*16*,

17). The similarity in the structures and functions of *Streptomyces* sp. N174 and barley chitinase led us to synthesize $(GlcN)_3$-UMB, because $(GlcNAc)_3$-UMB was successfully hydrolyzed by barley chitinase releasing the fluorescent UMB moiety (*9*). As shown in Fig. 3, the fluorescence intensity derived from free UMB molecules increased with reaction time, indicating that the chitosanase can hydrolyze the bond between the GlcN residue and the UMB moiety. From TLC analysis of the hydrolytic products from $(GlcN)_3$-UMB, chitosanase was found to hydrolyze not only the glycosidic linkage between the GlcN residue and UMB moiety, but also the linkage between GlcN residues. Nevertheless, the synthetic substrate allows analysis at low substrate concentrations, and thus should be useful for evaluating chitosanase activity. The mode of action of chitosanase toward $(GlcN)_n$ was investigated for several bacterial chitosanases. Among them, the enzymes from *Bacillus pumilus* (Honda *et al.*, unpublished) and *Nocardia orientalis* (*3*) have reaction mechanisms similar to that of *Streptomyces* sp. N174 chitosanase. Thus, $(GlcN)_3$-UMB would be a useful substrate for these enzymes as well.

The kinetic behaviour of chitosanase toward the substrate $(GlcN)_3$-UMB was investigated in comparison with the substrates $(GlcN)_n$ ($n=4$, 5, and 6). As shown in Fig. 6, substrate inhibition was observed with $(GlcN)_4$ and $(GlcN)_5$. The shorter the substrate chain length, the stronger the substrate inhibition. The shorter oligomeric substrate might be able to bind to vacant subsites (*18*) producing a deadend enzyme-substrate complex. In the substrate concentration range below 1 mM, the hydrolytic rate of chitosanase cannot be evaluated because of the low sensitivity of the refractive index detection used in HPLC analysis. Thus, kinetic constants could not be obtained with the shorter chain length substrates. On the other hand, no substrate inhibition was observed when $(GlcN)_3$-UMB was used as the substrate, and the steady state kinetic constants were successfully obtained with the synthetic substrate. The k_{cat} value obtained for $(GlcN)_3$-UMB was much lower than that for $(GlcN)_6$. The difference in the k_{cat} values might be rationalized as substrate size dependence. Substrate size dependence of the rate constant has been reported for hen egg white lysozyme, goose egg white lysozyme, and barley chitinase (*19, 20*). *Streptomyces* sp. N174 chitosanase, whose structure is similar to those of goose egg white lysozyme and barley chitinase, might have a similar substrate size dependence resulting in the much lower k_{cat} value for $(GlcN)_3$-UMB. Another explanation for the difference in k_{cat} values is the splitting specificity; that is, chitosanase can hardly attack the terminal glycosidic linkage of the $(GlcN)_n$ substrate. In fact, the chitosanase produces little GlcN monomer from any $(GlcN)_n$ substrate (*6*). The release of UMB from $(GlcN)_3$-UMB results from the terminal attack of the chitosanase. Thus, the k_{cat} value for UMB release from $(GlcN)_3$-UMB is much lower than that for $(GlcN)_6$ hydrolysis. We think that the latter explanation is more likely in this case.

We conclude that a novel substrate, $(GlcN)_3$-UMB, is convenient for kinetic analyses of chitosanases, especially at low substrate concentrations. Further kinetic studies of the *Streptomyces* sp. N174 chitosanase is now in progress in our laboratory using the novel substrate.

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