Reaction mechanism of chitosanase from *Streptomyces* sp. N174

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Chitosanase was produced by the strain of *Streptomyces lividans* TK24 bearing the *csn* gene from *Streptomyces* sp. N174, and purified by S-Sepharose and Bio-Gel A column chromatography. Partially (25–35%) N-acetylated chitosan was digested by the purified chitosanase, and structures of the products were analysed by NMR spectroscopy. The chitosanase produced hetero-oligosaccharides consisting of D-GlcN and GlcNAc in addition to glucosamine oligosaccharides [$(GlcN)_n$, $n = 1$, 2 and 3]. The reducing- and non-reducing-end residues of the hetero-oligosaccharide products were GlcNAc and GlcN respectively, indicating that the chitosanase can split the GlcNAc-GlcN linkage in addition to that of GlcN-GlcN. Time-dependent ^{1}H-NMR spectra showing hydrolysis of $(GlcN)_6$ by the chitosanase were obtained in order to determine the anomeric form of the reaction products. The chitosanase was found to produce only the α-form; therefore it is an inverting enzyme. Separation and quantification of $(GlcN)_n$ was achieved by HPLC, and the time course of the reaction catalysed by the chitosanase was studied using $(GlcN)_n$ ($n = 4$, 5 and 6) as the substrate. The chitosanase hydrolysed $(GlcN)_6$ in an endo-splitting manner producing $(GlcN)_2$, $(GlcN)_3$ and $(GlcN)_4$, and did not catalyse transglycosylation. Product distribution was $(GlcN)_3 \gg (GlcN)_2 > (GlcN)_4$. Cleavage to $(GlcN)_3 + (GlcN)_3$ predominated over that to $(GlcN)_2 + (GlcN)_4$. Time courses showed a decrease in rate of substrate degradation from $(GlcN)_6$ to $(GlcN)_5$ to $(GlcN)_4$. It is most likely that the substrate-binding cleft of the chitosanase can accommodate at least six GlcN residues, and that the cleavage point is located at the midpoint of the binding cleft.

INTRODUCTION

Chitinolytic enzymes have been recognized as pathogenesis-related proteins and applied to biological control of plant disease [1]. These enzymes are also useful for obtaining chito-oligosaccharides which have interesting biological properties, such as eliciting pathogenesis-related proteins in higher plants [2] and immunopotentiating effectors [3]. For the effective utilization of chitinolytic enzymes, however, enzymological information is required. Several investigations of the structure and function of chitinases have been performed. In particular, a site-directed mutagenesis study of chitinase from *Bacillus circulans* WL-12 identified the amino acid residues responsible for catalysis [4]. Three-dimensional structures of chitinases from *Hordeum vulgare* L. seeds [5] and *Serratia marcescens* [6] have been elucidated by X-ray crystallography. Further data on the structure and function of chitinases are being accumulated. On the other hand, information on chitosanases is quite limited. The primary structure has been deduced for only three chitosanases from microbes ([7,8]; J.-Y. Masson, I. Boucher, W. A. Neugebauer, D. Ramotar and R. Brzezinski, unpublished work). The mode of action toward partially *N*-acetylated chitosan has been investigated for only a few chitosanases [9,10]. In addition to these data, structural information from X-ray crystallography and functional information from protein engineering would be very useful.

Masson et al. [11] have reported efficient production of chitosanase by using recombinant *Streptomyces lividans* strains carrying the cloned *csn* (formerly *chs* but was modified to *csn* [8] to avoid confusion with chitin synthetase) gene from *Streptomyces* sp. N174. The chitosanase produced by this system has been crystallized, and the resolution of its three-dimensional structure from X-ray-diffraction data is in progress [12]. Thus *Streptomyces* sp. N174 chitosanase produced by *S. lividans* was considered to be the most appropriate for investigation of the structure and function of chitosanases. In the present study, the properties of the reaction catalysed by wild-type chitosanase from *Streptomyces* sp. N174 were investigated to obtain essential basic information for future studies by site-directed mutagenesis. Chitosanase digestion of partially *N*-acetylated chitosan or $(GlcN)_n$ ($n = 4$, 5 and 6) was carried out, and the structures of the products were analysed by HPLC and NMR spectroscopy. From these results, the active-site properties of the enzyme were estimated and the specificity was compared with those of other chitosanases.

MATERIALS AND METHODS

Materials

Partially *N*-acetylated chitosan (25–35% acetylated) prepared from crab shells was purchased from Wako. CM-Sephadex C-25 and S-Sepharose Fast Flow were from Pharmacia, Bio-Gel A-0.5m was from Bio-Rad, and Cellulofine Gcl-25m from Seikagaku Kogyo Co. $(GlcN)_n$ ($n = 2, 3, \ldots 6$) were also purchased from Seikagaku Kogyo Co. ^2H_2O was purchased from MSD Isotopes, and [^{2}H]acetic acid (99.5%) and its sodium salt (99%) were from Aldrich Chemical Inc. Other reagents were of analytical grade.

Bacterial strains and plasmids

The bacterial strain used for chitosanase production was *S. lividans* TK24, transformed with plasmid pRL270, a derivative of the vector pFD666 [13] carrying the wild-type chitosanase gene (*csn*) from *Streptomyces* sp. N174 [11]. Methods for

Abbreviations used: $(GlcN)_n$, β-1,4-linked oligosaccharide of GlcN with a degree of polymerization of n; $(GlcNAc)_n$, β-1,4-linked oligosaccharide of GlcNAc with a degree of polymerization of n.

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propagation and storage of this strain were as previously described [11].

Enzyme production and purification

S. lividans TK24 [pRL270] was grown in 50 ml of Tryptic Soy Broth (Difco) supplemented with 50 μg/ml kanamycin. This culture was centrifuged (10 min; 800 ***g***), and 5 ml of the mycelial pellet was used to inoculate 500 ml of glucosamine medium consisting of 1.5% D-glucosamine and 0.5% starch in minimal salts [11]. After 60–72 h of growth at 30 °C with vigorous shaking (300 rev./min), the culture was filtered twice through a Schleicher & Schuell filter paper no. 410. The culture filtrate was concentrated to 40 ml by tangential-flow ultrafiltration on Minitan (Millipore). The conductivity of the enzyme preparation was then adjusted to 3 mS with distilled water and the solution loaded on to a column (1.6 mm × 65 mm) of S-Sepharose Fast Flow, previously equilibrated with 50 mM sodium acetate buffer, pH 4.5 (buffer A). The column was washed with 20 ml of buffer A followed by 20 ml of buffer A containing 0.22 M NaCl, and chitosanase was eluted with 20 ml of buffer A containing 0.27 M NaCl; 2.5 ml fractions were collected. The four most active fractions were pooled and further purified by size-exclusion chromatography on a column (1.6 cm × 98 cm) packed with Bio-Gel A-0.5m (200–400 mesh) equilibrated with 100 mM sodium acetate buffer, pH 5.0. Fractions of volume 2.5 ml were collected, and those containing purified chitosanase were pooled and stored at −20 °C after addition of 1 vol. of sterile glycerol.

Determination of chitosanase

Chitosanase activity was measured as described [14], using soluble chitosan as substrate. Protein concentration was determined by the method of Bradford [15].

Digestion of partially *N*-acetylated chitosan

Partially *N*-acetylated chitosan (4.5 g) was dissolved in 500 ml of 0.1 M sodium acetate buffer, pH 5.3, and mixed with 45 nmol of *Streptomyces* sp. N174 chitosanase and 5 ml of 2% NaN_3. After 48 h of incubation (37 °C), an additional 23 nmol of the chitosanase was added to the solution and the mixture was further incubated for 48 h. The reaction mixture was boiled for 5 min to terminate the reaction and adjusted to pH 9.4 with 0.1 M NaOH to precipitate products with a high degree of polymerization. The supernatant was used as a chitosanase hydrolysate in the following experiments.

Purification of the products from partially *N*-acetylated chitosan

The chitosanase hydrolysate was loaded on a column (3.5 cm × 10 cm) of charcoal in order to separate $(GlcN)_n$ products [16]. The column was washed with distilled water, and the fraction that passed through the charcoal column was pooled and further applied to a column (2.0 cm × 25 cm) of Dowex 50W (X2). $(GlcN)_n$ products were eluted with a linear gradient from distilled water to 3 M HCl. Each of the reducing-sugar fractions obtained was pooled and used for structural analysis. The fraction that was adsorbed to the charcoal column was eluted with 60% ethanol. This fraction was then evaporated under reduced pressure, and dissolved in 10 mM sodium acetate buffer, pH 5.0 (buffer B) before being loaded on to a column of CM-Sephadex C-25 equilibrated with buffer B. After the column had been washed with buffer B, the oligosaccharides adsorbed to the CM-Sephadex resin were eluted with a gradient from buffer B to buffer B containing 1 M NaCl. Each of the reducing-sugar fractions was pooled and evaporated. The fractions containing an impurity were further purified on a gel-filtration column (2.2 cm × 170 cm) of Cellulofine Gcl-25m equilibrated with 0.1 M sodium acetate buffer, pH 5.0. After being desalted through an electronic dialyser (Micro Acilyzer G1; Asahikasei Kogyo), each fraction was used for structural analysis.

Reducing-sugar analysis

The reducing-sugar concentration in each fraction separated by column chromatography was measured by the method of Imoto and Yagishita [17].

HPLC

The oligosaccharide product obtained by CM-Sephadex C-25 chromatography was applied to a gel-filtration column (7.5 mm × 600 mm) of TSK-GEL G2000PW (Tosoh) using a Shimadzu LC-3A to examine its purity and apparent molecular mass. Elution was achieved with 0.1 M NaCl at room temperature and a flow rate of 0.3 ml/min. Oligosaccharides containing GlcNAc were monitored by UV absorption at 220 nm using a Shimadzu SPD-2A.

NMR spectroscopy

Several milligrams of the purified product were lyophilized three times from ^2H_2O, and then dissolved in 0.5 ml of ^2H_2O. The pH value of each sample was determined by a direct meter reading without correction for isotope effect, and the sample adjusted to pH 4.0–5.0 by addition of concentrated ^2HCl or NaO^2H. ^1H- and ^{13}C-NMR spectra were obtained using a 5 mm probe on a JEOL EX-270 instrument.

Determination of anomeric form of the hydrolytic products

The substrate, $(GlcN)_6$, was lyophilized three times from ^2H_2O, and then dissolved in 0.5 ml of 10 mM deuterated sodium acetate buffer, pH 4.5. The substrate solution was placed in a 5 mm NMR tube, and the enzyme (0.36 nmol) added. The NMR tube was immediately set into the NMR probe, which was thermostatically controlled at 30 °C. After an appropriate reaction time, accumulation of a ^1H-NMR spectra was started. The accumulation required 3 min. The substrate concentration was 8.3 mM.

Time course of hydrolysis of GlcN oligosaccharides by chitosanase

The substrate, $(GlcN)_n$ ($n = 4$, 5 or 6), was dissolved in 50 mM sodium acetate buffer, pH 5.5, to give a 16.6 mM solution. The enzyme (0.19 nmol) was added to 0.5 ml of the substrate solution, and the reaction mixture was incubated at 40 °C. After an appropriate reaction time, a portion of the reaction mixture was withdrawn and mixed with an equal volume of 0.1 M NaOH to terminate the reaction. The products obtained were analysed by HPLC. Separation of $(GlcN)_n$ was achieved using a gel-filtration column of TSK-GEL G2000PW in a similar manner to that described above. In this case, however, elution was carried out with 0.5 M NaCl, and $(GlcN)_n$ was detected with a differential refractometer (Shimadzu RID-2A). $(GlcN)_n$ concentrations were calculated using standard curves obtained with authentic saccharide solutions.

RESULTS

Chitosanase production and purification

Several modifications were introduced to the production and purification method used previously [11,14]. The chitosan

Table 1 Purification procedure for chitosanase from *Streptomyces* sp. N174

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification factor
Culture supernatant (500 ml)	396	7500	18.9	100	1.00
Ultrafiltration liquor (40 ml)	240	5790	24.1	77.2	1.27
S-Sepharose eluate (10 ml)	74	3920	53.0	52.2	2.80
Bio-Gel A eluate (10 ml)	58	3420	58.9	45.6	3.11

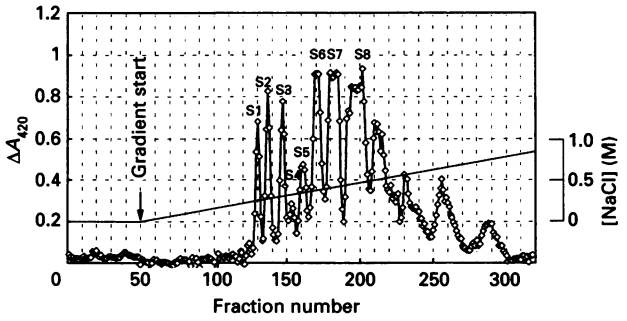

Figure 1 CM-Sephadex C-25 column (2.5 cm × 45 cm) chromatography of hetero-oligosaccharide products from the incubation of partially *N*-acetylated chitosan with *Streptomyces* sp. N174 chitosanase

The chitosanase hydrolysate was fractionated on a charcoal column, and the adsorbed fraction was applied to this column. Elution was performed with 10 mM sodium acetate buffer, pH 5.0. Linear gradient elution with NaCl was started at fraction number 50.

medium was replaced with one containing glucosamine as main carbon source. In this medium, a much lower level of proteolytic activity is observed (I. Boucher and R. Brzezinski, unpublished work). For enzyme concentration, ultrafiltration was used instead of precipitation with polyacrylic acid. In addition, stepwise elution was used instead of gradient elution during the ion-exchange chromatography step. Purification of chitosanase was improved by these modifications. The procedure is summarized in Table 1.

Products from incubation of partially *N*-acetylated chitosan with *Streptomyces* sp. N174 chitosanase

The purified chitosanase thus obtained was used to digest partially *N*-acetylated chitosan. The hydrolysate produced was first loaded on a charcoal column, and the fraction that passed through the column was applied to a column of Dowex 50W(X2). Three reducing-sugar fractions were separated and identified by ^{13}C-NMR analysis as GlcN, $(GlcN)_2$ and $(GlcN)_3$. Product distribution was $(GlcN)_2 \gg (GlcN)_3 > GlcN$. Thus the chitosanase was found to split the GlcN-GlcN linkage, producing $(GlcN)_2$ as the main product and small amounts of $(GlcN)_3$ and GlcN.

The fraction that was adsorbed to the charcoal was recovered by elution with 60% ethanol, and applied to a CM-Sephadex C-25 column. Gradient elution from 10 mM sodium acetate buffer to buffer containing 1 M NaCl gave many reducing-sugar fractions (S1–S8) as shown in Figure 1. No reducing-sugar fraction was obtained before the start of gradient elution, indicating that the chitosanase hydrolysate does not contain homo-oligosaccharides of GlcNAc [$(GlcNAc)_n$]. From the elution profile, S1, S2 and S3 were estimated to be of the highest purity. Therefore these three fractions were used for HPLC and NMR analysis after desalting with an electronic dialyser.

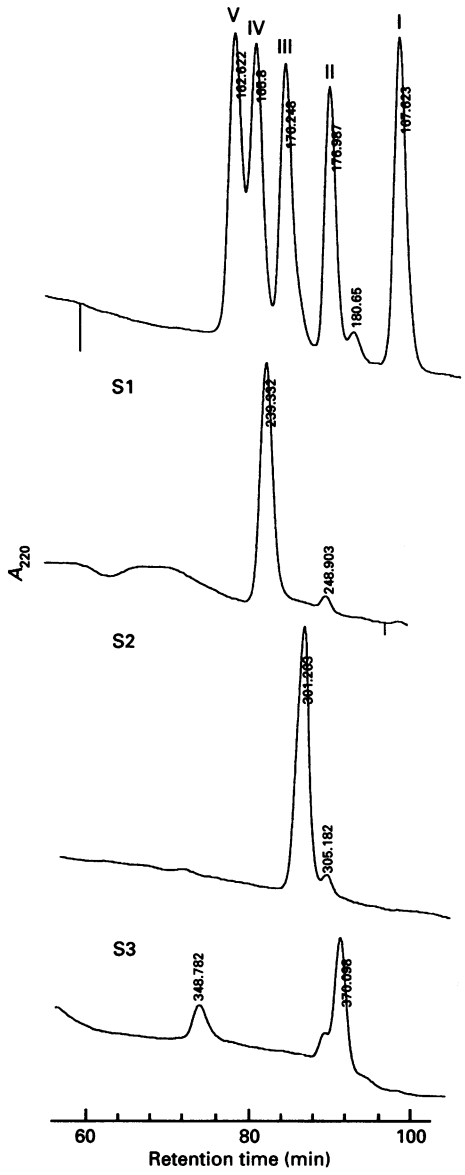

Figure 2 HPLC profiles of reducing-sugar fractions obtained by CM-Sephadex C-25 column chromatography

Fractions of S1, S2 and S3 (Figure 1) were directly applied to a column of TSK-GEL G2000PW, eluted with 0.1 M NaCl at a flow rate of 0.3 ml/min. The HPLC profile of the mixture of $(GlcNAc)_n$ (n = 1–5) is also shown at the top as a molecular-mass standard. Roman numerals indicate the n-value of $(GlcNAc)_n$.

HPLC profiles of S1, S2 and S3 are shown in Figure 2. For S1 and S2, although a very small amount of impurity was found at the retention time corresponding to $(GlcNAc)_2$, the main component of each sample exhibited a clear single peak. As judged from the retention time, S1 and S2 should be tetrasaccharide and

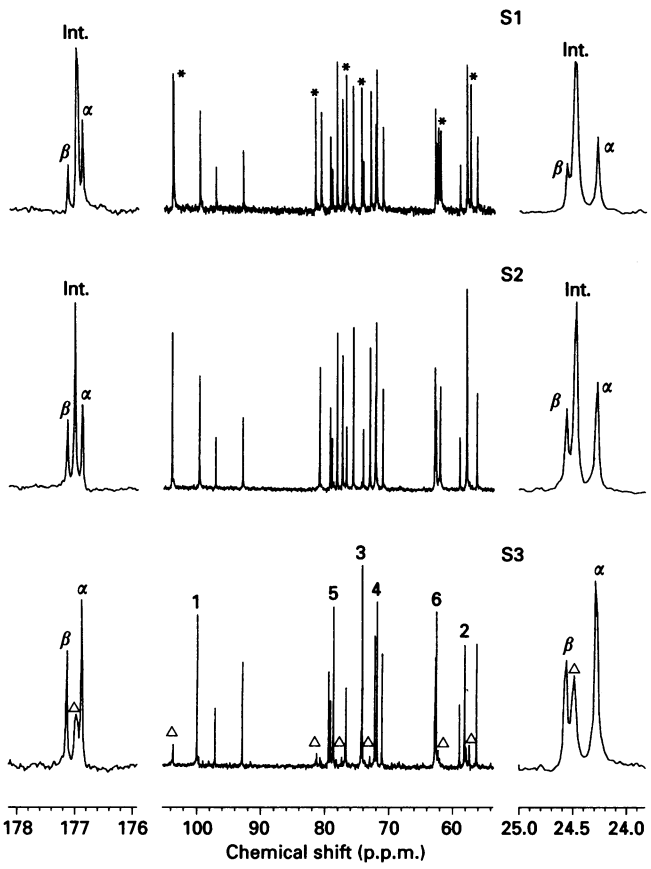

Figure 3 ^{13}C-NMR spectra (67.8 MHz) of S1, S2 and S3

α, β and Int. indicate the signals derived from the anomers of the reducing-end residue and internal residue respectively. For the chemical shifts and relative intensities, all signals of S2 are completely consistent with those of S1 except for those indicated by asterisks. The signals indicated by numbers in the spectrum of S3 are derived from the GlcN residue at the non-reducing end. The signals indicated by triangles are derived from impurities.

trisaccharide respectively. For S3, however, a considerable amount of impurity was found in addition to the main disaccharide component.

^{13}C-NMR spectra of these fractions are shown in Figure 3. According to previous assignments [18–20], the signals observed at 177 p.p.m. are derived from *N*-acetyl carbonyl carbons, and those at 24–25 p.p.m. are from *N*-acetyl methyl carbons. C-1 carbon signals appear in the region 90–105 p.p.m., and the rest are derived from C-2–C-6 carbons. The spectrum of S2 was exactly the same as that reported for GlcN-GlcNAc-GlcNAc obtained by digestion of partially *N*-acetylated chitosan by chitosanase from *Bacillus pumilus* BN-262 [16], thus this fraction must be GlcN-GlcNAc-GlcNAc. For S1, the profiles of the methyl and carbonyl carbon regions were similar to those of S2, but the peak area of the most central signal of each region was larger than that of S2. Therefore S1 must have an additional GlcNAc residue in its sequence. Thirty singlet signals are detected in the C-1–C-6 carbon region (55–105 p.p.m.) of the spectrum of S1. For 24 of these (the signals not indicated by asterisks), the chemical-shift values and the relative intensities are in accord with those of all signals in the same region of the spectrum for S2. Thus the sequence of S2, GlcN-GlcNAc-GlcNAc, must also exist in S1. The other six signals of S1 (indicated by asterisks in Figure 3) must be derived from an additional saccharide unit. The chemical-shift values of the six signals are 103.6, 81.3, 76.8, 74.0, 62.2 and 57.5 p.p.m., which are consistent with the chemical shifts reported for C-1, C-4, C-5, C-3, C-6 and C-2 carbon signals respectively of the internal GlcNAc residue after a constant +2.0 p.p.m. correction of the literature values [18]. Thus the additional unit is identified as an internal GlcNAc residue, and we concluded that S1 is GlcN-GlcNAc-GlcNAc-GlcNAc. The structures of S1 and S2 deduced from ^{13}C-NMR spectra are consistent with the HPLC results (Figure 2).

As shown in the HPLC profile of S3, the main component of S3 is a disaccharide. If the disaccharide has two *N*-acetyl groups, three singlet signals should be observed in each of the carbonyl and methyl regions, as seen in the spectrum of S2. For S3, although three signals were observed in each region, the intensity of the most central signal is quite a bit smaller than that of S2. Thus the midpoint signal must be derived from an impurity, and the disaccharide S3 must have only one *N*-acetyl group at its reducing end. The chemical-shift values of the signals indicated by numbers in the spectrum of S3 are consistent with the reported values of ^{13}C signals of a GlcN residue at the non-reducing end after a constant +1.5 p.p.m. correction of the literature values [20]. The other signals in the C-1–C-6 region could be assigned to the reducing-end GlcNAc residue. Thus the disaccharide component of S3 was identified as GlcN-GlcNAc.

S6, S7 and S8 were further purified by gel filtration on Cellulofine Gcl-25m, and the purified oligosaccharides were analysed by HPLC and NMR. HPLC analysis indicated that S6, S7 and S8 are a pentasaccharide, a tetrasaccharide and a trisaccharide respectively. The spectra of S7 and S8 exhibited exactly the same profiles as those of the spectra reported for GlcN-GlcN-GlcNAc-GlcNAc and GlcN-GlcN-GlcNAc respectively [16]. Thus we concluded that S7 and S8 are GlcN-GlcN-GlcNAc-GlcNAc and GlcN-GlcN-GlcNAc respectively. Identification of S6 was not achieved because the ^{13}C signals in its spectrum were too crowded. On careful comparison with the spectrum of S7, however, several ^{13}C signals that seemed to be derived from an additional GlcNAc unit were observed in the spectrum of S6. S6 therefore appears to be GlcN-GlcN-GlcNAc-GlcNAc-GlcNAc.

All of these results indicate that the chitosanase hydrolyses the partially *N*-acetylated chitosan, producing $(GlcN)_n$ and hetero-oligosaccharides in which the reducing- and non-reducing-end residues are GlcNAc and GlcN respectively. The chitosanase can split the glycosidic linkage GlcNAc-GlcN in addition to GlcN-GlcN.

Anomeric form of the reaction products

Determination of the anomeric structure of the hydrolytic products is important for understanding the reaction mechanism. Information on anomeric specificity has been obtained for several chitinases [21,22], but not for chitosanases. Therefore we set out to determine the anomeric form of hydrolytic products of the chitosanase from *Streptomyces* sp. N174 by ^{1}H-NMR spectroscopy. A time-dependent profile of the ^{1}H-NMR spectra showing hydrolysis of $(GlcN)_6$ by the chitosanase is shown in Figure 4. According to the assignments reported thus far [20], the signals at 5.43 p.p.m. (H1α) and 4.87 p.p.m. (H1β) are derived from the anomeric proton of reducing-end GlcN residue in the α-form and β-form respectively. The signals at 4.7–4.8 p.p.m. (H1) are from the anomeric protons of the other GlcN residues. With time, the relative intensities of the signals of H1 decreased and those of the reducing-end signals (H1α+H1β) increased. The peak areas of H1, H1α and H1β relative to the total peak area of

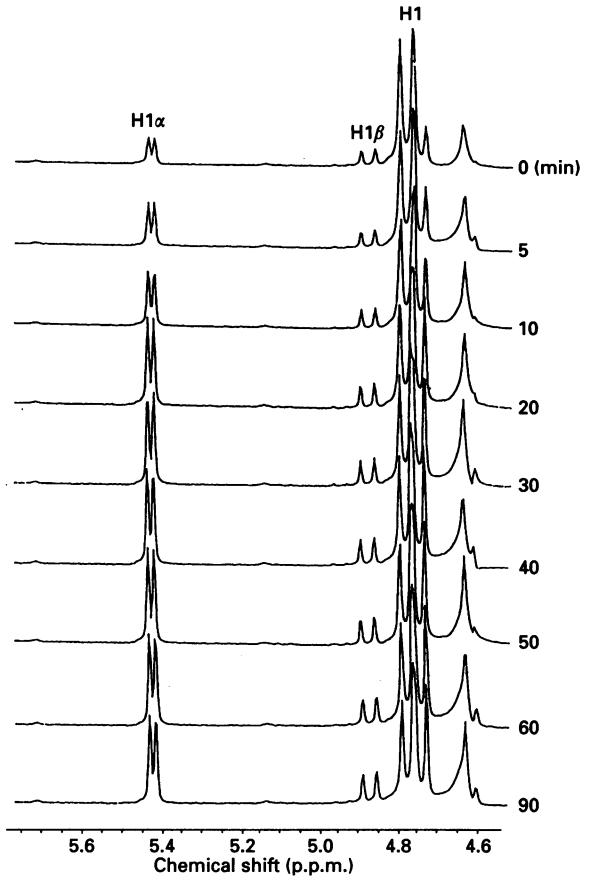

Figure 4 Time-dependent ^{1}H-NMR spectra showing hydrolysis of $(GlcN)_6$ by *Streptomyces* sp. N174 chitosanase

H1α, H1β and H1 indicate the signals derived from the anomeric protons of the reducing-end residue and those of the other GlcN residues respectively. The enzyme (0.36 nmol) was mixed with 0.5 ml of 8.3 mM $(GlcN)_6$ solution dissolved in 10 mM deuterated sodium acetate buffer, pH 4.5, in an NMR tube, and then the reaction was conducted in the NMR probe at 30 °C.

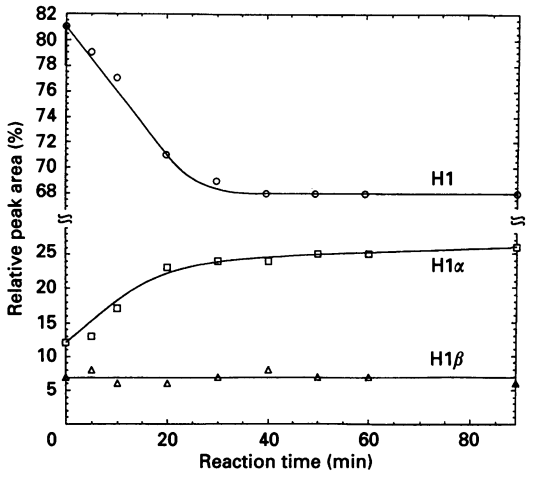

Figure 5 Time courses of $(GlcN)_6$ degradation and anomer formation catalysed by *Streptomyces* sp. N174 chitosanase

The relative peak areas of H1α (□), H1β (△) and H1 (○) to the total peak area of the anomeric protons (H1α + H1β + H1) were determined from the spectra shown in Figure 4, and plotted against reaction time.

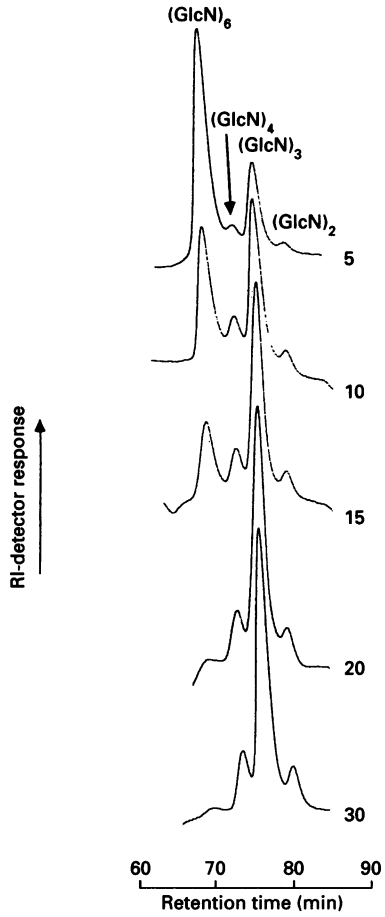

Figure 6 Time-dependent profile showing the hydrolysis of $(GlcN)_6$ by *Streptomyces* sp. N174 chitosanase

A gel-filtration column (7.5 mm × 600 mm) of TSK-GEL G2000PW was used for the separation, and elution was performed with 0.5 M NaCl at room temperature at a flow rate of 0.3 ml/min. $(GlcN)_n$ were detected with a differential refractometer. The enzyme (0.19 nmol) was added to 0.5 ml of 16.6 mM $(GlcN)_6$ solution dissolved in 50 mM sodium acetate buffer, pH 5.5. The reaction was carried out at 40 °C. The numbers indicate the reaction time in minutes. RI, refractive index

anomeric proton signals (H1α + H1β + H1) were calculated and plotted against reaction time (Figure 5). An increase in relative peak area was found only in H1α. Thus the anomeric form of the hydrolytic products was determined to be α, indicating that *Streptomyces* sp. N174 chitosanase is an inverting enzyme. It was also found that the rate of mutarotation of $(GlcN)_n$ is quite a bit lower than that of $(GlcNAc)_n$ [22,23].

Time course of GlcN oligosaccharide digestion by the chitosanase

When investigating the reaction mechanism of carbohydrolases, it is important to study the experimental time course of oligosaccharide digestion, because much information on the catalytic reaction, such as the splitting mode, subsite structure and transglycosylation, can be obtained [24]. Therefore we set about obtaining time course by separating and quantifying $(GlcN)_n$ using HPLC. As shown in Figure 6, a time-dependent profile of $(GlcN)_6$ hydrolysis was successfully obtained using an HPLC system composed of a gel-filtration column of TSK-GEL

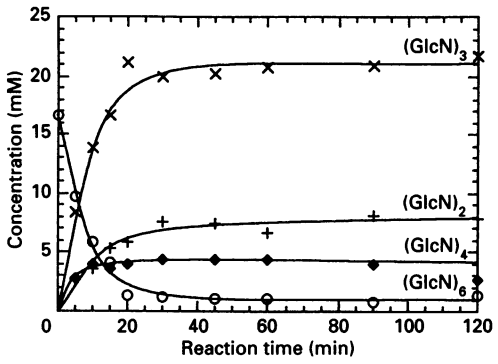

Figure 7 Time courses of $(GlcN)_6$ degradation catalysed by *Streptomyces* sp. N174 chitosanase

The $(GlcN)_n$ concentrations at each reaction time were calculated from peak areas in the HPLC profile. +, $(GlcN)_2$; ×, $(GlcN)_3$; ◆, $(GlcN)_4$; ○, $(GlcN)_6$.

Table 2 Relative rates of $(GlcN)_n$ (n = 4, 5 and 6) degradation catalysed by *Streptomyces* sp. N174 chitosanase

Substrate	Relative rate of degradation (%)
$(GlcN)_6$	100
$(GlcN)_5$	41.3
$(GlcN)_4$	3.0

G2000PW and a differential refractometer. Oligosaccharide products with degrees of polymerization higher than that of the substrate $(GlcN)_6$ were not detected, indicating that the chitosanase cannot catalyse transglycosylation. The concentrations of $(GlcN)_n$ at each reaction time were calculated from peak areas in the HPLC profile, and plotted against reaction time (Figure 7). The substrate $(GlcN)_6$ rapidly decreased and was almost completely hydrolysed in 20 min. The products were $(GlcN)_2$, $(GlcN)_3$ and $(GlcN)_4$, indicating that the chitosanase hydrolyses the substrate in an endo-splitting manner. Product distribution was $(GlcN)_3 \gg (GlcN)_2 > (GlcN)_4$. The substrates $(GlcN)_5$ and $(GlcN)_4$ were also digested by the chitosanase and time courses were obtained under the same conditions. From $(GlcN)_5$, equimolar concentrations of $(GlcN)_2$ and $(GlcN)_3$ were produced, and only $(GlcN)_2$ was produced from $(GlcN)_4$. The relative rates of degradation of the oligosaccharides are listed in Table 2. The rate of degradation decreased with a decrease in substrate size from $(GlcN)_6$ to $(GlcN)_5$ to $(GlcN)_4$.

DISCUSSION

Chitosanases can be classified into three classes according to their mode of action toward the substrate, partially *N*-acetylated chitosan [16]. In a previous paper, the mode of action of the chitosanase from *B. pumilus* BN-262 was investigated by structural analysis of the products from partially *N*-acetylated chitosan [16]. This enzyme splits the glycosidic linkage GlcNAc-GlcN as well as GlcN-GlcN. In the present study, chitosanase from *Streptomyces* sp. N174 exhibited the same specificity as that of the *B. pumilus* enzyme. These chitosanases should therefore belong to an identical specificity class. The specificity toward partially *N*-acetylated chitosan provided the following information on the active site: one of the two subsites adjacent to the catalytic site has an extremely low affinity for GlcNAc and can bind only GlcN residues, whereas the other adjacent subsite is rather more flexible, being able to bind GlcNAc as well as GlcN. In the deduced binding mode of $(GlcNAc)_6$ to hen egg-white lysozyme [25], *N*-acetyl groups of the sugar residues of $(GlcNAc)_6$ are alternately in contact with subsites A, C and E of the lysozyme binding cleft. The deduced binding mode is consistent with the fact that *N*-acetyl groups of $(GlcNAc)_6$ are alternately oriented to the opposite side because of the oligosaccharide conformation resulting from β-1,4-glycosidic linkage. As GlcN residues are also linked by β-1,4-linkages in $(GlcN)_n$, a similar situation may hold for the chitosanase–$(GlcN)_n$ interaction, i.e. six amino groups of $(GlcN)_6$ may be alternately in contact with the chitosanase binding cleft. Consequently, one of the two subsites adjacent to the catalytic site requires an amino group for sugar-residue binding, and the other is flexible with respect to this requirement.

Anomeric specificities of chitinolytic enzymes have been reported by several investigators [21,22,26]. The chitinases from microbes investigated thus far {*Streptomyces griseus* [22], *Bacillus circulans* [21], *Bacillus* sp. PI-7S (T. Fukamizo and Y. Honda, unpublished work)} exhibit anomer retention in their hydrolytic reaction. In the present study, however, the chitosanase from *Streptomyces* sp. N174 exhibits anomer inversion. Chitosanase from *B. pumilus* BN-262 was also investigated for anomeric specificity and found to be an inverting enzyme (T. Fukamizo and Y. Honda, unpublished work). Of the chitinolytic enzymes from microbes investigated thus far, chitinases have been found to be retaining enzymes and chitosanases inverting enzymes. The anomeric specificity presumably depends on the fine structure of the catalytic site. In carbohydrolases, the two catalytic carboxylates of the catalytic site are considered to be closely situated in retaining enzymes, such as lysozyme, but more distantly separated in inverting enzymes [27]. From a site-directed mutagenesis study of *Streptomyces* sp. N174 chitosanase, Glu-22 and Asp-40 appear to be the catalytic residues (I. Boucher, T. Fukamizo, Y. Honda, G. E. Willick, W. A. Neugebauer and R. Brzezinski, unpublished work). In the three-dimensional structure of the chitosanase, the two catalytic carboxylates are 1.38 nm apart (J. D. Robertus, personal communication). Thus the positions of Glu-22 and Asp-40 in the chitosanase are consistent with the fact that the chitosanase is an inverter. However, a chitinase from a plant (*Dioscorea opposita*) was found to be an inverting enzyme [22]. Similarly, there may be a chitosanase that exhibits anomer retention. In future investigations, the anomeric specificity of chitosanases from various origins should be examined.

For most carbohydrolases, there are some problems in evaluating enzyme activity. When a polymeric substrate is used, a precise explanation of the results is impossible because of the heterogeneity of the degree of polymerization of the substrate. An oligomeric substrate binds to the enzyme in various ways, producing several oligomeric products. This results in a complicated profile of the reaction time course. In addition, when an enzyme catalyses transglycosylation, enzyme activity cannot simply be evaluated from the rate of substrate degradation or product formation. In an investigation of the reaction mechanism of hen egg-white lysozyme, kinetic analysis of the experimental time courses of oligomeric substrate degradation and product formation was performed using a kinetic model in which transglycosylation and all possible binding modes were taken into consideration [28,29]. This method could be very useful for investigating the kinetics of chitosanase. In order to obtain

reliable data for the kinetic analysis, it is necessary to devise a method of separating and quantifying $(GlcN)_n$. Separation of $(GlcN)_n$ has so far been achieved by TLC or HPLC using a partition column [30,31]. However, it is difficult to quantify $(GlcN)_n$ by a TLC method. In addition, the partition column used for the separation is not as durable as a gel-filtration column, and cannot afford a quantitative yield. Therefore, in the present study, we attempted to separate and quantify $(GlcN)_n$ by HPLC with a gel-filtration column, detecting $(GlcN)_n$ with a differential refractometer. This method proved very successful, and experimental time courses of $(GlcN)_n$ hydrolysis were obtained. As seen in Figure 7, from $(GlcN)_6$, the chitosanase produced $(GlcN)_3$ abundantly and $(GlcN)_2$ and $(GlcN)_4$ in lesser amounts. Considering the fact that two molecules of $(GlcN)_3$ are produced from $(GlcN)_6$, the frequency of cleavage to $(GlcN)_3+(GlcN)_3$ appears to be considerably higher than that of cleavage to $(GlcN)_2+(GlcN)_4$. As the amount of $(GlcN)_4$ was smaller than that of $(GlcN)_2$, $(GlcN)_4$ was assumed to be split further to $(GlcN)_2+(GlcN)_2$. The chitosanase also hydrolyses $(GlcN)_5$ and $(GlcN)_4$ in an endo-splitting manner, but the degradation rates are very different (Table 2), decreasing concomitantly with a decrease in substrate size. This indicates that the substrate-binding cleft of chitosanase can accommodate at least six GlcN residues and that cleavage occurs at the midpoint of the binding cleft. In order to obtain further information on the active-site structure, time-course experiments and kinetic analysis are being conducted using site-directed mutants of the chitosanase.

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REFERENCES

1 Ouchi, S., Toyoda, H., Morimoto, M. et al. (1992) in Biological Control of Plant Diseases (Tjamos, E. S., G. C. Papavizas and R. J. Cook, eds.), pp. 335–339, Plenum Press, New York
2 Kendra, D. F. and Hadwiger, L. A. (1984) Exp. Mycol. **8**, 276–281
3 Suzuki, S., Suzuki, K., Tokoro, A., Okawa, Y. and Suzuki, M. (1986) in Chitin in Nature and Technology (Muzzarelli, R. A. A., Jeuniaux, C. and Gooday, G. W., eds.), pp. 485–492, Plenum Press, New York
4 Watanabe, T., Kobori, K., Miyashita, K. et al. (1993) J. Biol. Chem. **268**, 18567–18572
5 Hart, P. J., Monzingo, A. F., Ready, M. P., Ernst, S. R. and Robertus, J. D. (1993). J. Mol. Biol. **229**, 189–193
6 Perrakis, A., Tews, I., Dauter, Z. et al. (1994) Structure **2**, 1169–1180
7 Ando, A., Noguchi, K., Yanagi, M. et al. (1992) J. Gen. Appl. Microbiol. **38**, 135–144
8 Masson, J.-Y., Denis, F. and Brzezinski, R. (1994) Gene **140**, 103–107
9 Fenton, D. M. and Eveleigh, D. E. (1981) J. Gen. Microbiol. **126**, 151–165
10 Izume, M., Nagae, S., Kawagishi, H., Mitsutomi, M. and Ohtakara, A. (1992) Biosci. Biotechnol. Biochem. **56**, 448–453
11 Masson, J.-Y., Li, T., Boucher, I., Beaulieu, C. and Brzezinski, R. (1993) in Chitin Enzymology (Muzzarelli, R. A. A., ed.), pp. 423–430, European Chitin Society, Ancona
12 Marcotte, E., Hart, P. J., Boucher, I., Brzezinski, R. and Robertus, J. D. (1993) J. Mol. Biol. **232**, 995–996
13 Denis, F. and Brzezinski, R. (1992) Gene **111**, 115–118
14 Boucher, I., Dupuy, A., Vidal, P., Neugebauer, W. A. and Brzezinski, R. (1992) Appl. Microbiol. Biotechnol. **38**, 188–193
15 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
16 Fukamizo, T., Ohkawa, T., Ikeda, Y. and Goto, S. (1994) Biochim. Biophys. Acta **1205**, 183–188
17 Imoto, T. and Yagishita, K. (1971) Agric. Biol. Chem. **35**, 1154–1156
18 Saito, H., Mamizuka, T., Tabeta, R. and Hirano, S. (1981) Chem. Lett., 1483–1484
19 Fukamizo, T., Ohtakara, A., Mitsutomi, M. and Goto, S. (1991) Agric. Biol. Chem. **55**, 2653–2655
20 Tsukada, S. and Inoue, Y. (1981) Carbohydr. Res. **88**, 19–38
21 Armand, S., Tomita, H., Heylaud, A., Watanabe, T. and Henrissat, B. (1994) FEBS Lett. **343**, 177–180
22 Fukamizo, T., Koga, D. and Goto, S. (1995) Biosci. Biotechnol. Biochem. **59**, 311–313
23 Fukamizo, T. and Hayashi, K. (1982) J. Biochem. (Tokyo) **91**, 619–626
24 Masaki, A., Fukamizo, T., Otakara, A., Torikata, T., Hayashi, K. and Imoto, T. (1981) J. Biochem. (Tokyo) **90**, 527–533
25 Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C. and Rupley, J. A. (1972) in The Enzymes (Boyer, P. D., ed.), 3rd edn., vol. 7, pp. 665–868, Academic Press, New York
26 Dahlquist, F. W., Border, C. L., Jr., Jacobson, G. and Raftery, M. A. (1969) Biochemistry **8**, 694–700
27 Wang, Q., Graham, R. W., Trimbur, D., Warren, R. A. J. and Withers, S. G. (1994) J. Am. Chem. Soc. **116**, 11594–11595
28 Masaki, A., Fukamizo, T., Otakara, A., Torikata, T., Hayashi, K. and Imoto, T. (1981) J. Biochem. (Tokyo) **90**, 1167–1175
29 Fukamizo, T., Minematsu, T., Yanase, Y., Hayashi, K. and Goto, S. (1986) Arch. Biochem. Biophys. **250**, 312–321
30 Sakai, K., Katsumi, R., Isobe, A. and Nanjo, F. (1991) Biochim. Biophys. Acta **1079**, 65–72
31 Izume, M. and Ohtakara, A. (1987) Agric. Biol. Chem. **51**, 1189–1191

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