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Efficient preparation of β-(1→6)-$(GlcNAc)_2$ by enzymatic conversion of chitin and chito-oligosaccharides

Kayo Shimoda[a], Kohji Nakajima[a], Yoshiharu Hiratsuka[a], Shin-Ichiro Nishimura[b] & Keisuke Kurita[c]

[a]*Toyo Suisan Kaisha, Ltd, 2-13-40, Kohnan, Minato-ku, Tokyo 108, Japan*
[b]*Division of Biological Science, Graduate School of Science, Hokkaido University, Sapporo 060, Japan*
[c]*Department of Industrial Chemistry, Faculty of Engineering, Seikei University, Musashino-shi, Tokyo 180, Japan*

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A chitin-degrading marine bacterium strain, OK2607, was isolated from the sea water of Okinawa, Japan. The bacterium was identified as *Alteromonas* sp. The extracellular enzyme preparation obtained from the culture media of the bacterium effectively degraded chitin, and the degradation behavior was examined in detail. Unexpectedly, the major product was a β-(1→6)-linked disaccharide of *N*-acetylglucosamine (GlcNAc), and only small amounts of *N,N'*-diacetylchitobiose and GlcNAc were detected. High transglycosylation activity of the enzyme preparation was also confirmed with *N*-acetyl-chito-oligosaccharides giving rise to the same β-(1→6) disaccharide. The results indicate that the enzyme preparation is quite efficient to synthesize the unusual β-(1→6) oligosaccharide starting from the β-(1→6) polysaccharide or the oligosaccharides and would serve as a useful tool to prepare various β-(1→6) oligosaccharides. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Chitin is a β-(1→6)-linked homopolysaccharide of GlcNAc, and is a main constituent of arthropod shells and cell walls of microorganisms such as fungi. It is the second most abundant organic compound on the earth, next to cellulose, but remains an almost unutilized biomass resource. Of various approaches to the utilization of chitin, that based on chitin and chitosan oligomers is now attracting a great deal of attention because of their characteristic bioactivities. The oligomers are commonly prepared by acid hydrolysis and are promising as medicines, diagnostic drugs, fungicides and virucides, elicitors of plants, food additives, and so on. Chitinolytic enzymes are also often conveniently used to produce the oligosaccharides, and many kinds of chitinase-producing microorganisms have been isolated (Roberts, 1992; Zikakis, 1984).

We have been interested in the efficient production of *N*-acetyl-chito-oligosaccharides by enzymatic processes and tried to isolate marine bacteria that produce chitinolytic enzymes. Interestingly, the extracellular enzyme system of a chitinolytic strain OK2607, isolated from the coastal sea water of Okinawa, Japan, has turned out to have both chitinolytic and transglycosylating activities. Here we report the highly efficient formation of a unique β-(1→6)-linked disaccharide of GlcNAc from either chitin or chitin oligomers with the enzyme preparation.

MATERIALS AND METHODS

General

Chitin was isolated from squid pens of *Ommastrephes bartrami* by the previously reported method (Kurita *et al.*, 1993), and pulverized to 0.5 mm mesh with an ultra-centrifugal mill, Retsch ZM-1 (Germany). *N*-Acetyl-chito-oligosaccharides $(GlcNAc)_n$ were purchased from Seikagaku Kogyo (Tokyo, Japan). As a buffer solution, 20 mM Tris–HCl of pH 7.0 was used. Degradation products of chitin were analyzed with HPLC with a Shodex SE-61 RI detector (column, Asahipak NH2P-50 (4.6φ×250 mm); mobile phase, acetonitrile/water (75:25); flow rate, 1.0 ml/min). For isolation of the products, a preparative Asahipak NH2P-50 HPLC column was used. Fast atom bombardment mass spectra (FAB-MS) were recorded with a JMS-DX303 mass spectrometer (JEOL, Tokyo, Japan), glycerol being used as the matrix. ^1H–^1H NMR correlation spectroscopy (COSY) and ^{13}C NMR spectroscopy were performed with a JEOL JNM-GX500 spectrometer.

Isolation of chitinolytic bacteria

Sea water samples were collected in 10 ml lidded plastic tubes at various locations of the pelagic and coastal areas near Okinawa Island. The sea water was diluted 100 times with sterilized physiological saline, and a 50 μl aliquot was spread on a ZoBell 2216E agar medium containing 0.5% powdered chitin. After incubation at 25°C for 3–7 days, colonies forming clear zones as a result of degradation of chitin were selected to give strain OK2607. Detailed experiments to determine morphological, physiological, and biochemical characteristics of the bacterium were carried out primarily according to the method of Baumann *et al.* (1984).

Formation of enzyme preparation

Strain OK2607 was cultured in a 5 l jar fermenter containing 3 l of modified ZoBell's medium (0.5% polypeptone, 0.1% yeast extract, 0.001% $MgSO_4$, and 0.001% KH_2PO_4 in the sea water, pH 7.6) with 15 g of powdered chitin at 25°C for 72 h. The culture medium was centrifuged at 4°C at 19000 **g** for 20 min, and the supernatant was treated with aqueous ammonium sulfate (concentration, 40% of saturation). The precipitate was dissolved in a small amount of Tris–HCl buffer (pH 7.0), and the solution was dialyzed against the same buffer. The disaltant solution was diluted with the Tris–HCl buffer to a total volume of 100 ml, and the resulting solution was used as the enzyme preparation.

Degradation of chitin with the enzyme preparation

Chitin powder (5 mg), 100 μl of the Tris–HCl buffer, and 100 μl of the enzyme preparation were mixed in a 1.5 ml microtube. After incubation at 37°C for 48 h, the mixture was filtered, and the filtrate was directly applied to analytical HPLC.

Preparative degradation

The enzymatic reaction was similarly performed on a larger scale (powdered chitin, 100 mg; Tris–HCl buffer, 2.5 ml; enzyme preparation, 2.5 ml) to enable the isolation and characterization of the degraded products. After 60 h of incubation at 37°C, 5.0 ml of acetonitrile was added to the mixture, and insoluble materials were removed by filtration. The filtrate was concentrated under reduced pressure. After removal of most of the solvent, 1 ml of acetonitrile/water (75:25 v/v) was added, and insoluble materials were removed with a 0.45 μm membrane filter. The resulting filtrate was applied to preparative HPLC to give 22.3 mg of a major product as a white crystalline powder that was assumed to be a β-(1→6)-linked disaccharide of GlcNAc from spectral data. It was very hygroscopic and started to decompose at around 163°C (163°C (dec), Defaye *et al.*, 1989). IR (KBr):ν 1656 (amide I), 1549 (amide II), and 1150–1000 cm^{-1} (pyranose).

For further structural identification, the product was acetylated in the usual manner with acetic anhydride in pyridine at room temperature overnight. The peracetylated derivative was isolated with water, chromatographed on silica gel with chloroform/methanol (20:1), and recrystallized from ethanol to give colorless small crystals, IR (KBr):ν 1741 (ester C=O), 1670 (amide I), 1522 (amide II), and 1224 cm^{-1} (C–O).

Anal. Calcd for $C_{28}H_{40}N_2O_{17}\cdot H_2O$: C, 48.41; H, 6.09; N, 4.03. Found: C, 48.11; H, 5.94; N, 4.01.

Transglycosylation of oligosaccharides with the enzyme preparation

N-Acetylchito-oligosaccharides $(GlcNAc)_n$ where $n=1$–6 were dissolved in the Tris–HCl buffer at a concentration of 1%. An aliquot of 500 μl of the oligosaccharide solution was pipetted into a 1.5 ml microtube, and 300 μl of the Tris–HCl buffer and 200 μl of the enzyme preparation were added. The reaction mixture was incubated at 37°C for 48 h and filtered. The filtrate was applied to analytical HPLC.

RESULTS AND DISCUSSION

Identification of strain OK2607

Strain OK2607 was a Gram-negative bacillus, and the cell had a polar flagellum. The physiological and biological characteristics of the strain are summarized in Table 1. The mol% of G+C of DNA was 46.3%. Based on these properties, strain OK2607 was identified as *Alteromonas* species. The strain has been deposited in National Institute of Bioscience and Human Technology, Tsukuba, Japan, with an accession number FERM P-13194.

Degradation of chitin

The strain was cultured with modified ZoBell's medium containing chitin at 25°C. The supernatant was treated with aqueous ammonium sulfate, dialyzed, and diluted with a Tris–HCl buffer of pH 7.0 to give an enzyme preparation. Chitin was then incubated with the enzyme preparation at 37°C, and the degraded products were analyzed by HPLC. As shown in Fig. 1, a large peak was observed immediately following those of GlcNAc and *N,N'*-diacetylchitobiose at a retention time of 11 min. It was not ascribable to any of *N*-acetylchito-oligosaccharides.

In order to identify the compound of the large peak in Fig. 1, it was isolated by preparative HPLC as a white crystalline powder. Figure 2 shows FAB-MS data of the compound. The $[M+Na]^+$ ion peak at *m/z* 447

Table 1. Physiological and biological characteristics of strain OK2607

Gram stain	–
Cell shape	Straight
O–F test	Oxidation
Polar flagellum	+
Pigmentation	+ (yellow)
Oxidase	+
Reduction of NO_3^- to NO_2^-	–
Gas produced from D-glucose	–
Na^+ required for growth	+
Production of:	
Gelatinase	+
Chitinase	+
DNase	+
Utilization of:	
D-Mannose	+
Sucrose	+
D-Fructose	+
Maltose	+
DL-Malate	–
Citrate	–
α-Ketoglutarate	–
Succinate	–
Fumarate	+
D-Sorbitol	–
Erythritol	–
N-Acetylglucosamine	+
Glycerol	+
G + C content (mol%)	46.3

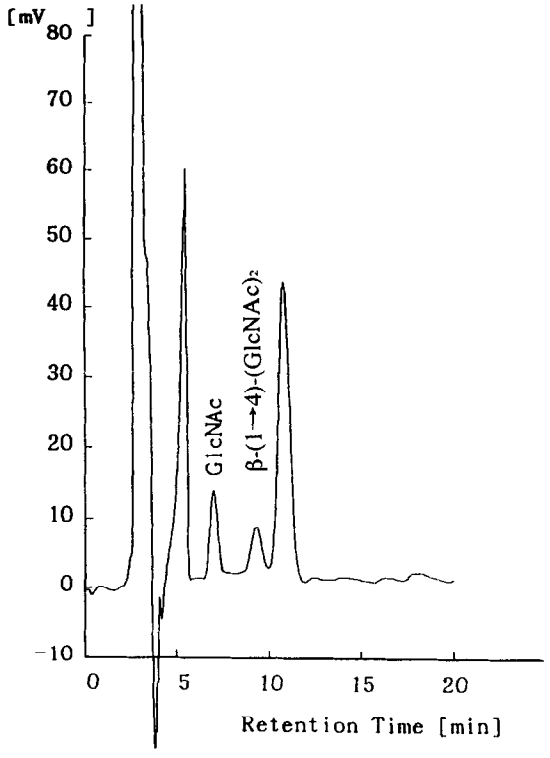

Fig. 1. High-performance liquid chromatogram of reaction products obtained by treating chitin with the enzyme preparation.

and the $[M+H]^+$ ion peak at *m/z* 425 in the spectrum indicate the molecular weight to be 424, suggesting the formation of a disaccharide of GlcNAc. It was then peracetylated with acetic anhydride (Nishimura *et al.*, 1989), in order to enable structural identification by elemental analysis and IR and NMR spectroscopies. The elemental analysis data of the peracetylated product again supported the disaccharide structure. The

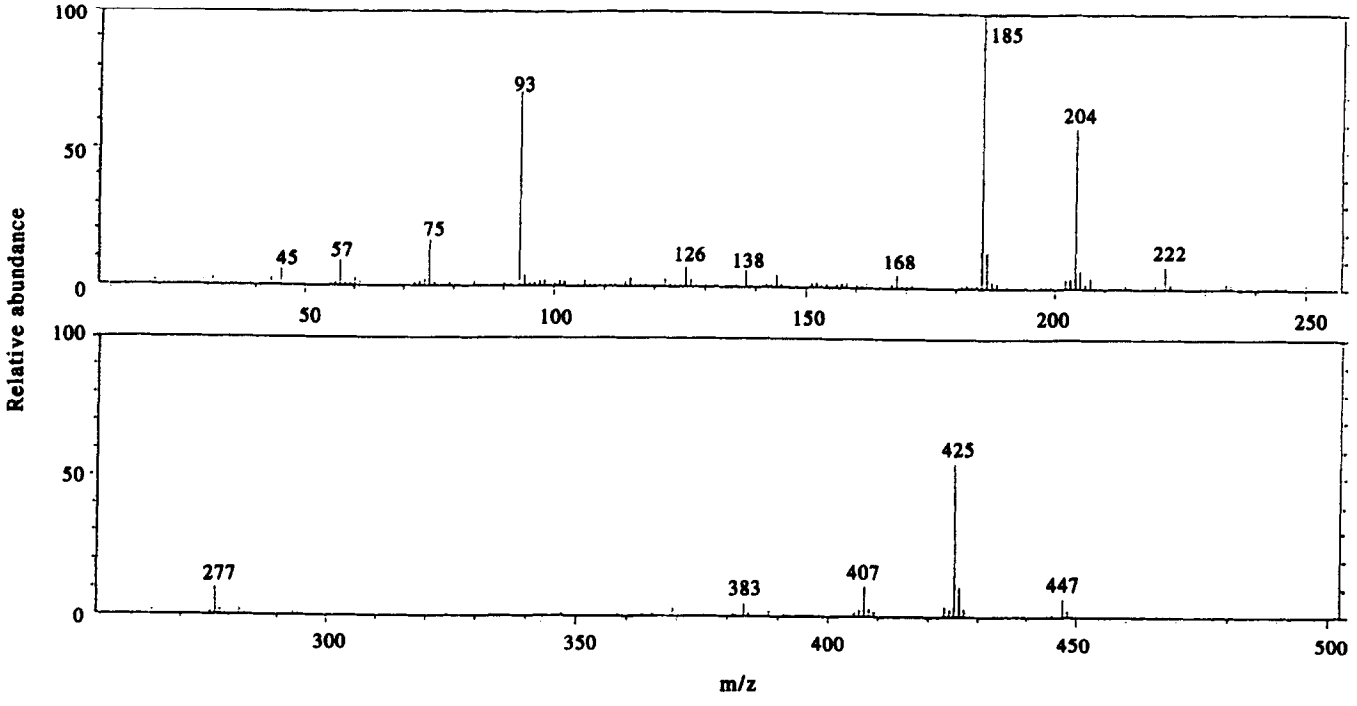

Fig. 2. FAB-MS spectrum of the major product in the positive mode.

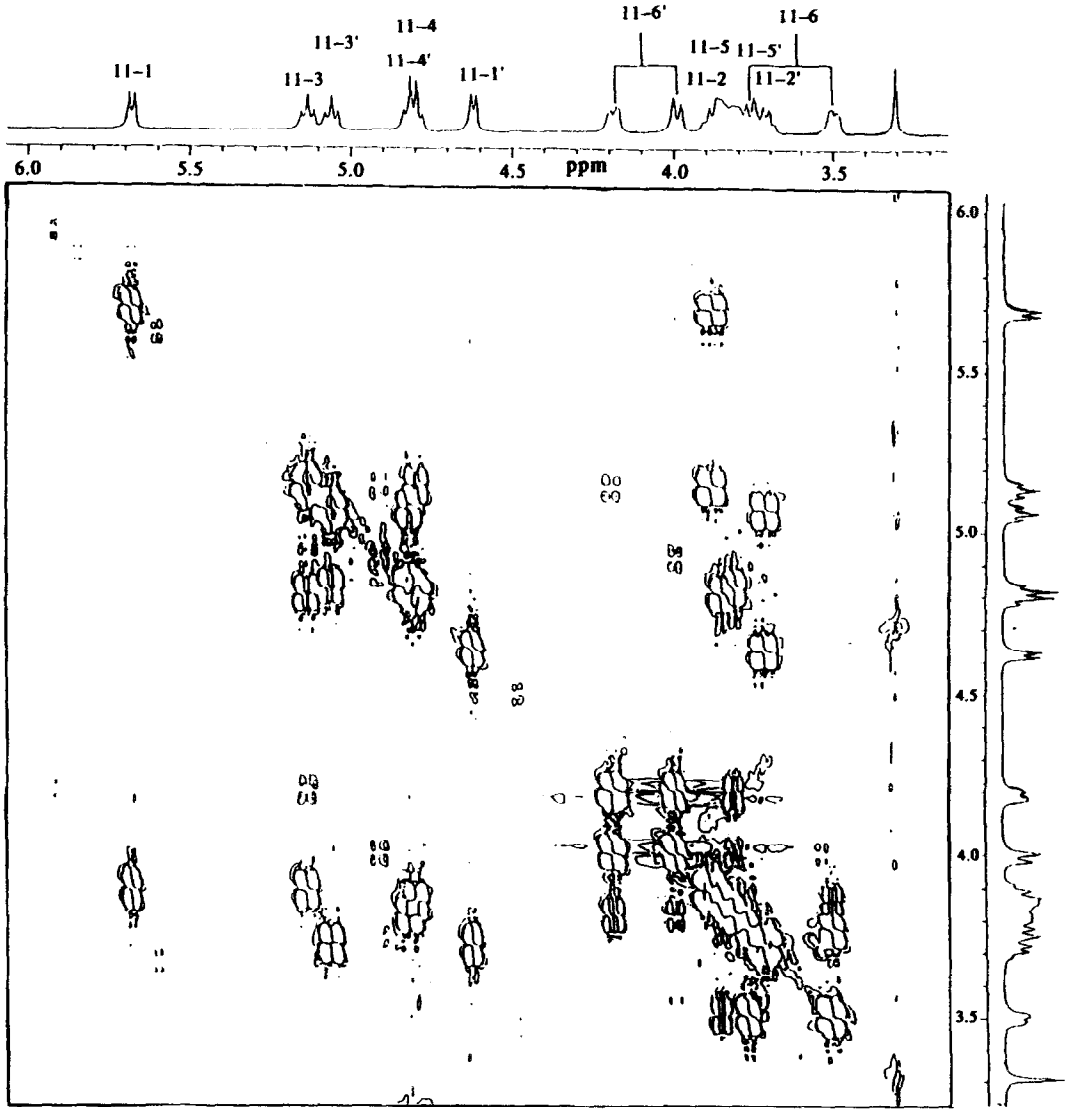

Fig. 3. ^{1}H–^{1}H COSY spectrum of the peracetylated major product measured in DMSO-d_6 at 500 MHz.

IR spectrum showed characteristic absorption bands due to ester, amide, and pyranose groups and resembled that of peracetylated *N,N'*-diacetylchitobiose. The ^{1}H–^{1}H COSY and ^{13}C NMR spectra are shown in Figs 3 and 4, and the assigments of peaks are given in the figures. The ^{1}H–^{1}H COSY spectrum shows nearby signals of H-4 and H-4′ at around 4.8 ppm. In the ^{13}C NMR spectrum, C-4 and C-4′ signals appeared at exactly the same position (δ 68.54 ppm), whereas C-6 and C-6′ signals were observed at 66.86 and 61.72 ppm (Blunberg *et al.*, 1982). These chemical shifts are characteristic of disaccharides having a (1→6)-linkage such as isomaltose and gentiobiose. Furthermore, the J_{H1-H1} value of 8.9 Hz supports the presence of a β-linkage. These results indicate that the compound is a β-(1→6)-linked GlcNAc dimer, 2-acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxyglucopyranose. Under appropriate conditions, this product was obtained in a 22.3% yield.

Transglycosylation of oligosaccharides by the enzyme preparation

The enzyme preparation was then evaluated in terms of the possible transglycosylation activity. When *N*-acetylchito-oligosaccharides up to the hexamer were treated with the enzyme preparation at 37°C, the starting oligosaccharides were consumed completely in 4–5 h. The incubation was continued up to 48 h, and the results are summarized in Table 2.

As evident in the table, the major product was always the β-(1→6) disaccharide except in the case of GlcNAc where no higher oligosaccharides were formed. In no cases were oligosaccharides with degrees of polymerization of three and above detected. From the oligosaccharides constituted of even numbers of GlcNAc units ($n=2$, 4 and 6), only small amounts of GlcNAc were formed. The yield of the β-(1→6) disaccharide was as high as 71.7% starting from *N,N'*-diacetylchitobiose.

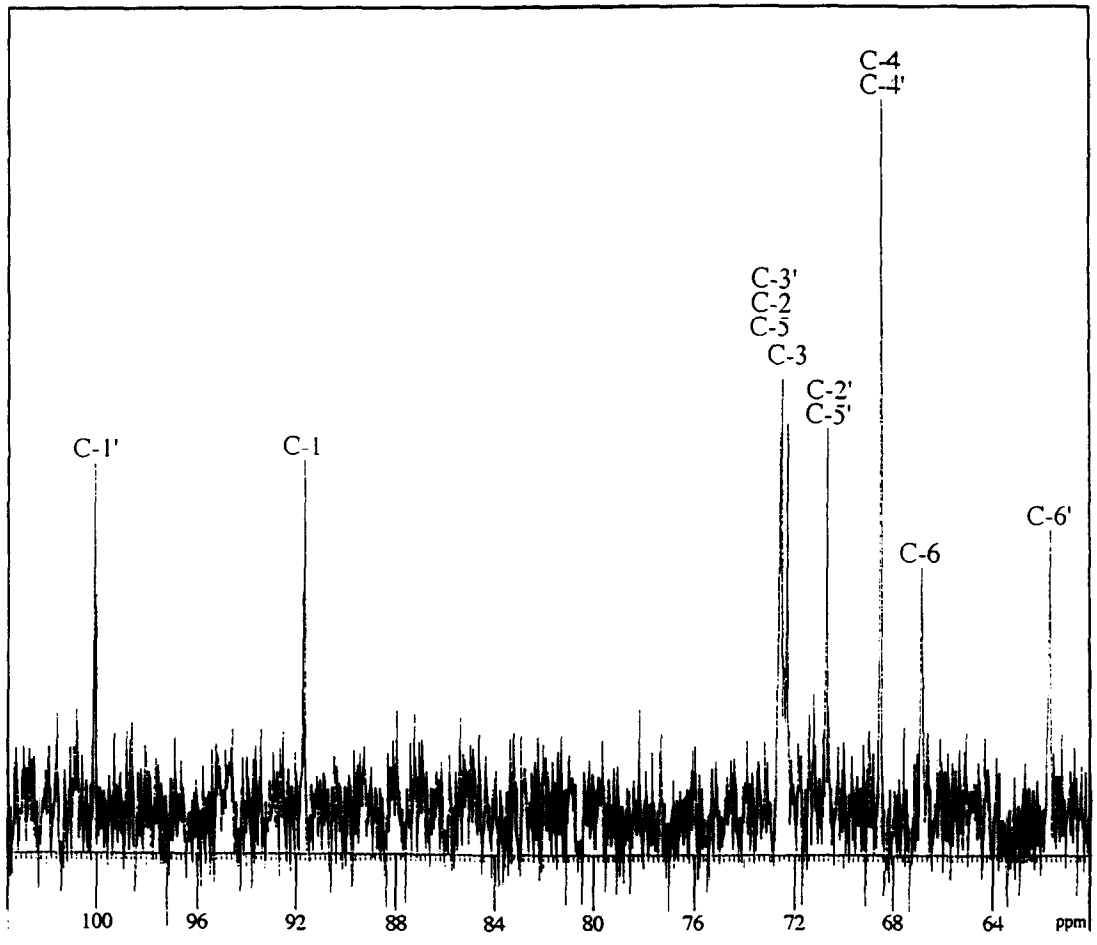

Fig. 4. ^{13}C-NMR spectrum of the peracetylated major product DMSO-d_6 at 125.6 MHz.

On the other hand, the oligosaccharides having odd numbers of GlcNAc units gave larger amounts of GlcNAc, on account of the consumption of *N,N'*-diacetylchitobiose units for the formation of the β-(1→6) disaccharide, resulting in the accumulation of GlcNAc. The β-(1→6) disaccharide was, for example, obtained in a 59.6% yield as shown in Table 2.

These results show that the enzyme preparation is composed of at least two types of enzymes; one is a chitinase which degrades chitin and the oligomers to *N,N'*-diacetylchitobiose, and the other is a transglycosylative enzyme which produces β-(1→6)-(GlcNAc)$_2$.

The time course of transglycosylation was thus examined, and a typical example starting from the tetrasaccharide is illustrated in Fig. 5. As shown there, the peak due to *N,N'*-diacetylchitobiose was reduced with a rise of the peak due to the β-(1→6) disaccharide. This implies that the transglycosylation to the β-(1→6) disaccharide occurred primarily from *N,N'*-diacetylchitobiose preformed by chitinolytic degradation.

CONCLUSION

The enzyme preparation from *Alteromonas* sp. OK2607 was confirmed to have both chitinolytic and transgly-

Table 2. Transglycosylation of *N*-acetylchito-oligosaccharides

Substrate	Reaction products (yield %)			
	GlcNAc	β-(1 → 4)-(GlcNAc)$_2$	β-(1 → 6)-(GlcNAc)$_2$	β-(1 → 4)-(GlcNAc)$_3$ and higher oligomers
GlcNAc	100.0	0	0	0
β-(1 → 4)-(GlcNAc)$_2$	10.7	17.6	71.7	0
β-(1 → 4)-(GlcNAc)$_3$	40.3	8.1	51.6	0
β-(1 → 4)-(GlcNAc)$_4$	15.4	14.4	70.2	0
β-(1 → 4)-(GlcNAc)$_5$	29.4	11.0	59.6	0
β-(1 → 4)-(GlcNAc)$_6$	20.2	13.0	66.8	0

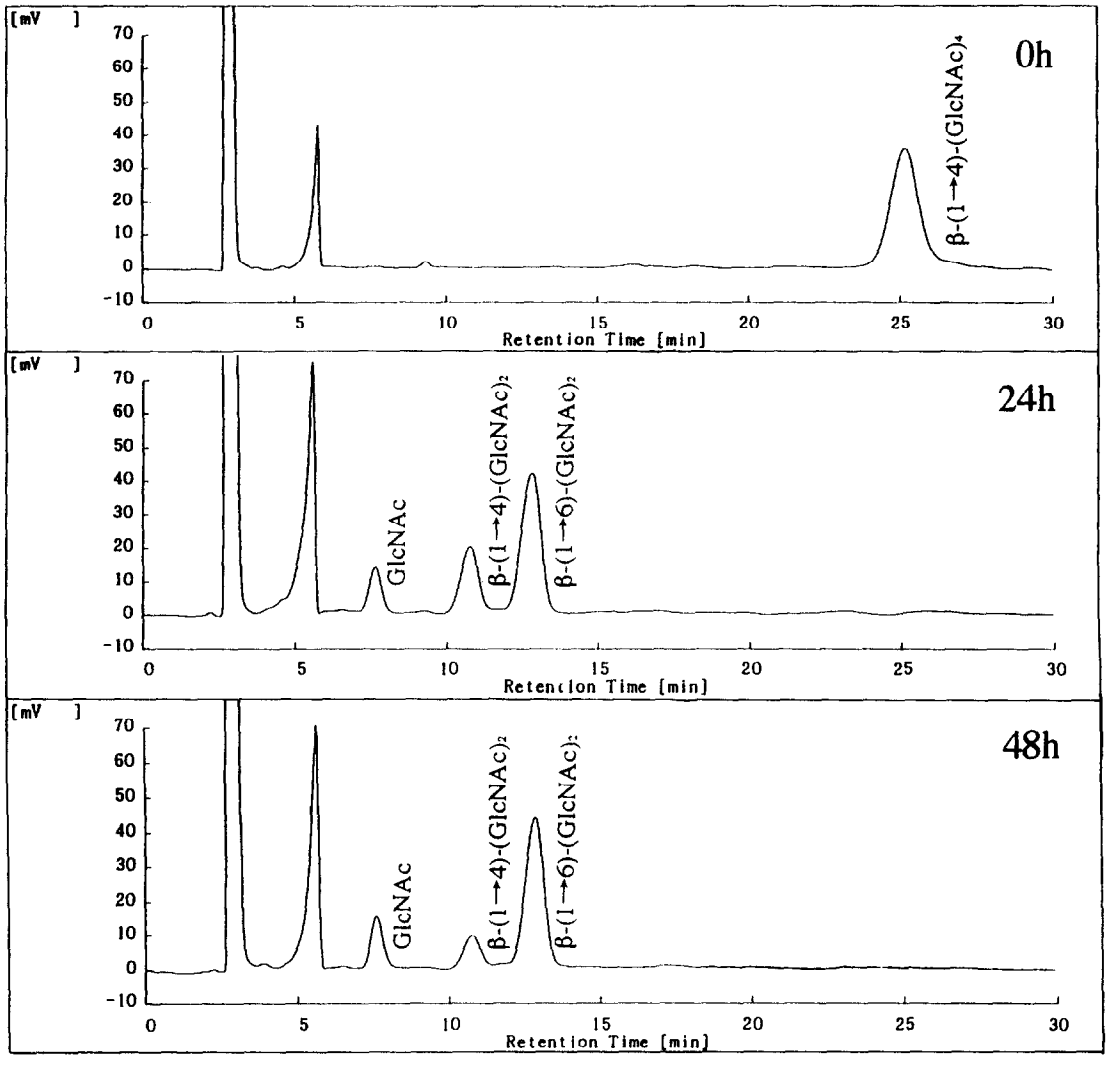

Fig. 5. Time course of transglycosylation of β-(1→4)-(GlcNAc)$_4$ with the enzyme preparation.

cosylative activities. The preparation efficiently produced a β-(1→6) disaccharide of GlcNAc from either chitin or *N*-acetylchito-oligosaccharides. This is the first example of a transferase forming β-(1→6)-linkages from *Alteromonas* sp. Although the same product was obtained from GlcNAc and hydrogen fluoride (Defaye *et al.*, 1989), the reaction was not specific for producing the disaccharide, and moreover, the reaction conditions were rather harsh. Formation of β-(1→6)-(GlcNAc)$_2$ was also reported through transglycosylation of *N*,*N*′-diacetylchitobiose with an *N*-acetylglucosaminidase (Nanjo *et al.*, 1990). The transglycosylation with the preparation from *Alteromonas* sp. OK2607, however, proceeds at a much lower concentration, e.g. 0.5% *N*,*N*′-diacetylchitobiose, and moreover, the yield of β-(1→6)-(GlcNAc)$_2$ was more than 71%, indicating high efficiency and specificity for the production of the β-(1→6) disaccharide. These results suggest the possibility of preparing versatile oligosaccharides having a β-(1→6)-linkage as well as β-(1→6)-(GlcNAc)$_2$, which are important in view of the high potential in biological, biotechnological, and pharmacological fields. Further purification and characterization of the enzymes are now underway.

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