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Enzymic synthesis of useful chito-oligosaccharides utilizing transglycosylation by chitinolytic enzymes in a buffer containing ammonium sulfate*

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ABSTRACT

A chitinase purified from culture filtrates of *Trichoderma reesei* KDR-11 efficiently catalyzed a transglycosylation reaction on tetra-*N*-acetylchitotetraoside in a buffer medium containing ammonium sulfate, converting the tetrasaccharide into hexa-*N*-acetylchitohexaose (39.6%) and di-*N*-acetylchitobiose (55.7%) as the major products. Sugar-chain elongation from di-*N*-acetylchitobiose as the initial substrate to hexa-*N*-acetyl-chitohexaose and hepta-*N*-acetylchitoheptaose was also efficiently induced through lysozyme catalysis in the presence of ammonium sulfate at high (30%) concentration. In this case, the addition of ammonium sulfate to the reaction system resulted in a remarkable increase of the hexamer and heptamer productions, which are desirable as biologically active oligosaccharides.

INTRODUCTION

There is a growing appreciation of the potential of biologically active oligosaccharides. Thus $(GlcNAc)_6$ and $(GlcNAc)_7$ show notable antitumor activity against Sarcoma 180 solid tumors in Balb/c mice[1] and are efficient elicitors of chitinase (EC 3.2.1.14) activity in melon plants[2]. Such aspects suggest further potential for these chito-oligomers in medicine and biology. Conventional methods for obtaining chito-oligomers by partial acid hydrolysis of chitin and chitosan give only low yields of the desired hexamer and heptamer[3,4]. Fluorohydrolysis of chitin in anhydrous hydrogen fluoride affords a more convenient route to chito-oligomers than conventional chemical degradations[5]. We have recently reported that the transferase activity of a chitinase purified from *Nocardia orientalis* IFO 12806 can be used for the preparative-scale synthesis of $(GlcNAc)_6$ and $(GlcNAc)_7$ from $(GlcNAc)_4$ and $(GlcNAc)_5$, respectively[6]. Furthermore, we tried to improve the yield of the hexamer from the tetramer by utilizing the transglycosylation reaction of a chitinase from *T. reesei*. However, this is only a

* Abbreviations: GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; $(GlcNAc)_n$(n = 2–7), β-(1→4)-linked *n*-mer of GlcNAc; *p*NP-GlcNAc, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside; *p*NP-$(GlcNAc)_2$, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside.

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two-sugar chain-elongation from the initial $(GlcNAc)_4$ substrate. It would be more significant if we could prepare $(GlcNAc)_6$ or $(GlcNAc)_7$ from a lower oligomer such as $(GlcNAc)_2$. Accordingly, hen egg-white lysozyme (EC 3.2.1.17) was chosen, even though its transglycosylating activity has been hitherto neglected because its rate constant for hydrolysis in water[7] is higher.

This paper describes an efficient enzymic synthesis of $(GlcNAc)_6$ or $(GlcNAc)_7$ by utilizing the sugar chain-elongation reaction of chitinolytic enzymes in a buffer medium containing ammonium sulfate, and the lysozyme-mediated reaction from $(GlcNAc)_2$ to the hexamer and heptamer.

EXPERIMENTAL

Materials. — A series of *N*-acetyl chito-oligosaccharides (degree of polymerization, 2–6) were kindly provided from Yaizu Suisan Kagaku Industry. $(GlcNAc)_7$ was prepared by our method[6]. *p*NP-$(GlcNAc)_2$ was synthesized by the autocatalytic–fusion reaction[8]. The hen egg-white lysozyme used in these experiments was recrystallized six times (lot E83Z05), supplied by Seikagaku Kogyo Co. Ltd.; Tokyo. Colloidal chitin was prepared by the method of Jeuniaux[9]. All other chemicals were obtained from commercial sources.

Enzyme assays. — Chitinase activity was assayed with colloidal chitin as a substrate. The mixture (contained 1 mL of colloidal chitin suspended in 0.1M acetate buffer (pH 3.6) to give an absorbance of 1.00 at 660 nm, and an enzyme solution of 2.0 mL total volume) was incubated for 20 min at 50°. After incubation, the percentage decrease in turbidity was determined. One unit of enzyme activity was defined as the amount that caused a 5% decrease of the absorbance at 660 nm of the cell suspension per 10 min by the modified Jeuniaux method[10].

N-Acetyl-β-D-hexosaminidase (EC 3.2.1.52) activity was assayed with *p*NP-GlcNAc as the substrate, as previously described[11]. One unit of activity was defined as the amount of enzyme liberating 1 μmol *p*-nitrophenol per min.

Preparation of affinity adsorbent. — To prepare the aminated compound according to the method of Harpaz and Flowers[12], a solution of 100 mg of *p*NP-$(GlcNAc)_2$ in 20 mL of 50% aq. MeOH containing 10 mg of palladium-on-charcoal was hydrogenated at atmospheric pressure for 2 h at room temperature. After filtration and removal of the solvent under diminished pressure, the resultant syrup was dissolved in 10 mL of 0.1M acetate buffer (pH 6.0). To this solution, 5 g of suction-dried formyl-Sepharose CL-4B, prepared according to the method of Itoh *et al.*[13], was added, and the mixture was preincubated for 1 h at 40° with gentle shaking. To the suspended solution, 100 mg of $NaCNBH_3$ was added and the mixture was incubated overnight at 40° with gentle shaking. After washing of the conjugated Sepharose with 50 mL of distilled water, the remaining formyl groups were blocked by treatment with 2 mL of M Tris-HCl (pH 7.4) and 5 mg of $NaCNBH_3$ for 1 h at room temperature. The gel was finally filtered and washed again with 50 mL of distilled water to give the product used for affinity chromatography [tentatively named $(GlcNAc)_2$-Sepharose CL 4B].

Purification of chitinase from T. reesei. — Crude chitinase from the culture filtrates of *T. reesei* KDR-11 mutant, prepared by precipitation with ammonium sulfate followed by desalting on Sephadex G-25 according to the method of Kawamori *et al.*[14], was kindly provided by Kyowa Hakko Kogyo Co. Ltd.; Tokyo. Enzyme purification was carried out at 4° unless otherwise stated.

(*A*) *CM-Sepharose Fast Flow ion-exchange chromatography.* The crude enzyme (0.82 g) from *T. reesei* was dissolved in 30 mL of 50 mM acetate buffer at pH 4.0 (buffer *A*). The enzyme solution was applied to a column (3.0 × 44 cm) of CM-Sepharose Fast Flow equilibrated with buffer *A*. The column was washed with buffer *A* and then eluted with a linear gradient (0–0.5M) of NaCl in buffer *A* (total vol. 3 L). The eluate was collected in 20 mL fractions. Most of the chitinase activity (tubes 27–35) emerged from the column a little behind the major protein peak during washing with several volumes of the starting buffer. Eluates of this fraction were combined and concentrated to low volume (2 mL) using an Amicon Diaflo unit equipped with a PM-10 membrane operating at 50 lb. in^{-2} pressure.

(*B*) *Affinity chromatography.* The enzyme solution from step (*A*) was directly loaded onto a $(GlcNAc)_2$-Sepharose CL-4B column (1.3 × 4.5 cm) equilibrated with 20 mM phosphate buffer at pH 7.0. No chitinase activity was observed when the column was first washed with the equilibrated buffer. When the column was changed to 20mM acetate buffer at pH 5.0, a single peak of chitinase activity associated with the protein peak (tubes 32–38) was eluted. The eluate was collected in 2 mL fraction. The combined solutions were concentrated to 1–2-mL using the Amicon Diaflo unit, lyophilized, and then stored over $CaSO_4$ at 4°.

General properties of the purified chitinase. — (*A*) *Effects of pH on activity and stability.* Buffer solutions were prepared to cover the pH range 2.5–10; 0.1M citrate buffer (pH 2.5–3.5), 0.1M acetate buffer (pH 3.5–6.0), 0.1M phosphate buffer (pH 6.0–9.0), and 0.1M borate buffer (pH 9.0–10.0). The activity was measured at various pH values under the standard assay conditions already described. The effects of pH on the stability of the enzyme were observed by assaying the enzyme activity after incubation for 24 h at 25° in the absence of substrate at each of the pH values just described.

(*B*) *Effects of temperature on activity and stability.* The activity was measured at various temperatures (20–80°) under the standard assay conditions. The heat stability of the enzyme was observed by assaying enzyme activity after incubation of the enzyme for 1 h at various temperatures in the absence of substrate.

(*C*) *Effects of metal ions.* The enzyme was incubated for 15 min at 40° in the presence of mM concentrations of each of the following salts: $MgCl_2 \cdot 6H_2O$, $CaCl_2$, $ZnCl_2$, $FeSO_4 \cdot 7H_2O$, $Pb(OAc)_2 \cdot 3H_2O$, $CuSO_4 \cdot 5H_2O$, and $HgCl_2$ and the residual activity was measured by the standard assay procedures.

Analytical method. — Protein concentration was determined spectrophotometrically at 280 nm or by the method of Lowry *et al.*[15], using bovine serum albumin as standard. Sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis was carried out according to Laemmli[16], using 10% gel. MW-Marker (Oriental Yeast Co. Ltd.) containing an oligomer series of cytochrome c, was used as a molecular-weight stan-

dard. Protein bands were detected by staning with Coomassie Brilliant Blue R-250. H.p.l.c. was performed with a YMC packed column Type A.014 (SJL) column(6 mm i.d. × 30 cm) in a Shimadzu LC-6A liquid-chromatography apparatus equipped with an SPD-6A u.v. detector. Elution was effected with water–MeCN (3:7, v/v). The flow rate was 0.8 mL/min at a pressure of 60 $kg.cm^{-2}$ and the detector measured u.v. absorbance at 210 nm (characteristic absorption of the *N*-acetyl group). ^{13}C-N.m.r. spectra were recorded in deuterium oxide using 1% oligosaccharide solution in 5-mm tubes at room temperature on a JNM–GX 500 spectrometer operating at 125 MHz in the pulsed Fourier-transform mode with complete proton decoupling. F.a.b.-mass spectra of oligosaccharides were recorded with a Jeol DX-303 HF mass spectrometer, operating at the full accelerating potential (3 kV) and coupled to a Jeol DA-500 mass-data system. The sample (3 μL) in distilled water was added to the glycerol matrix, and 0.1M HCl (1μL) was added. Specific rotation was determined with a Digital Automatic Polarimeter PM-101 apparatus (Union Giken Corp., Ltd.). Elemental analyses were performed using a Perkin–Elmer 240C apparatus.

RESULTS AND DISCUSSION

I *Chitinase.* (a) *Purification of chitinase.* A chitinase of *T. reesei* was purified to apparent homogeneity by chromatography on CM-Sepharose Fast Flow followed by affinity chromatography on $(GlcNAc)_2$-Sepharose CL-4B. A peak showing chitinase activity was completely separated from *N*-acetyl-β-D-hexosaminidase activity on CM-Sepharose Fast Flow [Fig. 1(a)]. The chitinase-activity fraction was further purified by

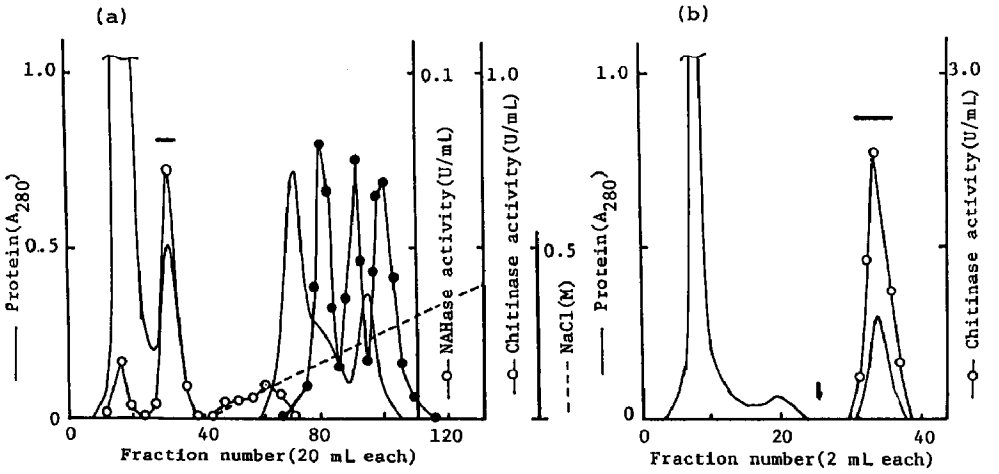

Fig. 1. Chromatography of crude chitinase from *T. reesei* on CM-Sepharose Fast Flow followed by $(GlcNAc)_2$-Sepharose CL 4B affinity chromatography of a chitinase partially purified from CM-Sepharose: (*a*) the enzyme solution (30 mL) having an activity of 183.7 unit was placed on a column (3.0 × 44 cm) of CM-Sepharose Fast Flow at a flow rate of 120 mL/h; (*b*) the enzyme solution (2 mL) having an activity of 32.1 unit was placed on a column (1.3 × 4.5 cm) of $(GlcNAc)_2$-Sepharose CL 4B at a flow rate of 2 mL/h.

TABLE I

Purification of a chitinase from *T. reesei*

Step	*Protein (mg)*	*Total activity (U)*	*Specific activity (U/mg)*
Crude	820.0	183.7	0.22
CM-Sepharose	68.3	32.1	0.47
$(GlcNAc)_2$-Sepharose	11.4	15.0	1.32

affinity chromatography with the agarose derivative of the substrate analogue, *p*-aminophenyl di-*N*-acetyl-*β*-chitobioside [Fig. 1(b)]. The enzyme gave a single protein band on SDS–polyacrylamide gel electrophoresis and was found to be homogeneous on gel filtration on Bio-Gel P-60. The molecular weight was estimated to be 58 000, as judged by SDS–polyacrylamide gel electrophoresis. This purified enzyme possessed strong transglycosylating ability, as shown later in this paper. A summary of the purification procedures of the chitinase is presented in Table I. The enzyme was purified only 6-fold using the present assay, with a 8.1% yield.

Some hydrolytic properties of the purified enzyme on colloidal chitin were further examined. The optimum pH for the enzyme was found to be 4.0 in 0.1M acetate buffer and it was stable in the range 3.5–9. The maximum activity of the enzyme was at 60°, and it was stable in the range 20–50°. The enzyme was completely inactivated after 1 h at 70° in 0.1M acetate buffer (pH 4.0). The enzyme incubated in the presence of mM concentrations of metal ions was not affected substantially by Mg^{+2}, Ca^{+2}, Zn^{+2}, Fe^{+2}, Pb^{+2}, Ba^{+2}, Ni^{+2}, Cu^{+2}, Sr^{+2}, and Mn^{+2} and was slightly inhibited by Hg^{+2} (93% of initial activity retained). It was not inhibited by EDTA at 10mM.

(b) *Preparation of $(GlcNAc)_6$ by transglycosylation.* $(GlcNAc)_4$ (100 mg) was dissolved in 1 mL of 0.1M phosphate buffer (pH 7.0) containing 20% ammonium sulfate. To this solution was added 1 unit of chitinase and the mixture was kept for 48 h at 60°. As time progressed, the medium became turbid, and eventually a precipitate was formed. The precipitate was collected by centrifugation, washed, and centrifuged 3 times with 3 mL of 50% aq. MeOH. The washed precipitate was dissolved in 5 mL of water and the insolubilized portion was removed by centrifugation (3000 r.p.m.). The insoluble material (4 mg) might be higher oligomers formed by transglycosylation. The supernatant solution was directly applied to a Bio-Gel P-4 column. Elution was monitored by measuring the absorbance of *N*-acetyl groups at 210 nm. The chromatogram shows a remarkably high hexaose peak deriving from the action of the enzyme on $(GlcNAc)_4$ (Fig. 2). Fraction G_6, which appeared at the same filtration volume as authentic $(GlcNAc)_6$, was collected and lyophilized to give 24 mg, and was subjected to structural analysis as previously described[6].

Fraction G_6 showed only a single peak on l.c., consistent with that of a standard sample of $(GlcNAc)_6$. The ^{13}C-n.m.r. spectrum could be superimposed on that of authentic $(GlcNAc)_6$ reported in our previous paper[6]. The linkage nature of the hexamer was further investigated by digesting it with lysozyme (from hen egg-white). The

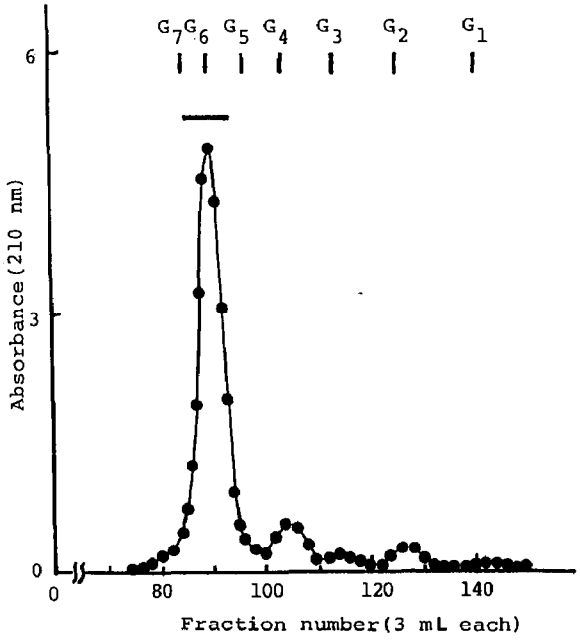

Fig. 2. Gel-chromatographic separation of transglycosylation products by the action of chitinase on $(GlcNAc)_4$. Chromatography of carbohydrates was carried out on a column (2.6 × 95 cm) of Bio-Gel P-4, 200–400 mesh at 50°. The column was eluted with degassed water at a flow rate of 60 mL/h. The elution pattern was monitored by u.v. absorbance at 210 nm. The elution positions of G_2–G_7, a series of chito-oligosaccharides (d.p., 2–7) and G_1 (GlcNAc) are shown.

substrate (1 mg per mL) was digested with 50 μg of enzyme in 0.1M acetate buffer (pH 5.5) at 40°, and aliquots were removed at various times for l.c. The initial substrate (fraction G_6) had disappeared completely after 10 min. The order of products appearing at 24 h was $(GlcNAc)_2$ > GlcNAc > $(GlcNAc)_3$. If linkages other than β-D-(1→4) had been present in the substrate, other oligosaccharides would have been present in the mixture. Yields, and physico-chemical analytical data, are shown in Table II. F.a.b.-m.s. of fraction G_6 showed the protonated molecular ion, $[MH]^+$, together with fragments at intervals of m/z 203 corresponding to the sequence of the glycosidic bonds as reported[5] for $(GlcNAc)_6$. From these results, fraction G_6 was identified as $(GlcNAc)_6$.

(c) *Effects of ammonium sulfate concentration, temperature, and pH on transglycosylation.* To investigate the effects of various conditions on transglycosylation, it was first necessary to establish conditions for maximal yield of the desired compound. The l.c. method has been reported to be the most efficient and sensitive means of monitoring the chitinolytic enzyme-reaction[6,17]. The effect of ammonium sulfate concentration on transglycosylation from the initial $(GlcNAc)_4$ substrate was first examined on the 1-mL scale in a buffer medium containing different concentrations of ammonium sulfate as shown in Fig. 3(a). The suspended samples (100 μL) were taken at intervals during the incubation, and then diluted with 9 volumes of water for analysis by l.c. The maximum $(GlcNAc)_6$ production at 20° ammonium sulfate was ~1.6-fold that

TABLE II

Yields, optical rotations, f.a.b., and analytical data for chito-oligosaccharides produced by chitinolytic enzymes

Fraction	*Yield (%)*	$[\alpha]_D^{25}$ *(degrees)*	m/z[a] *(M + H)*	*Mol. Formula*	*Analytical data*					
					Calc			*Found*		
					C	H	N	C	H	N
G_6 from chitinase	24[b]	−14.6 (1.0)[c]	1237	$C_{48}H_{80}N_6O_{31}\cdot H_2O$	45.90	6.53	6.69	46.23	6.67	6.55
G_6 from lysozyme	9.5[d]	−14.8 (1.0)	1237	$C_{56}H_{93}N_7O_{36}\cdot H_2O$	45.90	6.53	6.69	46.30	6.70	6.50
G_7 from lysozyme	6.5[d]	−17.5 (0.5)	1440	$C_{56}H_{93}N_7O_{36}\cdot H_2O$	46.09	6.52	6.72	46.22	6.60	6.57

[a] In f.a.b. + ionization mode. [b] From $(GlcNAc)_4$ (100 mg). [c] For a solution in water. [d] From $(GlcNAc)_2$ (200 mg).

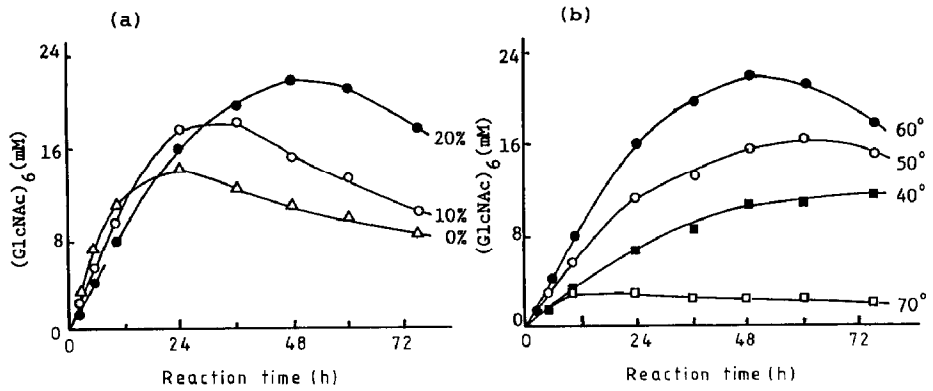

Fig. 3. Effects of ammonium sulfate concentration and temperature for chitinase-mediated $(GlcNAc)_6$ production: (*a*) the enzyme reaction was performed with a 10% substrate solution at 60° in 0.1M phosphate buffer (pH 7.0) containing different concentrations of ammonium sulfate (0,10, and 20%) and samples were taken during the incubation for analysis by l.c.; (*b*) Conditions were the same as those in (*a*): $(GlcNAc)_4$ was dissolved in a buffer containing 20% ammonium sulfate at 40, 50, 60, and 70°, respectively.

in the absence of this salt. In this case, the enzyme acts on $(GlcNAc)_4$ to form $(GlcNAc)_6$ (39.6%) and $(GlcNAc)_2$ (55.7%) as the major products. The effects of different temperatures in media containing 20% ammonium sulfate on the transglycosylation reaction are shown in Fig. 3(b); the rate of $(GlcNAc)_6$ production increased with temperature to a maximum at 60°, and thus reaction at elevated temperature was suited for the effective synthesis of $(GlcNAc)_6$. The effect of buffers of different on transglycosylation at 60° was also examined (data not shown). Acetate (100mM, pH 4.0 and 5.5) and phosphate buffers (100mM, pH 7.0 and 9.0) were used. At pH 7.0, the maximum $(GlcNAc)_6$ production was much larger than that at pH 5.5 or 4.0 (the optimum for chitin-degrading activity), and the efficiency of transglycosylation was very dependent on the pH. Reaction at pH 9.0 considerably decreased the transferase activity. The optimum pH for hydrolase activity does not necessarily correspond to that for transferase activity.

II *Lysozyme.* (a) *Preparation of* $(GlcNAc)_6$ *and* $(GlcNAc)_7$ *by transglycosylation.* To a solution of $(GlcNAc)_2$ (200 mg) in 2 mL of 0.1M acetate buffer (pH 4.0) containing 30% ammonium sulfate was added 20 mg of hen egg-white lysozyme and the mixture was kept for 8 h at 70°. As time progressed, the medium became turbid, and eventually formed a precipitate, which was centrifuged off, washed 3 times with 5 mL of 50% aqueous methanol, and then suspended in 20 mL of water with cooling in an ice-bath. The insoluble portion was removed by centrifugation. The insoluble material (14 mg) might be higher oligomers formed in the reaction, as an aqueous suspension became completely clear on digestion by lysozyme. The supernatant was desalted by using a Micro Acilyzer (Asahikasei Kogyo Co. Ltd.), concentrated to one-fourth volume under diminished pressure, and lyophilized to afford 42 mg of a fraction named F-1.

L.c. analysis of F-I showed mainly two peaks (G_6 and G_7) whose elution times

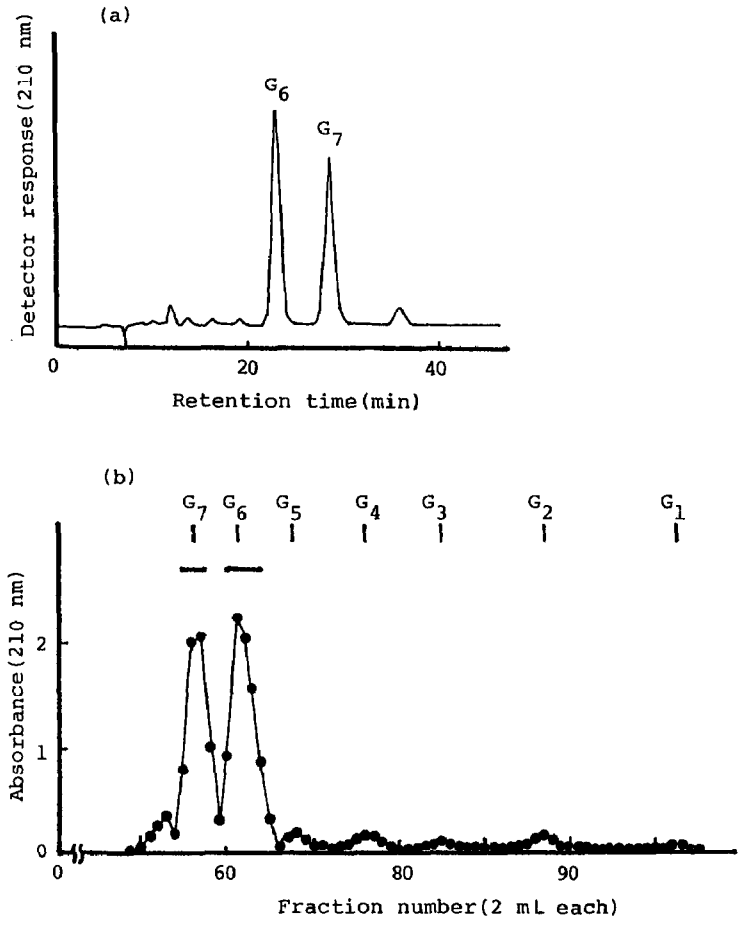

Fig. 4. L.c. analysis and chromatographic separation of transglycosylation products by the action of lysozyme on $(GlcNAc)_2$: (*a*) l.c. analysis performed as described in Materials and Methods; (*b*) chromatography of carbohydrates was carried out on a column (2 × 100 cm) of Bio-Gel P-4, < 400 mesh, at 50°. The column was eluted with degassed water at a flow rate of 12 mL/h. The elution positions of G_2–G_7, a series of chito-oligosaccharides (d.p., 2–7) and G_1 (GlcNAc) are shown.

corresponded to those of authentic $(GlcNAc)_6$ and $(GlcNAc)_7$, respectively [Fig. 4(a)]. Accordingly, F-I was dissolved in 9 mL of water, and one third of this solution was loaded onto a Bio-Gel P-4 column [Fig. 4(b)]. The chromatogram shows remarkably high hexaose and heptaose peaks deriving from the action of the enzyme on $(GlcNAc)_2$. Fractions G_6 and G_7 were each combined from three runs on Bio-Gel P-4 and lyophilized to give 19 and 13 mg, respectively. They were shown to be $(GlcNAc)_6$ and $(GlcNAc)_7$ by the methods just described. Their ^{13}C n.m.r. spectra could be superimposed on those of authentic $(GlcNAc)_6$ and $(GlcNAc)_7$. The hydrolytic action of hen egg-white lysozyme on fractions G_6 and G_7 was also identical to those on corresponding authentic samples. Furthermore, f.a.b.-m.s. of fraction G_6 was consistent with that of $(GlcNAc)_6$ already mentioned. For fraction G_7, the quasi-molecular ion, m/z 1440 $[MH]^+$, was observed as the only a detectable signal (Table II).

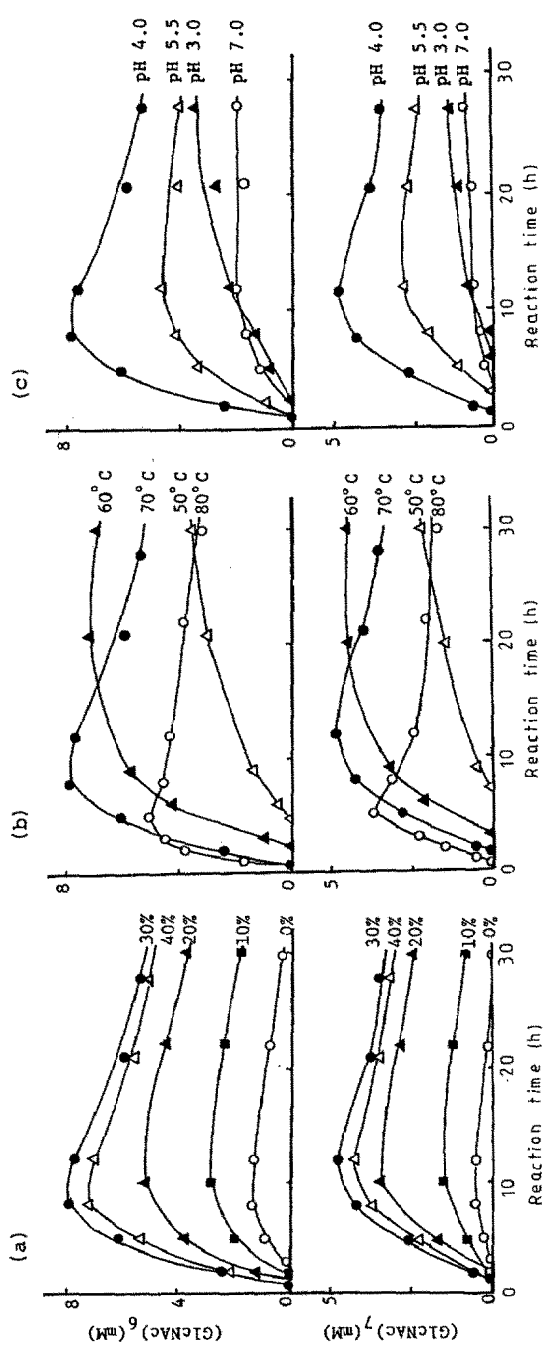

Fig. 5. Effects of ammonium sulfate concentration, temperature, and pH for lysozyme-mediated production of $(GlcNAc)_6$ and $(GlcNAc)_7$: (*a*) the enzyme reaction was performed with 10% substrate at 70° in 0.1M acetate buffer containing different concentrations (0, 10, 20, 30, and 40%) of ammonium sulfate, and samples were taken during incubation for analysis by l.c.; (*b*) $(GlcNAc)_2$ was dissolved in a buffer containing 30% ammonium sulfate at 50, 60, 70, and 80°, respectively. Other conditions were the same as those in (*a*).

(b) *Effects of ammonium sulfate concentration, temperature, pH, and enzyme concentration on transglycosylation.* The effects of various conditions on lysozyme-mediated transglycosylation were investigated by the l.c. method described in Section I(c). The time-course of $(GlcNAc)_6$ and $(GlcNAc)_7$ production from $(GlcNAc)_2$ incubated at different ammonium sulfate concentrations was first examined by l.c. [Fig. 5(a)]. The maximum rates of hexamer and heptamer formation increased with rising ammonium sulfate concentration below 40%. At 30% ammonium sulfate, the times at which maximum $(GlcNAc)_6$ and $(GlcNAc)_7$ production was reached were 8 and 12 h, respectively, and the maximum amounts of $(GlcNAc)_6$ and $(GlcNAc)_7$ produced were $\sim$6 and 8 times respectively those in a reaction medium in the absence of this salt. In this case, the products appearing at 8 h were GlcNAc, $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$, $(GlcNAc)_6$, and $(GlcNAc)_7$ in the proportions of 50.2, 13.2, 3.5, 3.3, 17.4, and 12.1%. The effect of temperature on the reaction at 30% ammonium sulfate was similarly examined [Fig. 5(b)]. The maximum rates of hexamer and heptamer production increased markedly with rising temperature, but fell at 80°. Reaction at 70° was best suited for the rapid production of the hexamer and heptamer. The times for maximum $(GlcNAc)_6$ production at 60 and 70°, were $\sim$20 and 8 h, respectively. Figure 5(c) shows the effects of buffers of different pH at 70°. Production of $(GlcNAc)_6$ and $(GlcNAc)_7$ was fastest at pH 4.0; the efficiency of transglycosylation was very dependent on pH. At pH 7.0 the transferase activity is considerably weakened. We observed that the optimum conditions (temperature 50° and pH 5.5) for chitin-hydrolyzing activity did not correspond to those for transferase activity. The rate of transglycosylation was very dependent on the enzyme concentration under the same conditions (data not shown). Thus, the times at which maximum $(GlcNAc)_6$ production was reached at 1, 0.5, and 0.2% enzyme concentrations were 8, 22, and 60 h, respectively. Consequently the efficiency of transglycosylation was improved not only by a high concentration of ammonium sulfate, but also by controlling the temperature, pH, and enzyme concentration.

(c) *Proposed mechanism of the transglycosylation mode of chitinolytic enzymes.* As already described, the efficiency of the transglycosylation by chitinolytic enzymes was apparently improved in a medium containing ammonium sulfate, especially for the lysozyme-mediated reaction. In the transglycosylation mode, the present chitinase may transfer the di-*N*-acetyl chitobiosyl residue to $(GlcNAc)_4$ acceptor molecules to form $(GlcNAc)_6$, in a similar manner to that of the *N. orientalis* enzyme[6]. The sugar chain-elongation reaction from the $(GlcNAc)_2$ substrate to $(GlcNAc)_6$ and $(GlcNAc)_7$ by lysozyme was estimated (Fig. 6) by analysis of the change in composition with reaction time in mixtures of lysozyme and $(GlcNAc)_2$. From earlier data[18], the rate of hydrolytic attack on $(GlcNAc)_2$ compared with $(GlcNAc)_3$ (at 1.0) is 0.003, whereas that on $(GlcNAc)_6$ is 30 000. $(GlcNAc)_2$ itself is cleaved only very slowly because it is bound largely in a nonproductive mode[18,19]. Thus, $(GlcNAc)_3$ formation through transfer of the *N*-acetylglucosaminyl residue to $(GlcNAc)_2$ is a rate-limiting step in the overall process of transglycosylation, because as large amounts of $(GlcNAc)_3$ build up, the formation of $(GlcNAc)_6$ and $(GlcNAc)_7$ is initiated. In this case, once $(GlcNAc)_3$ formation reaches its maximum, the amount rapidly decreases during the subsequent reaction. As a result,

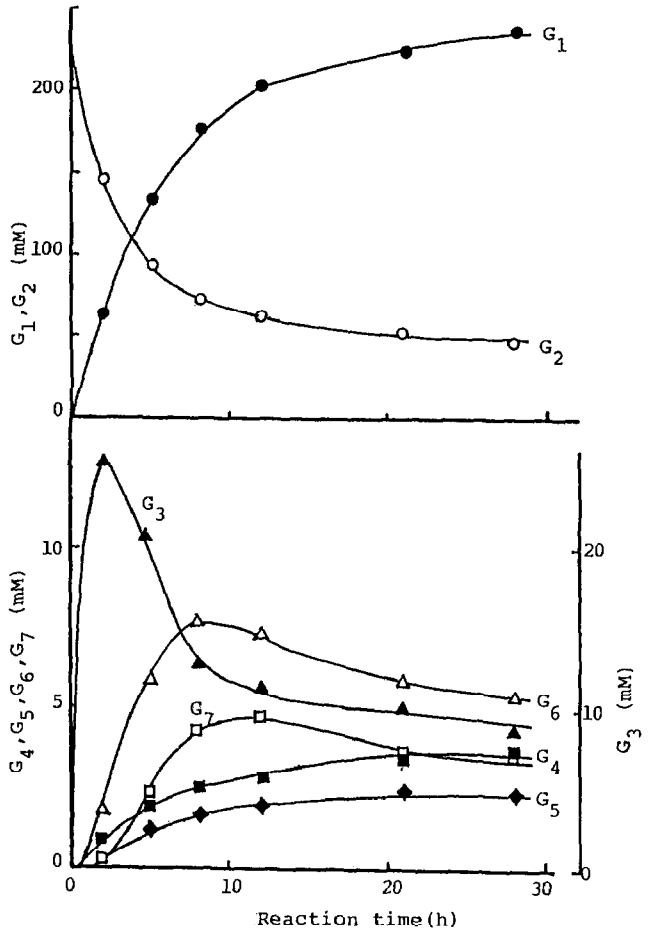

Fig. 6. Formation of products of the lysozyme-mediated reaction on $(GlcNAc)_2$. The enzyme reaction was performed with a 10% substrate solution at 70° as described in Section II(a), and samples were taken during the incubation for analysis by l.c. G_1, GlcNAc; G_2–G_7, a series of chito-oligosaccharides (d.p., 2–7).

the sugar chain-elongation reaction from the trisaccharide proceeds in sequence to produce $(GlcNAc)_4$ and $(GlcNAc)_5$, which may act as chain carriers in a series of reactions for formation of hexamer and heptamer. Finally, when $(GlcNAc)_6$ and $(GlcNAc)_7$ are produced by transglycosylation, most of them precipitate out to separate appreciable amounts of the desired compounds during the reaction. Because the hexamer and heptamer are only slightly soluble in the described medium, there is less probability that they are attacked by the enzyme, which is saturated with an excess of $(GlcNAc)_2$ or $(GlcNAc)_3$. To clarify this point, the solubilities of $(GlcNAc)_6$ in the presence and absence of ammonium sulfate were investigated at different temperatures. The hexamer shows only 0.1% solubility at 70° in a medium containing 30% ammonium sulfate, and its solubility is only 12.5% of that in the absence of the salt, indicating that addition of ammonium sulfate to the buffer dramatically lowers the solubility of

$(GlcNAc)_6$. In this way, the efficiency of the transglycosylation process is collectively reinforced by the presence of a minimal amount of water, an excess of substrate[20,21] and by the presence of a high concentration of ammonium sulfate.

In conclusion, an enzymic reaction using commercially available lysozyme provides a novel and simple technique for preparation of the biologically active oligosaccharides $(GlcNAc)_6$ and $(GlcNAc)_7$ from $(GlcNAc)_2$, a readily available precursor or obtainable enzymically[22] or chemically[3,5] in substantial quantities from chitin.

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