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Note

Production of *N*-acetyl-D-glucosamine from α-chitin by crude enzymes from *Aeromonas hydrophila* H-2330

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Abstract

The selective and efficient production of *N*-acetyl-D-glucosamine (GlcNAc) was achieved from flake type of α-chitin by using crude enzymes derived from *Aeromonas hydrophila* H-2330. © 2002 Elsevier Science Ltd. All rights reserved.

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D-Glucosamine and *N*-acetyl-D-glucosamine (GlcNAc) have attracted much attention owing to their therapeutic activity in osteoarthritis. They have also been evaluated as a food supplement.[1–3] GlcNAc is more suitable than D-glucosamine for oral administration because of its sweet taste. GlcNAc is mainly produced by acid (concd HCl) hydrolysis of chitin. This procedure, however, has some problems such as low yield (below 65%)[4] or acidic wastes. Most recently, we have reported the production of GlcNAc in good yield from β-chitin derived from squid pen by use of cellulases.[5] These crude enzymes, however, did not efficiently hydrolyze α-chitin derived from crab or shrimp shell, which is more available, at low cost and more widely distributed in nature compared with β-chitin. The present work describes the effective production of GlcNAc from α-chitin by crude enzymes derived from *Aeromonas hydrophila* (A).[6] Fig. 1 shows a typical HPLC chromatogram of the hydrolyzate. Since the activity of the crude enzyme preparation tends to decrease at higher temperature, the hydrolysis was carried out at 17 °C. The selective production of GlcNAc was observed during continuous hydrolysis. The production of GlcNAc oligomers (G2–G7: dimer–heptamer) was negligible. This fact suggests that both endo- and exo-type chitinases exist in crude enzymes A and produce GlcNAc selectively by cooperation. To achieve selective production of GlcNAc, the enzymatic activity (mU/mg) of endo- and exo-type chitinase was evaluated (Scheme 1). Higher exo-chitinase activity was observed than that of endo-chitinase, thus demonstrating that chitin was hydrolyzed slowly by endo-chitinase and produced GlcNAc oligomers.

Fig. 2 shows the time courses on the production of GlcNAc from various α-chitins by crude enzymes A. In all cases, similar behavior was observed on the hydrolysis of α-chitin and GlcNAc was produced in 66–77% (mol/mol) yield from these α-chitins after 10 days (Table 1). The M_w of α-chitin measured by GPC with hexafluoro-2-propanol (HFP) as eluent was independent of the degradability. To confirm the chemical structure of hydrolyzate, crude enzymes A was dialyzed before hydrolysis to remove low M_w of components. From the ^{1}H NMR analysis, the hydrolyzate by crude enzymes A showed the good agreement in ^{1}H NMR signals with authentic GlcNAc.

Most recently, we found out that three kinds of enzyme ($M_w = 90$, 72, and 62 kDa from SDS-PAGE analysis) were included in crude enzymes A. The de-

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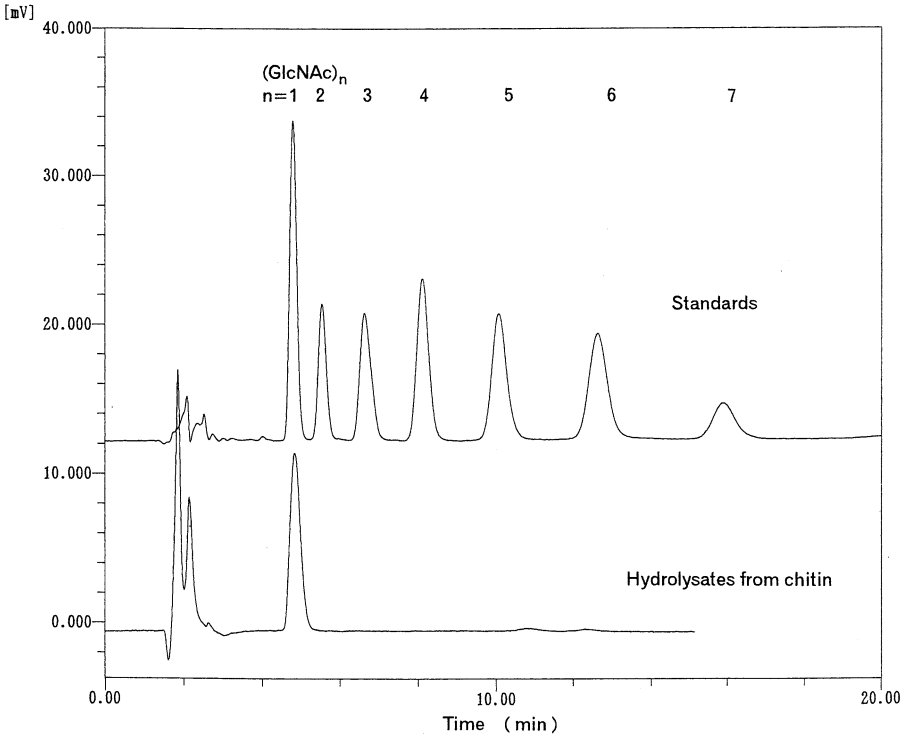
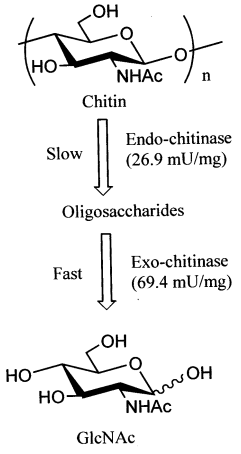


Fig. 1. HPLC chromatogram of the hydrolyzate of α-chitin by crude enzymes A. Condition, see Section 1.

tailed study on the enzymatic property of separated three enzymes will be published in near future. The selective production of GlcNAc from α-chitin could be achieved by crude enzymatic hydrolysis in good yield (77%). This process will be useful for the efficient production of GlcNAc.

1. Experimental

Materials.—α-Chitin (derived from crab shell) of flake A (> 50 μm,) and flake B (> 50 μm) were purchased from Wako Pure Chemical Industries Ltd. and Koyo Chemical Co., Ltd., respectively. Powdered α-chitin (pulverized to 3.8 μm, crab shell) was supplied from Sunfive Co., Ltd. Crude enzymes derived from *A. hydrophila* H-2330 was prepared in our laboratory (Kyoto Institute of Technology) according to the previous report.[6] Chitin (20 mg) was suspended in 4 mL of 20 mM phosphate buffer (pH 7.0) containing crude enzymes A (7.4 mg) and EDTA 2 Na (5 mM). The suspension was shaken at 17 °C. After the prescribed time, a part of the reaction mixture (10–20 μL) was taken out, diluted with water (0.48–0.49 mL) and CH_3CN (1.0 mL), filtered, and analyzed by HPLC. The amount of GlcNAc in the reaction mixture was estimated from the calibration curve of standard GlcNAc. Yield of GlcNAc was calculated as follows.



Scheme 1.

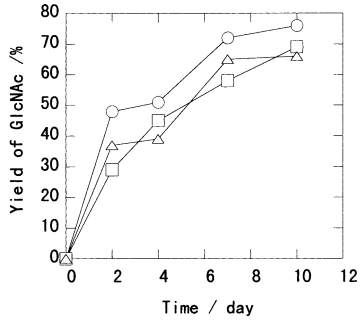


Fig. 2. Time courses on the production of GlcNAc from α-chitin by crude enzymes A. ○, flake-A (> 50 μm); □, flake-B (> 50 μm); △, fine powder (3.8 μm). α-Chitin: [S] = 5 mg/mL; crude enzymes A: [E] = 1.85 mg/mL; pH 7.0; 17 °C.

Table 1
Production of GlcNAc from α-chitin by crude enzymes A [a]

Entry	α-Chitin [b] (mg/mL)	M_n	M_w	Yield (%)
1	flake-A (5)	180,000	1,200,000	77
2	flake-A (20)	180,000	1,200,000	77
3	flake-B (5)	130,000	390,000	69
4	flake-B (20)	130,000	390,000	66
5 [c]	flake-B (20)	130,000	390,000	64
6	powder (5)	50,000	180,000	66
7	powder (20)	50,000	180,000	64

[a] Enzymes A, 1.85 mg/mL; pH 7.0 (4 mL); 17 °C; 10 days.
[b] Flake-A, Wako; flake-B, Koyo TCL; powder, fine powder (3.8 μm).
[c] Enzymes A was dialyzed before hydrolysis (see Section 1).

Yield (%) = GlcNAc produced (mol)/
repeating unit (mol) of chitin added × 100

Measurements.—HPLC analysis was performed on a Tosoh LC-8020 apparatus (column, Shodex Asahipak NH2P-50; rt; 7:3 CH_3CN–water; flow rate = 1.0 mL/min; injection, 0.1 mL; detection, UV at 210 nm). GPC analysis of α-chitin was performed as follows. Chitin (10 mg) was suspended in hexafuluoro-2-propanol (HFP: 2 mL) containing 0.1 mM CF_3CO_2Na. After 3 days, the solution was filtered (0.45 μm) and applied for GPC (apparatus, Tosho HLC-8220; column, Tosho TSK-gel super H-RC and HM-N; eluent, HFP containing 0.1 mM CF_3CO_2Na; standard, polymethyl methacrylate; flow rate, 0.2 mL/min; temp, 40 °C; detector IR).

Analysis of enzymatic activity.-.—endo-Chitinase activity was estimated from increasing the reducing-end groups by the modification of Schale's procedure.[7] Since chitin was insoluble in aqueous solvent, 70% deacetylated chitin which was soluble in acidic water (pH 4) was used as substrate. One unit means 1 μmol of reducing-end group produced after 1 min of hydrolysis per 1 mg of protein. The exo-chitinase activity was estimated by HPLC from the production of GlcNAc using $(GlcNAc)_2$ as substrate. One unit means 1 μmol of GlcNAc produced after 1 min of hydrolysis per 1 mg of protein.

Chemical analysis of hydrolyzate from α-chitin.—Crude enzymes A containing EDTA 2 Na (5 mM) in 20 mM phosphate buffer (25 mL) was dialyzed using Vivapore dialysis kit (M_w cut off = 7500 of polyethersulfone membrane, Vivascience Co.) to remove low M_w of components. Remained high M_w of fraction (2 mL) was diluted to 25 mL with water. To a solution was added α-chitin (500 mg) and shaken at 17 °C. After 10 days, the hydrolyzate was dialyzed by cellulose membrane (M_w cut off = 12,000) and low M_w of fraction was collected, evaporated, and dried (350 mg, 70% (w/w) from chitin). Finally, the hydrolyzate was dissolved in D_2O and the ^1H NMR spectrum taken (JEOL Alpha-500 NMR spectrophotometer).

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