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Enzymatic production of *N*-acetyl-D-glucosamine from chitin. Degradation study of *N*-acetylchitooligosaccharide and the effect of mixing of crude enzymes

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Abstract

N-Acetyl-D-glucosamine (GlcNAc) was produced from chitin by use of crude enzyme preparations. The efficient production of GlcNAc by cellulases derived from *Trichoderma viride* (T) and *Acremonium cellulolyticus* (A) was observed by HPLC analysis compared to lipase, hemicellulase, and pectinase. β-Chitin showed higher degradability than α-chitin when using cellulase T. The optimum pH of cellulase T was 4.0 on the hydrolysis of β-chitin. The yield of GlcNAc was enhanced by mixing of cellulase T and A.

Keywords: *N*-Acetyl-D-glucosamine; Chitin; Crude enzyme

1. Introduction

N-Acetyl-D-glucosamine (GlcNAc) has been a focusing material for the improvement of osteoarthritis as well as D-glucosamine (GlcN), which has attracted much attention owing to its therapeutic activity in osteoarthritis and been evaluated as a food supplement (Crolle & D'Este, 1980; Drovanti, Bignamini, & Rovati, 1980; Kajimoto et al., 1998; Kim & Conrad, 1974; Setnikar, Giacchetti, & Zanolo, 1986; Setnikar, Palumbo, Canali, & Zanolo, 1993; Tapadinhas, Rivera, & Bignamini, 1982; Vaz, 1982). Although sulfate and hydrochloride salts of GlcN are already commercialized for this disease, these are not suitable for oral administration owing to its bitter taste. On the other hand, GlcNAc shows a sweet taste and will be able to be applied for orally administrated supplement. Since GlcNAc is also a component of proteoglycan and a part of GlcN is transformed to GlcNAc by metabolism procedure, the therapeutic activity on osteoarthritis for GlcNAc would be similar to that of GlcN. Although GlcNAc is produced by acid hydrolysis of chitin, this procedure has some problems such as high cost, low yield (Sakai, 1995), and acidic wastes by use of conc. HCl, etc. *N*-acetylation of GlcN is also possible to produce GlcNAc. This product, however, is not approval as a natural type material owing to its chemical process.

Recently, we have reported the production of GlcNAc from α-chitin or β-chitin by use of crude enzymes and obtained GlcNAc in good yields (Sashiwa et al., 2001a,b, 2002). The present article describes the detailed study on the production of GlcNAc from various chitins and *N*-acetylchitooligosaccharides by enzymatic hydrolysis as conclusion. The quantitative production of GlcNAc could be achieved from β-chitin by the mixing of two crude enzymes such as cellulases derived from *Trichoderma viride* (T) and *Acremonium cellulolyticus* (A).

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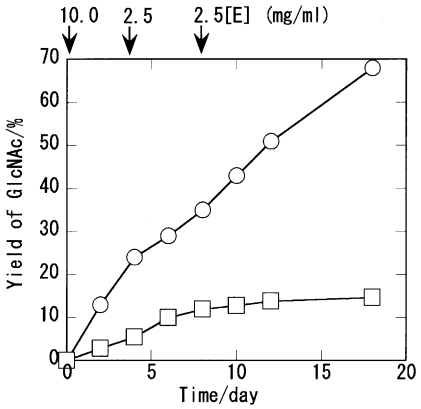

Fig. 1. Time courses on the production of GlcNAc from chitins by cellulase T. ○, β-chitin (3.1 μm); □, α-chitin (14.3 μm). Chitin: [S] = 10 mg/ml; cellulase T: [E] = 10 mg/ml (0–4 days), after 4 and 8 days, enzyme were added; [E] = 12.5 mg/ml (4–8 days); [E] = 15.0 mg/ml (8–18 days); pH 4.8 (AcOH buffer: 10 ml); 37 °C.

2. Experimental

2.1. Materials

Powdered β-chitin (particle size = 3.1 μm, derived from squid pen) and α-chitin (3.8 and 14.3 μm, derived from crab shell) were supplied from Sunfive Co. Ltd, Japan. *N*-acetylchitooligosaccharides were purchased from Seikagaku Corporation, Japan. Cellulase T and flake chitin (>50 μm) were purchased from Wako Pure Chemical Industries Ltd, Japan. Cellulase A was supplied from Meiji Seika Co., Japan. Lipase, hemicellulase, and pectinase were also supplied from Amano Pharmaceutical Co., Japan. These crude enzymes are essentially active for non-chitinous substrates such as cellulose, hemicellulose, and pectin. However, these enzymes include chitinolytic enzymes as contaminants such as chitinase (EC 3.2.1.14) and β-*N*-acetylhexosaminidase (EC 3.2.1.52).

2.2. General methods

Typical procedure is as follows: chitin (100 mg) was suspended in 10 ml of 0.1 M AcOH buffer (pH 4.0). To a suspension, enzyme (100 mg) was added and shaken at 37 °C. After the prescribed time, a part of reaction mixture (10–100 μl) was taken out, diluted with H_2O (0.4–0.49 ml) and CH_3CN (1.0 ml), filtered, and analyzed to measure the amount of GlcNAc in the reaction mixture by HPLC. The amount of GlcNAc $(GlcNAc)_2$ and was estimated from the calibration curve of standard GlcNAc and $(GlcNAc)_2$. The yield of GlcNAc was calculated by the following equation.

Yield(%) = [GlcNAc produced (mol)/

repeating unit of chitin (mol) added] × 100

The yield of $(GlcNAc)_2$ was also estimated by HPLC in a similar way for GlcNAc as follows.

Yield(%) = [$(GlcNAc)_2$ produced (mol)/repeating unit of $(GlcNAc)_2$ in *N*-acetylchitooligosaccharides or chitin (mol) added] × 100.

2.3. Analysis of hydrolyzate by HPLC and NMR

HPLC analysis of hydrolyzate was performed on a Tosoh LC-8020 apparatus (column, Shodex Asahipak NH2P-50; rt; CH_3CN/H_2O = 7/3; flow rate = 1.0 ml/min; injection, 0.1 ml; detection, UV at 210 nm). After the enzymatic hydrolysis, insoluble materials were removed by centrifugation (3000 rpm). Water-soluble fraction was purified by HPLC under the same conditions as described above. A part of eluted fractions corresponding to the peak area of GlcNAc (Rt = 5.3 min) was collected, dried, and weighed (5 mg). The product (5 mg) was dissolved in D_2O and ^1H and ^{13}C NMR spectra were taken on JEOL A-500 NMR spectrometer.

2.4. Enzymatic activity

The activity of endo-type enzyme (mU) was estimated by reducing end group determination according to the modified Schale's method (Imoto & Yagishita, 1971) using 70% deacetylated chitin as a substrate at pH 4.0 and 37 °C (one unit means 1 μmol of reducing end group produced per 1 min for 1 mg of enzyme). That of exo-type enzyme (mU) was determined by the production of GlcNAc from $(GlcNAc)_2$ at pH 4.0 and 37 °C, which was monitored by HPLC as described above.

3. Results and discussion

3.1. Hydrolysis of β-chitin by various crude enzymes

Chitosan was degraded by crude enzymes such as hemicellulase and lipase (Aiba & Muraki, 1999; Muzzarelli, Tomasetti, & Ilari, 1994; Muzzarelli, Xia, Tomasetti, & Ilari, 1995; Yalpani & Pantaleone, 1994). Moreover, non-chitinolytic crude enzymes such as cellulase, which essentially hydrolyze cellulose as a substrate, also degraded both chitin and partially *N*-acetylated chitosans owing to the presence of endo- and exo-type chitinases in crude enzyme preparations (Aiba & Muraki, 1999; Sashiwa et al., 2001a; Sukwattanasinitt, Zhu, Sashiwa, & Aiba, 2002; Zhu et al., 2001). In our previous report, the positive production of GlcNAc from β-chitin (derived from squid pen), which shows good swelling property compared with α-chitin (derived from crab or shrimp shell), was accomplished by crude enzymes such as cellulase T and A (Sashiwa et al., 2001a). Fig. 1 shows the time courses on the production of GlcNAc by cellulase T in comparison with α-chitin (14.3 μm) and β-chitin (3.1 μm). To maintain the hydrolysis reaction, cellulase T was added again after

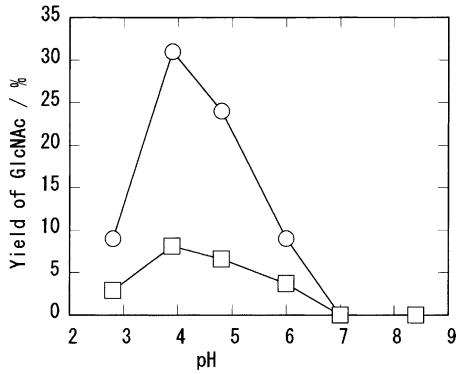

Fig. 2. Effect of pH on the production of GlcNAc from β-chitin. ○, 4 days; □, 1 day; chitin: [S] = 10 mg/ml; cellulase T: [E] = 10 mg/ml; 4 ml; 37 °C; pH 2.8–6.0, 0.1 M AcOH buffer; pH 7.0–8.4, 0.1 M phosphate buffer.

4 and 8 days. β-Chitin was continuously hydrolyzed even after 19 days and 74% of GlcNAc was produced. In the case of α-chitin, however, the production of GlcNAc was almost stopped after 8 days and the yield of GlcNAc was low (16%) even after 19 days. Fig. 2 shows the effect of pH on the hydrolysis of β-chitin. The optimum pH was around 4.0 under these conditions and hydrolysis did not proceed over pH 7. Although some results were shown under the moderate hydrolysis conditions (pH 4.8), the following study toward the most efficient production of GlcNAc was mainly performed at the optimum conditions (pH 4.0).

The effect of particle size on the hydrolysis of α-chitin was tested in Table 1. In comparison with 3.8, 14.3, and over 50 μm, the particle size or surface area was slightly effective on the hydrolysis of α-chitin. In contrast, β-chitin was degraded by cellulase T at quite higher rate than α-chitin at the similar level of particle sizes. These results suggest that the difference of the crystalline structure (α or β) would be a more important factor than surface area on the production of GlcNAc from chitin by cellulase T. From the HPLC chromatogram of the hydrolyzate of any chitins, quite selective production of GlcNAc was observed and no production of GlcNAc oligomers was found except for cellulase A. The NMR analysis of hydrolyzate of β-chitin by cellulase T was performed to confirm its chemical structure. From the ^{1}H and ^{13}C NMR analysis, hydrolyzate showed the same ^{1}H and ^{13}C NMR signals compared with authentic GlcNAc, thus indicates that the chemical structure of the hydrolyzate was confirmed as GlcNAc.

Table 1
Effect of particle size on the production of GlcNAc by cellulase T

Chitin	Size (μm)	Yield of GlcNAc (%)
β-Chitin	3.1	14.0
α-Chitin	3.8	6.0
α-Chitin	14.3	3.6
α-Chitin[a]	>50	1.1

Chitin [S] = 10 mg/ml; cellulase T [E] = 10 mg/ml; pH 4.8 (2 ml); 37 °C; 3 days.
[a] Flake chitin.

Table 2
Production of GlcNAc from *N*-acetylchitooligosaccharide by various enzymes

Enzyme	Yield[a] (%) of GlcNAc		
	$(GlcNAc)_2$	$(GlcNAc)_4$	$(GlcNAc)_6$
Cellulase T	33	23	21
Cellulase A	3	0[b]	0[b]
Hemicellulase	100	87	89
Lipase	100	100	84
Pectinase	100	87	100

[S] = 0.8 mg/ml; [E] = 0.4 mg/ml; pH 4.8 (2 ml); 37 °C; 1 day.
[a] Yield(%) = [GlcNAc produced (mmol)/repeating unit in *N*-acetylchitooligosacchride (mmol) added] × 100.
[b] Only $(GlcNAc)_2$ was obtained quantitatively.

3.2. Hydrolysis of N-acetylchitooligosaccharides

Table 2 shows the production of GlcNAc from *N*-acetylchitooligosaccharides by various crude enzymes. The quantitative production of GlcNAc was observed by lipase, hemicellulase, and pectinase, though that by cellulase T was not so effective. While cellulase A did not produce GlcNAc but quantitatively produce $(GlcNAc)_2$ from $(GlcNAc)_4$ and $(GlcNAc)_6$. This result could be explained as follows. Crude lipase has higher activity of exo-chitinase than endo-chitinase from results of the high production of GlcNAc from oligomers but low yield from chitin. Cellulase T has a moderate activity of exo-chitinase and the selective production of GlcNAc from chitin was accomplished by the cooperative action of endo- and exo-chitinases. In the case of cellulase A, it includes high activity of endo- and exo-chitinase activity would be quite low. To confirm the production of GlcNAc monomer from chitin by cellulase A, we tested the hydrolysis of *N*-acetylchitooligosaccharides at

Table 3
Hydrolysis of *N*-acetylchitooligosaccharide by cellulase A

Substrate	Time (day)	Yield (%)	
		$(GlcNAc)_2$	GlcNAc
$(GlcNAc)_2$	2	53	47
$(GlcNAc)_2$	3	36	64
$(GlcNAc)_2$	8	0	100
$(GlcNAc)_3$	2	58	42
$(GlcNAc)_3$	3	51	49
$(GlcNAc)_3$	8	14	86
$(GlcNAc)_5$	2	28	72
$(GlcNAc)_5$	3	5	95
$(GlcNAc)_5$	8	0	100

[S] = 0.8 mg/ml; [E] = 3.2 mg/ml; pH 4.0 (2 ml); 37 °C.

Table 4
Enzymatic activity of endo- and exo-chitinases in crude enzyme preparations

Enzyme	Endo (mU/mg)	Exo (mU/mg)
Cellulase T	3.7	1.4
Cellulase A	12.3	0.18
Lipase	2.7	9.8

Sashiwa et al. (2001b).

high concentration of the enzyme. As shown in Table 3, $(GlcNAc)_2$ was gradually hydrolyzed to monomer and perfectly hydrolyzed after 8 days. In the case of $(GlcNAc)_3$ and $(GlcNAc)_5$, similar results were given. These results suggest that a small amount of exo-chitinase was included in cellulase A and gave GlcNAc monomer from *N*-acetylchitooligosaccharides.

3.3. Effect of mixing of enzymes

Since the activities of endo- and exo-chitinases were different in each crude enzymes (Table 4, Sashiwa et al., 2001b), the mixing of two crude enzymes will allow to enhance the production of GlcNAc. As shown in Table 5, the good enhancement of the GlcNAc production (73%) was observed by mixing of cellulase T and A compared with these enzymes alone (58 and 59%). In the case of mixing cellulase A/hemicellulase (58%), cellulase A/lipase (62%), and cellulase A/pectinase (59%), the yield of GlcNAc were slightly enhanced. In Table 6, these mixing effects were also observed on the hydrolysis of α-chitin and the production of GlcNAc was most enhanced by mixing of cellulase T and A. Table 7 shows the mixing effect of endo- and exo-chitinases on the production of GlcNAc. With mixing cellulase A and lipase, over 42% yield of GlcNAc was shown at 5–50 of endo/exo ratio (mU/mU). In our previous report (Sashiwa et al., 2001b), similar enhancement was also observed by mixing of cellulase T and A. These results suggest that the optimum ratio of endo- and exo-chitinases in the crude enzyme preparations would be around these ranges (5–50) for the effective production of GlcNAc.

Table 5
Effect of mixing of enzyme on the production of GlcNAc from β-chitin

Cellulase T (mg/ml)	Cellulase A (mg/ml)	Hemicellulase (mg/ml)	Lipase (mg/ml)	Pectinase (mg/ml)	Yield (%)
40	–	–	–	–	58
–	40	–	–	–	59[a]
–	–	40	–	–	12
–	–	–	40	–	16
–	–	–	–	40	9
20	20	–	–	–	73
20	–	20	–	–	44
20	–	–	20	–	54
20	–	–	–	20	43
–	20	20	–	–	58
–	20	–	20	–	62
–	20	–	–	20	59

β-Chitin: [S] = 10 mg/ml; pH 4.0 (2 ml); 37 °C; 3 days.
[a] $(GlcNAc)_2$ was detected (Y = 11%).

Table 6
Effect of mixing enzymes on the production of GlcNAc from α-chitin

Cellulase T (mg/ml)	Cellulase A (mg/ml)	Yield (%)
40	–	16
–	40	22
20	20	33

α-Chitin: [S] = 10 mg/ml; pH 4.0 (2 ml); 37 °C; 3 days.

In conclusion, selective production of GlcNAc from both α- and β-chitin could be achieved by the crude enzyme preparations such as cellulase T, A, and lipase. Since these crude enzymes are quite advantage for cost (0.11–0.13 US$/g), the use of equal weight of enzyme to chitin is economically feasible. Another cost problem is that β-chitin (5.6–24 US$/g) is too expensive than α-chitin (0.14 US$/g). Recently, we reported that more effective hydrolysis of α-chitin could be achieved by crude bacterial enzymes. For example highly production of GlcNAc from α-chitin was accomplished by crude enzymes from *Aeromonas hydrophila* H-2330 in 77% yield (Sashiwa et al., 2002), and those from Burkholderia cepacia in 85% yield (Pichyangkura, Kudan, Kuttiyawong, Sukwattanasinitt, & Aiba, 2002). Needless to say, enzymatic hydrolysis is conducted under quite mild conditions and high selectivity is achieved in the production of GlcNAc compared with chemical process. This method would be useful for the industrial production of GlcNAc, which will be commercialized as a food supplement.

Acknowledgements

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Table 7
Effect of endo- and exo-chitinases on the production of GlcNAc

Enzyme/enzyme (mg/mg)	Endo (mU)	Exo (mU)	Endo/Exo (mU/mU)	Yield (%)	
				GlcNAc	$(GlcNAc)_2$
Cell A only (10/0)	12.3	0.18	68.3	23	10
Cell A/Lipase (9.5/0.5)	11.8	0.66	47.9	42	0
Cell A/Lipase (8.5/1.5)	10.9	1.62	6.7	44	0
Cell A/Lipase (8/2)	10.4	2.1	4.9	43	0
Cell A/Lipase (7/3)	9.4	3.1	3.1	35	0
Cell A/Lipase (5/5)	7.5	5.0	1.5	30	0
Cell A/Lipase (3/7)	5.6	6.9	0.8	25	0
Lipase only (0/10)	2.7	9.8	0.23	13	0

β-chitin: [S] = 10 mg/ml; [E] = 10 mg/ml; pH 4.0 (2 ml); 37 °C; 5 days. Endo- and exo-activities (mU) for mixed enzymes were calculated on the bases of the summation of those (mU) for cellulase A and lipase.

References

Aiba, S., & Muraki, E. (1999). Preparation of higher *N*-acetylchitooligosaccharides in high yields. In R. H. Chen, & H.-C. Chen (Eds.), (*Vol. III*) (pp. 89–96). *Advances in chitin science*, Taiwan: Rita Advertising Co. Ltd.

Crolle, G., & D'Este, E. (1980). Glucosamine sulphate for the management of arthrosis a controlled clinical investigation. *Current Medical Research and Opinion*, *7*, 104–109.

Drovanti, A., Bignamini, A. A., & Rovati, A. L. (1980). Therapeutic activity of oral glucosamine sulfate in osteoarthrosis: A placebo-controlled double-blind investigation. *Clinical Therapeutics*, *3*, 260–272.

Imoto, T., & Yagishita, K. (1971). A simple activity measurement of lysozyme. *Agriculture of Biological Chemistry*, *35*, 1154–1156.

Kajimoto, O., Sakamoto, K., Takamori, Y., Kajitani, N., Imanishi, T., Matsuo, R., & Kajitani, Y. (1998). Therapeutic activity of oral glucosamine hydrochloride in osteoarthritis of the knee: A placebo-controlled, double-blind, cross-over study. *Nippon Rinsho Eiyo Gakkaishi*, *20*, 41–47.

Kim, J. J., & Conrad, E. H. (1974). Effect of D-glucosamine concentration on the kinetics of mucopolysaccharide biosynthesis in cultured chick embryo vertebral cartilage. *Journal of Biological Chemistry*, *249*, 3091–3097.

Muzzarelli, R. A. A., Tomasetti, M., & Ilari, P. (1994). Depolymerization of chitosan with the aid of papain. *Enzyme Microbiology and Technology*, *16*, 110–114.

Muzzarelli, R. A. A., Xia, W., Tomasetti, M., & Ilari, P. (1995). Depolymerization of chitosan and substituted chitosans with the aid of a wheat germ lipase preparation. *Enzyme Microbiology and Technology*, *17*, 541–545.

Pichyangkura, R., Kudan, S., Kuttiyawong, K., Sukwattanasinitt, M., & Aiba, S. (2002). Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. *Carbohydrate Research*, *337*, 557–559.

Sakai, K. (1995). *Chitin and chitosan handbook*. Gihodo, Tokyo: Japanese Society of Chitin and Chitosan, pp. 209–218.

Sashiwa, H., Fujishima, S., Yamano, N., Kawasaki, N., Nakayama, A., Muraki, E., & Aiba, S. (2001a). Production of *N*-acetyl-D-glucosamine from β-chitin by enzymatic hydrolysis. *Chemistry Letter*, 308–309.

Sashiwa, H., Fujishima, S., Yamano, N., Kawasaki, N., Nakayama, A., Muraki, E., Hiraga, K., Oda, K., & Aiba, S. (2002). Production of *N*-acetyl-D-glucosamine from α-chitin by crude enzymes from *Aeromonas hydrophila* H-2330. *Carbohydrate Research*, *337*, 761–763.

Sashiwa, H., Yamano, N., Fujishima, S., Muraki, E., Kawasaki, N., Nakayama, A., Sakamoto, K., Ohta, H., Nishikata, Y., Hiraga, K., Oda, K., & Aiba, S. (2001b). Production of *N*-acetyl-D-glucosamine from chitin by enzymatic hydrolysis. *Chitin and Chitosan Research*, *27*, 257–260.

Setnikar, I., Giacchetti, C., & Zanolo, G. (1986). Pharmacokinetics of glucosamine in the dog and man. *Arznein-Forsch/Drug Ressearch*, *36*, 729–735.

Setnikar, I., Palumbo, R., Canali, S., & Zanolo, G. (1993). Pharmacokinetics of glucosamine in the man. *Arznein-Forsch/Drug Reserach*, *43*, 1109–1113.

Sukwattanasinitt, M., Zhu, H., Sashiwa, H., & Aiba, S. (2002). Utilization of commercial non-chitinase enzymes from fungi for preparation of 2-acetamido-2-deoxy-D-glucose from β-chitin. *Carbohydrate Research*, *337*, 133–137.

Tapadinhas, M. J., Rivera, I. C., & Bignamini, A. A. (1982). Oral glucosamine sulphate in the management of arthrosis: Report on a multi-centre open investigation in Portugal. *Pharmatherapeutica*, *3*, 157–168.

Vaz, A. L. (1982). Double-blind clinical evaluation of the relative efficacy of ibuprofen and glucosamine sulphate in the management of osteoarthrosis of the knee in out-patients. *Current Medical Research and Opinion*, *8*, 145–149.

Yalpani, M., & Pantaleone, D. (1994). An examination of the unusual susceptibilities of aminoglycans to enzymatic hydrolysis. *Carbohydrate Research*, *256*, 159–175.

Zhu, H., Sukwattanasinitt, M., Pichyangkura, R., Miyaoka, S., Yunoue, M., Muraki, E., & Aiba, S. (2001). Preparation of *N*-acetyl-D-glucosamine from chitin by enzymatic hydrolysis. In T. Uragami, K. Kurita, & T. Fukamizo (Eds.), *Chitin and chitosan–chitin and chitosan in life science* (pp. 330–331). Tokyo: Kodansha Scientific Ltd.