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Utilization of commercial non-chitinase enzymes from fungi for preparation of 2-acetamido-2-deoxy-D-glucose from β-chitin

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Abstract

Commercial non-chitinase enzymes from *Aspergilus niger*, *Acremonium cellulolyticus* and *Trichoderma viride* were investigated for potential utilization in the preparation of 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine, GlcNAc) from chitin. Among the tested enzymes, cellulase *A. cellulolyticus* exhibited highest chitinolytic activity per weight toward amorphous chitin and β-chitin from squid pen. The optimum pH of the enzyme was 3 where it produced two major hydrolytic products, GlcNAc and *N*,*N*′-diacetylchitobiose ([GlcNAc]$_2$). The product ratio, GlcNAc:[GlcNAc]$_2$, increased while the total yield decreased as the pH was raised from 3. All of the [GlcNAc]$_2$ produced at pH 3 can be converted in situ to GlcNAc by mixing cellulase *A. cellulolyticus* with one of several other enzymes from *A. niger* resulting in a higher yield of GlcNAc. An appropriate mixing ratio of cellulase *A. cellulolyticus* to another enzyme was 9:1 (w/w) and an optimum substrate concentration was 20 mg/mL.

Keywords: *N*-Acetyl-D-glucosamine; Chitin; Chitinase; *Acremonium cellulolyticus*; *Aspergillus niger*; *Trichoderma viride*

1. Introduction

2-Acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine, GlcNAc) is a basic unit in many biologically important oligosaccharides, glycoproteins and glycolipids. GlcNAc has also been reported to have therapeutic potential in the treatments of various diseases including osteoarthristis,[1] inflammatory bowel disease[2] and gastritis.[3] The preparation of GlcNAc is currently carried out by reacetylation of D-glucosamine produced from an acid hydrolysis of chitin.[4] The acid hydrolysis is usually performed by heating chitin in concentrated hydrochloric acid, which poses technical and environmental concerns. It is also virtually impossible to avoid decomposition of the desired product without having an uncompleted hydrolysis under such harsh conditions. The reaction thus usually gives a limited yield of D-glucosamine. Enzymatic hydrolyses usually proceed under much milder conditions with less toxic reagents. A continuous two-step process for industrial production of GlcNAc using chitinase from *Serratia marcesens* has been developed.[5] Recently, we, however, relied on another approach using inexpensive, commercially available, non-chitinase enzymes for the preparation of GlcNAc.[6] A high yield of GlcNAc can be produced cleanly by hydrolysis of amorphous[7] or β-chitin[8] with cellulase *Tv* (*Trichoderma viride*). The present drawback of this approach is a relatively low chitinolytic activity of this enzyme. We report here a systematic study centering on another commercial enzyme, cellulase *Ac* (*Acremonium cellulolyticus*), which has led to higher efficiency of GlcNAc production from β-chitin by commercial crude enzymes.

2. Results and discussion

Six commercial non-chitinase enzymes from three species of fungi, *Aspergilus niger* (*An*), *A. cellulolyticus* (*Ac*) and *T. viride* (*Tv*), were tested for hydrolytic activity of amorphous chitin at pH 4.5. Cellulase *Ac*

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exhibited the highest chitinolytic activity among the six enzymes evaluated (Table 1). This enzyme, however, produced two major hydrolytic products, GlcNAc and $[GlcNAc]_2$. Thus this enzyme was not suitable for the preparation of GlcNAc in one step. For a one-step hydrolysis of chitin to GlcNAc, the enzyme preparation must contain both chitinase (EC 3.2.1.14) and β-*N*-acetylhexosaminidase (EC 3.2.1.52) activities. The chitinase is responsible for hydrolyzing polymeric chitin into smaller oligosaccharides, which are in turn further hydrolyzed by β-*N*-acetylhexosaminidase to GlcNAc. To our delight, cellulase *Ac* also showed chitinolytic activity on β-chitin comparable to that observed on amorphous chitin. The use of β-chitin in place of amorphous chitin can simplify substrate preparation since amorphous chitin must be prepared from a highly basic solution of chitin. Further experiments were thus performed on β-chitin. Our pH dependence study revealed that significant yield of $[GlcNAc]_2$ was observed at pH 3 (Table 2), which was the optimum pH, suggesting that cellulase *Ac* contained only a limited amount of β-*N*-acetylhexosaminidase. The results also indicated that the chitinase activity in cellulase *Ac* was highly acidophilic. The total yields of GlcNAc and $[GlcNAc]_2$ dropped sharply as the pH increased from 3 to 4, but decreased only slightly as the pH lowered to 2. The β-*N*-acetylhexosaminidase activity in this enzyme was, however, less acidophilic as the yield of GlcNAc decreased only slightly as the pH increased to pH 4.

To improve the production of GlcNAc in a one-step hydrolysis, cellulase *Ac* should be combined with another enzyme possessing high β-*N*-acetylhexosaminidase activity. All six enzymes were tested again for β-*N*-acetylhexosaminidase activity using $[GlcNAc]_2$ as a substrate. Despite possessing lower chitinolytic activity (Table 1), the enzymes from *An* and *Tv* displayed higher β-*N*-acetylhexosaminidase activity than cellulase *Ac* (Table 3). Furthermore, the enzymes from *An* showed considerably higher β-*N*-acetylhexosaminidase activity than that of the enzyme from *Tv*. The slow hydrolysis of $[GlcNAc]_2$ with cellulase *Ac* also confirmed that this crude enzyme contained a limited amount of β-*N*-acetylhexosaminidase.

The yield of GlcNAc was significantly increased when the enzymes from *Tv* and *An* were mixed with cellulase *Ac* (Table 4). The mixed enzymes from cellulase *Tv* nonetheless gave a significant amount of $[GlcNAc]_2$ as a hydrolytic product owing to lower β-*N*-acetylhexosaminidase activity of cellulase *Tv* compared to those of enzymes from *An* (Table 3). There was no $[GlcNAc]_2$ observed up to the enzyme mixing ratio of 9:1 cellulase *Ac*–another enzyme, when each of the enzymes from *An* was used in the mixing. The yield of GlcNAc was also increased significantly when the mixing ratio was changed from 1:1 to 9:1. As the mixing ratio increased further to 19:1, trace amounts of

Table 1
Chitinolytic activity of some commercial enzymes on amorphous chitin [a]

Enzyme [b]	% Yield of GlcNAc [c]
Cellulase *An*	less than 1%
Hemicellulase *An*	less than 1%
Lipase *An*	less than 1%
Pectinase *An*	less than 1%
Cellulase *Ac*	13.1
Cellulase *Tv*	8.6

[a] [S] = 1.4 mg/mL; [E] = 1.5 mg/mL in acetate buffer pH 4.5.
[b] All enzymes were obtained from Amano Enzyme, Inc. except cellulase *Ac* which was obtained from Meiji Seika Co.
[c] Percentage yields were determined by HPLC after incubation at 37 °C for 3 days.

Table 2
pH dependence of chitinolytic activity of cellulase *Ac* on β-chitin [a]

pH [b]	% Yield [c]		
	GlcNAc	$(GlcNAc)_2$	Total
2	25	25	50
3	28	28	56
4	24	11	35
5	18	3	21
6	9	0	9
7	0	0	0

[a] [S] = 10 mg/mL; [E] = 10 mg/mL.
[b] Citrate buffer was used for pH 2 and citrate–phosphate buffer was used for pH 3–7.
[c] Percentage yields were determined by HPLC after incubation at 37 °C for 4 days.

Table 3
β-*N*-Acetylhexosaminidase activity of some commercial non-chitinase enzymes [a]

Enzyme	% Yield [b] GlcNAc	Relative activity
Cellulase *An*	100	25
Hemicellulase *An*	96	24
Lipase *An*	95	24
Pectinase *An*	100	25
Cellulase *Ac*	4	1
Cellulase *Tv*	13	3

[a] *N*,*N*′-Diacetylchitobiose was used as a substrate, [S] = 1 mg/mL; [E] = 1 mg/mL in citrate–phosphate buffer pH 3.
[b] Percentage yields were determined by HPLC after incubation at 37 °C for 6 h.

Table 4
Effect of mixing ratio on GlcNAc preparation [a]

Enzyme	Mixing ratio [b]	% Yield [c]	
		GlcNAc	$[GlcNAc]_2$
Cellulase *Ac*		28	28
Cellulase *Ac*:	1	55	7
Cellulase *Tv*	9	37	24
Cellulase *Ac*:	1	43	0
Cellulase *An*	9	61	0
	19	62	trace
Cellulase *Ac*:	1	45	0
Hemicellulase *An*	9	61	0
	19	64	0
Cellulase *Ac*:	1	48	0
Lipase *An*	9	61	0
	19	60	trace
Cellulase *Ac*:	1	44	0
Pectinase *An*	9	61	0
	19	64	0

[a] β-chitin was used as a substrate; [S] = 10 mg/mL; [E] = 10 mg/mL in citrate–phosphate buffer pH 3.
[b] Weight by weight ratio.
[c] Percentage yields were determined by HPLC after incubation at 37 °C for 4 days.

$[GlcNAc]_2$ were observed in some cases, but the changes in GlcNAc yields were insignificant. These results were reasonable since the amount of cellulase *Ac*, which possessed most of the chitinase activity essential for the first slow step in the hydrolysis of insoluble polymeric chitin, increased by 80% when the mixing ratio was changed from 1:1 to 9:1. On the other hand, the amount of cellulase *Ac* increased by only a mere 5% upon changing of the mixing ratio from 9:1 to 19:1.

Table 6
Time dependence of rate of GlcNAc production [a]

Time (day)	Yield of GlcNAc (mg/mL)	Relative production rate [b]
1	0.50	100
2	0.66	86
4	1.20	66
8	2.32	58
12	3.46	58

[a] The reaction was carried out by using 9:1 cellulase *Ac*:pectinase *An* and β-chitin in citrate–phosphate buffer pH 3. [E] = 2 mg/mL [S] = 20 mg/ml at 37 °C.
[b] The relative production rate on the *n* date was calculated from $100 \times Yield_n/(Yield_1 \times time)$.

The effects of substrate and enzyme concentrations were investigated by using the mixed enzyme, 9:1 cellulase *Ac* and lipase *An*. The increase of substrate concentration while keeping the enzyme concentration constant resulted in higher GlcNAc yield but lower percentage yield (Table 5). When both substrate and enzyme concentrations were raised from 10 to 20 mg/mL, the GlcNAc yield doubled and the percentage yield was not altered. The percentage yield was decreased significantly when both substrate and enzyme concentrations were further increased to 40 mg/mL. The reduction of the percentage yield of GlcNAc at high concentration of substrate and enzyme conceivably attributed to a poor mixing as the reaction media became very thick at this concentration.

Under conditions of excess substrate, the rate of GlcNAc production by a mixed enzyme, cellulase *Ac*–Pectinase *An*, decreased by over 40% during 12 days of incubation (Table 6). In most of the enzymatic reactions, there are two major causes for a decrease in the reaction rate: enzyme denaturation and product inhibition. The extent of the product inhibition was investi-

Table 5
Effect of concentrations of enzyme and substrate on GlcNAc production [a]

Concentration of enzyme (mg/mL)	Concentration of β-chitin (mg/mL)	% Yield [b]		Yield of GlcNAc (mg/mL)
		GlcNAc	$[GlcNAc]_2$	
10	10	61	0	6.1
10	20	48	trace	9.6
10	40	28	2	11.2
20	20	62	trace	12.4
40	40	33	4	12.2

[a] The reaction was carried out by using 9:1 cellulase *Ac*:lipase *An* mixed enzymes and β-chitin in citrate–phosphate buffer pH 3.
[b] Percentage yields were determined by HPLC after incubation at 37 °C for 4 days.

Table 7
Effect of GlcNAc concentration on the production rate [a]

Initial concentration of GlcNAc (mg/mL)	Yield of GlcNAc (mg/mL)	Relative production of GlcNAc [b]
0	4.9	100
2.0	4.6	94
5.0	4.1	84
8.0	4.0	82

[a] The reaction was carried out by using 9:1 cellulase *A*:pectinase *An* mixed enzymes and β-chitin in citrate–phosphate buffer pH 3. [E] = 10 mg/mL [S] = 10 mg/mL at 37 °C.
[b] The relative production of GlcNAc in the presence of *x* mg/mL GlcNAc was calculated from $100 \times \text{Yield}_x/\text{Yield}_0$.

gated by carrying out the reaction in the presence of GlcNAc intentionally added in various concentrations at the beginning of the reaction. The results showed that the production rate decreased by less than 5% when the initial concentration of GlcNAc was 2 mg/mL (Table 7), which was the average concentration range in the previous experiment. The results clearly indicated that the product inhibition was the only minor cause of the reduction in the production rate. However, enzyme denaturation should not be the only reason accounting for the rest of the rate decline since the rate looked rather constant after 8 days (Table 6). We speculated that the chitin substrate itself maybe the main reason for the decrease in the GlcNAc production rate. The substrate, β-chitin from squid pen, must contain both amorphous and crystalline domains integrated in the materials. The amorphous domains of the β-chitin particle are far more susceptible to enzymatic hydrolysis than the crystalline domains. The hydrolysis rate was thus faster at the beginning and slowed down as less of the amorphous volume remained in the substrate. Nonetheless, the β-chitin used mostly in this study showed hydrolytic susceptibility to cellulase *Ac* notably higher than the more natural abundant α-chitin,[7] but comparable to the amorphous base-regenerated chitin. In summary, the studies described here have shown the potential use of some commercial non-chitinase enzymes from fungi for a convenient preparation of GlcNAc from β-chitin. We are currently developing a simple and low-cost process for preparation of GlcNAc and $[\text{GlcNAc}]_2$ at multigram scale using β-chitin and these commercial enzymes from fungi.

3. Experimental

Amorphous chitin or β-chitin powder (particle size 3 μm) or $[\text{GlcNAc}]_2$ were used as a substrate for hydrolysis with six commercial enzymes (cellulase *An*, hemicellulase *An*, lipase *An*, pectinase *An*, cellulase *Ac* and cellulase *Tv*). The amorphous chitin and β-chitin contained less than 1% ash content. The degrees of acetylation of the chitins determined from elemental analysis were over 90%. The enzymes had protein ($M_W >$ 10,000) content of around 10%. A typical reaction was carried out by incubating a mixture of a known concentration of enzyme and the substrate in a suitable buffer solution in the presence of NaN_3 (500 ppm) preservative at 37 °C. The exact set of conditions for each experiment was specified in the footnote of the table. At each time point, a portion of the reaction mixture was sampled, diluted with water, then mixed with CH_3CN (at the ratio of 30:70, v/v), filtered, and analyzed by HPLC (column: Shodex Asahipak NH_2P-50; flow rate: 1 mL/min; mobile phase: 3:7 water–CH_3CN; detection: UV at 210 nm). The amounts of GlcNAc and $[\text{GlcNAc}]_2$ in the reaction mixtures were determined from the calibration lines of the corresponding standards. The percentage yields of GlcNAc or $[\text{GlcNAc}]_2$ were calculated based on the number of moles of the products produced against the number of moles of such units presented in the starting chitin.

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