

Regular Paper

Characterization of a GH Family 20 Exo- β -*N*-acetylhexosaminidase with Antifungal Activity from *Streptomyces avermitilis*

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Abstract: We characterized SaHEX, which is a glycoside hydrolase (GH) family 20 exo- β -*N*-acetylhexosaminidase found in *Streptomyces avermitilis*. SaHEX exolytically hydrolyzed chitin oligosaccharides from their non-reducing ends, and yielded *N*-acetylglucosamine (GlcNAc) as the end product. According to the initial rate of substrate hydrolysis, the rates of (GlcNAc)₃ and (GlcNAc)₅ hydrolysis were greater than the rates for the other oligosaccharides. The enzyme exhibited antifungal activity against *Aspergillus niger*, which was probably due to hydrolytic activity with regard to chitin in the hyphal tips. Therefore, SaHEX has potential for use in GlcNAc production and food preservation.

Key words: *N*-acetylhexosaminidase, chitin, antifungal activity, biopreservation

INTRODUCTION

Chitin, a β 1,4-linked homopolymer of *N*-acetylglucosamine (GlcNAc) residues, is the second most abundant biomass on earth.¹⁾ Chitin constitutes the structural scaffold of a wide range of living organisms, including fungal cell walls; the exoskeletons of insects, arthropods, and crustaceans; and the beaks of cephalopods.^{2,3,4,5)} Chitinases (EC 3.2.1.14) are enzymes that hydrolyze the β -1,4-glycosidic bonds of chitin, yielding soluble, low-molecular-weight chitin oligosaccharides. Exo- β -*N*-acetylhexosaminidases (HEXase, EC 3.2.1.52) decompose these oligosaccharides to the monosaccharide GlcNAc. According to the CAZy database (<http://www.cazy.org/>),⁶⁾ chitinases belong to glycoside hydrolase (GH) families 18 and 19, and HEXases belong to GH families 3, 20, and 84.^{7,8,9)} These enzymes contribute to chitin recycling, and maintain the natural carbon and nitrogen cycles. Industrially, chitin is purified from crab or shrimp waste, and is subsequently converted into valuable materials including GlcNAc. Although chitin has

been converted to GlcNAc chemically, enzyme-mediated conversion is regarded as a green and environmentally friendly process. Another distinguishing feature of these enzymes is their antifungal activity. Chitinases and HEXases hydrolytically degrade the chitin in the cell walls and septa of pathogenic fungi, and inhibit the growth of fungal mycelia.^{7,8,10,11,12)} Fungi are a major cause of microbial food spoilage, which has a huge economic cost. Therefore, fungal contamination of foods remains a significant problem in the food industry.

In common with other actinobacteria, members of the genus *Streptomyces* are aerobic, saprophytic, and gram-positive. They produce the majority of clinically important antibiotics and industrially important enzymes.¹³⁾ Moreover, they can also assimilate almost any natural polysaccharide, and produce enzymes that decompose them, such as amylase, glucanase, chitinase, chitosanase, xylanase, and various glycosidases,^{14,15,16,17)} some of which are commercially available. Therefore, because they are generally considered safe biodegrading natural products, there has been some focus on chitinolytic enzymes from *Streptomyces* as possible agents for GlcNAc production and the biopreservation of food. In the present study, we describe the enzymatic characterization of a GH20 enzyme from *Streptomyces avermitilis* called SaHEX. SaHEX is a commercial enzyme preparation (Nagase ChemteX Corporation, Osaka, Japan), and is licensed for use as a food additive in Japan.

MATERIALS AND METHODS

Materials. SaHEX, an exo- β -*N*-acetylhexosaminidase from

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Abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; GlcNAcase, exo- β -*N*-acetylglucosaminidase; (GlcNAc)_n, β -1,4-linked oligosaccharide of GlcNAc with a polymerization degree of *n*; GH, glycoside hydrolase; GlcNAc-GlcN, β -*N*-acetyl-D-glucosaminyl-(1,4)-D-glucosamine; HEXase, exo- β -*N*-acetylhexosaminidase; pNP-GlcNAc, *p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside; SaHEX, exo- β -*N*-acetylhexosaminidase from *Streptomyces avermitilis*.

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Streptomyces avermitilis was obtained from Nagase ChemteX Corp. The *nagZ4* gene (SAV_5268) encodes SaHEX in *S. avermitilis*. The amino acid sequence of this protein can be accessed through NCBI Protein Database under NCBI accession number BAC72980. GlcNAc and chitin oligosaccharides, (GlcNAc)_n (*n* = 2–6), were obtained by the acid hydrolysis of chitin from crab shells,¹⁸⁾ followed by gel filtration on Cellufine Gcl-25m (JNC Co., Tokyo, Japan). Hetero-disaccharide GlcNAc-GlcN was prepared by the method of Mitsutomi *et al.*¹⁹⁾ pNP-GlcNAc was obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). TSK-GEL G2000PW and TSK Amide-80 columns used for (GlcNAc)_n separation were from Tosoh Corporation (Tokyo, Japan). All other reagents were of analytical grade.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein determination. SDS-PAGE was done by the method of Laemmli using a 15 % acrylamide gel.²⁰⁾ Proteins on the gel were stained with Coomassie brilliant blue R250. The concentration of SaHEX was determined by reading absorbance at 280 nm using an extinction coefficient (106,230 M⁻¹ cm⁻¹) calculated from the equation proposed by Pace *et al.*²¹⁾

Enzyme assays. We investigated GlcNAcase activity of SaHEX spectrophotometrically by a *p*-nitrophenol (pNP) assay using pNP-GlcNAc as a substrate, and by a reducing sugar assay using (GlcNAc)_n (*n* = 2–6) or GlcNAc-GlcN as a substrate. For the pNP assay, we added the enzyme solution (1.5 μL) to 0.2 mL of 2 mM pNP-GlcNAc in 20 mM sodium acetate buffer (pH 5.0). After incubating the reaction mixture at 37 °C for 15 min, we added 0.8 mL of 0.25 M NaOH, and measured the absorbance of the released pNP spectrophotometrically at 405 nm. One enzyme unit (U) was defined as the amount of enzyme required to release 1 μmol of pNP per min at 37 °C. For the reducing sugar assay, we added the enzyme solution (5 μL) to 0.25 mL of 0.2 mM (GlcNAc)_n (*n* = 2–6) or GlcNAc-GlcN in 20 mM sodium acetate buffer (pH 5.0). After incubating the reaction mixture at 37 °C for 6 min, we determined the reducing power of the mixture with ferricyanide/ferrocyanide reagent according to the method described by Imoto and Yagishita.²²⁾ One enzyme unit (U) was defined as the enzyme activity that produced 1 μmol of GlcNAc per min at 37 °C.

Thin layer chromatography (TLC) of the reaction products produced by SaHEX. We incubated a reaction mixture comprising 2.1 μM SaHEX and 9.0 mM (GlcNAc)_n (*n* = 2–6) or GlcNAc-GlcN in 20 mM sodium acetate buffer (pH 5.0) at 37 °C for 0–30 min. The reaction products were analyzed by TLC using a silica gel 60 aluminum sheet (Merck, Darmstadt, Germany). The plate was developed in a solvent system comprising butan-1-ol/methanol/28.8 % ammonium hydroxide (5:4:2, by vol.), and the carbohydrates were visualized by heating the plate after dipping it in vanillin/H₂SO₄ reagent.

Anomer analysis of the reaction products produced by SaHEX. We used high-performance liquid chromatography (HPLC) to investigate the mode of cleavage of (GlcNAc)₃ and (GlcNAc)₄ by SaHEX by determining the anomeric forms of the enzymatic products. The enzymatic hydrolysis

of (GlcNAc)₃ and (GlcNAc)₄ was carried out in 20 mM sodium acetate buffer (pH 5.0), and the enzymatic reaction was conducted at low temperature (25 °C), to suppress mutarotation of the reaction products. The initial enzyme and substrate concentrations were 1.7 μM and 3.9 mM, respectively. After 2, 5, 15, and 30 min of incubation, we directly injected a portion of the reaction mixture into a TSK Amide-80 column, which was eluted with a mobile phase comprising 70 % acetonitrile and 30 % water at a flow rate of 0.7 mL/min. We detected the substrate and enzymatic products by ultraviolet absorption at 220 nm. The hydrolysis site of the oligosaccharide substrates was estimated from the product distribution and anomer ratio (α/β) of the individual oligosaccharide products.²³⁾ Because SaHEX is a retaining enzyme, the β-anomer should be abundant at the newly produced reducing ends. Therefore, the products, which are rich in the β-form, correspond to the glycon side of the substrate.

HPLC determination of the reaction time-course. We investigated the time-course of oligosaccharide degradation by quantitatively determining the reaction products from the SaHEX-catalyzed hydrolysis of (GlcNAc)_n (*n* = 2–6) using gel-filtration HPLC. The enzymatic reaction was performed in 20 mM sodium acetate buffer (pH 5.0) at 37 °C. The enzyme and substrate concentrations were 1.28 μM and 4.5 mM, respectively. To terminate the enzymatic reaction, we added an equal volume of 0.1 M NaOH solution to the reaction mixture, and immediately froze the solution in liquid nitrogen. After thawing, we applied a portion of the solution to a TSKgel G2000PW gel filtration column. Elution was carried out with distilled water at a flow rate of 0.3 mL/min. We detected the oligosaccharides by measuring the absorption of ultraviolet radiation at 220 nm. Peak areas obtained for the individual oligosaccharides were converted to molar concentrations, which were then plotted against the reaction time to obtain the reaction time-course.

Antifungal activity. We investigated the antifungal activity of SaHEX using *Aspergillus niger* as the test fungus. *A. niger* was grown on a potato dextrose agar (PDA) plate at 25 °C for 1 day. We deposited the fungal mycelia growing on the surface of the thin layer of PDA on the surfaces of microscope slides, and treated the slides with 20 mM sodium acetate buffer (pH 5.0; control) or the same buffer containing SaHEX (586 pmole). Within 5 min of treatment, we examined and photographed the slides using a light microscope (Olympus CH-2, Tokyo, Japan) at 400× magnification.

RESULTS AND DISCUSSION

Enzyme preparation and enzyme activity of SaHEX.

We dissolved 1 g of freeze-dried SaHEX obtained from Nagase ChemteX Corporation in 20 mL of 20 mM sodium acetate buffer (pH 5.0). Undissolved substances were removed by centrifugation at 14,000 × G for 15 min, and the supernatant was dialyzed against the same buffer. The SaHEX in the supernatant produced a single band on the SDS-PAGE, which indicates that the enzyme preparation was pure enough for biochemical characterization studies.

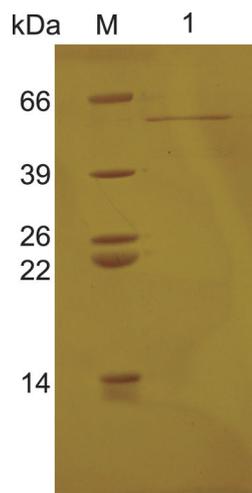


Fig. 1. SDS-PAGE analysis of *SaHEX*.
Lane M, molecular weight markers; 1, *SaHEX*.

Table 1. Specific activity of *SaHEX*.

Substrate	Specific activity (U/mg)*
pNP-GlcNAc	9.97**
GlcNAc-GlcN	3.84
(GlcNAc) ₂	8.45
(GlcNAc) ₃	6.74
(GlcNAc) ₄	22.2
(GlcNAc) ₅	24.0
(GlcNAc) ₆	23.3

*Specific activity was defined as $\mu\text{mol}/\text{min}$ of reducing sugar equivalent to GlcNAc released per milligram of enzyme. **Specific activity was defined as $\mu\text{mol}/\text{min}$ of pNP released per milligram of enzyme.

According to the SDS-PAGE analysis, the *SaHEX* had a molecular mass of 54 kDa, which corresponds to the molecular mass calculated from the amino acid sequence ($M_r = 53942.76$) (Fig. 1). The *SaHEX* was capable of hydrolyzing pNP-GlcNAc, (GlcNAc)_{*n*} (*n* = 2–6), and GlcNAc-GlcN. Among the natural glycosides tested, *SaHEX* produced more reducing sugars from (GlcNAc)_{*n*} (*n* = 4–6) than the others (Table 1).

TLC analysis of the reaction products produced by *SaHEX*.

We analyzed the patterns of (GlcNAc)_{*n*} (*n* = 2–6) degradation by *SaHEX* using TLC (Fig. 2A–E). The TLC analysis clearly indicated that *SaHEX* acted exolytically, yielding GlcNAc as the end product from all the chitin oligosaccharides; that is, (GlcNAc)_{*n*} (*n* = 2–6) was hydrolyzed to (GlcNAc)_{*n-1*} + (GlcNAc). The intermediate products—which were one GlcNAc unit shorter than the starting substrates—were further hydrolyzed to the final product GlcNAc. We also investigated the hydrolytic activity of *SaHEX* with regard to GlcNAc-GlcN. This hetero-disaccharide is a cell wall component of pathogenic fungi, and is a potential substrate for HEXase in nature.²⁴ It was shown that *SaHEX* is able to hydrolyze not only the β -1,4-glycosidic bond between GlcNAc-GlcNAc but also the bond between GlcNAc-GlcN (Fig. 2F). GH20 enzymes have been shown to use substrate-assisted mechanism involving the 2-

acetamidogroup of the –1 sugar.²⁵ Therefore, these results suggest that *SaHEX* has a specificity for the *N*-acetyl group of GlcNAc at subsite (–1), but not at subsite (+1).

Anomer analysis of the reaction products produced by *SaHEX*.

The anomeric forms of the enzymatic products provide information about the cleavage site of the oligosaccharide substrates. HPLC profiles showing the enzymatic hydrolysis of (GlcNAc)₃ and (GlcNAc)₄ are shown in Fig. 3. The GlcNAc product obtained from substrates (GlcNAc)₃ and (GlcNAc)₄ was rich in the β -anomer. Because the enzymatic reaction takes place through a substrate-assisted catalysis mechanism in GH20 family enzymes, *SaHEX* should produce the β -anomer during hydrolysis. Therefore, the predominance of the β -anomeric form of GlcNAc suggests that the first glycosidic linkage from the non-reducing end of the substrates was hydrolyzed by the enzyme. The α/β ratio of (GlcNAc)₂ to (GlcNAc)₃ in the hydrolysate of (GlcNAc)₄ was in a state of equilibrium ($\alpha/\beta = 1.6$), indicating that the product (GlcNAc)₃ from (GlcNAc)₄ was further hydrolyzed into GlcNAc and (GlcNAc)₂, just as the substrate (GlcNAc)₃ was. All of these results revealed that *SaHEX* hydrolyzes the first linkage from the non-reducing end of the chitin oligosaccharide substrates, indicating that *SaHEX* has GlcNAcase activity.

Time-course of chitin oligosaccharide hydrolysis by HPLC.

To determine the hydrolysis mechanism of *SaHEX*, we quantitatively investigated the enzymatic products by gel filtration HPLC. *SaHEX* rapidly released GlcNAc as the main product from (GlcNAc)_{*n*} (*n* = 2–6) (Fig. 4). (GlcNAc)_{*n*} was first converted to (GlcNAc)_{*n-1*} and an equivalent amount of GlcNAc. The intermediate product (GlcNAc)_{*n-1*} was further converted to (GlcNAc)_{*n-2*} and an equivalent amount of GlcNAc, and the reaction continued until all the intermediate products had been converted to the final end product GlcNAc. According to the initial rate of substrate hydrolysis, the rates of (GlcNAc)₃ and (GlcNAc)₅ hydrolysis were greater than the rates for the other oligosaccharides. *SaHEX* might degrade the odd-numbered chitin oligosaccharides faster than the even-ones except for (GlcNAc)₂. The rate of (GlcNAc)₂ hydrolysis was much lower than the rates of the other oligosaccharides. *ScHEX*, a β -*N*-acetylhexosaminidase from *S. coelicolor* A3(2) showed the highest activity against (GlcNAc)₆. In this case, the worst substrate was (GlcNAc)₄.²⁶ On the other hand, a β -*N*-acetylhexosaminidase from *S. alfalfae* showed the highest activity against (GlcNAc)₄, followed by (GlcNAc)₅, (GlcNAc)₆, (GlcNAc)₃, and (GlcNAc)₂.²⁷ The amino acid sequences of these enzymes were found to share more than 70 % identity with that of *SaHEX*. NagC, a β -*N*-acetylglucosaminidase from *S. thermoviolaceus* OPC-520, which has low sequence identity with *SaHEX* (22 %), hydrolyzed (GlcNAc)₂ faster than the other oligosaccharides, but did not hydrolyze (GlcNAc)₆.²⁸ Substrate specificity of GH20 enzymes from *Streptomyces* species is quite variable.

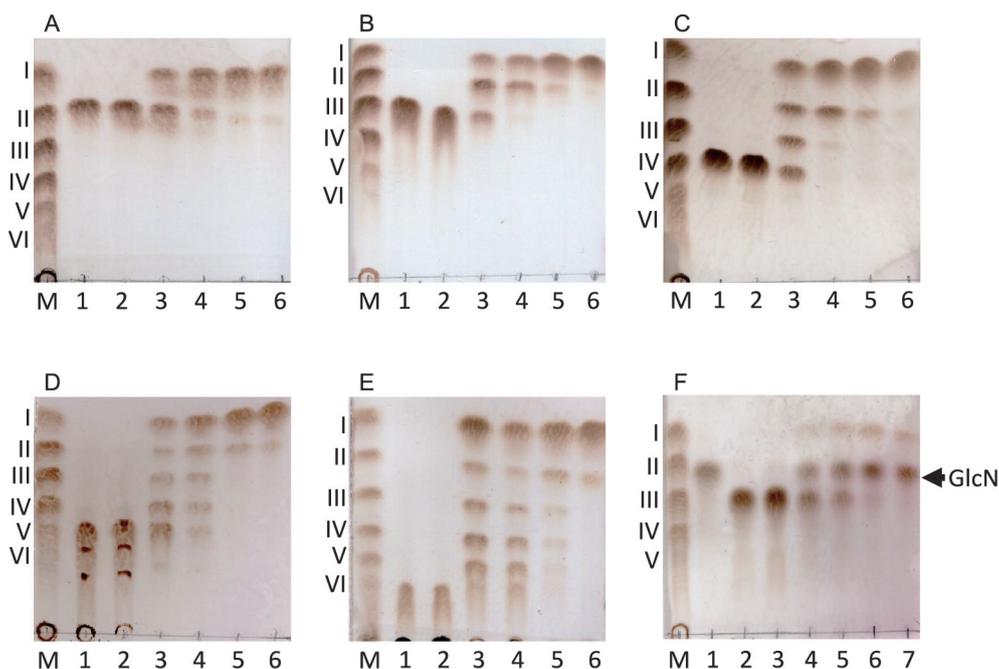


Fig. 2. TLC analysis of $(\text{GlcNAc})_n$ ($n = 2-6$) and GlcNAc-GlcN hydrolysis by *SaHEX*.

A reaction mixture comprising $2.1 \mu\text{M}$ *SaHEX* and 9.0 mM of: (A) $(\text{GlcNAc})_2$; (B) $(\text{GlcNAc})_3$; (C) $(\text{GlcNAc})_4$; (D) $(\text{GlcNAc})_5$; (E) $(\text{GlcNAc})_6$; or (F) GlcNAc-GlcN in 20 mM sodium acetate buffer ($\text{pH } 5.0$) was incubated at various times (from 0 to 30 min) at 37°C . (A)–(E) Lanes: M, a standard mixture of $(\text{GlcNAc})_n$ ($n = 1-6$); 1, substrate; 2–6, incubation for 0, 2, 5, 15, and 30 min, respectively. (F) Lanes: M, a standard mixture of $(\text{GlcNAc})_n$ ($n = 1-6$); 1, GlcN ; 2, substrate; 3–7, incubation for 0, 2, 5, 15, and 30 min, respectively.

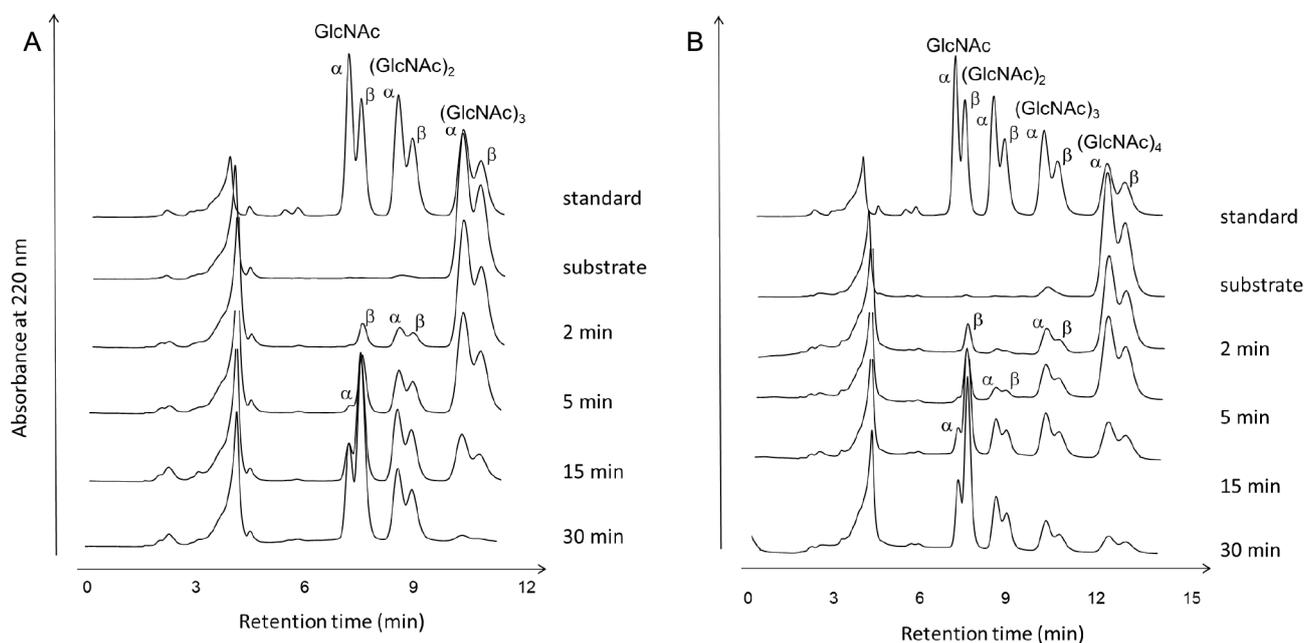


Fig. 3. Time-dependent HPLC profiles showing the hydrolysis of $(\text{GlcNAc})_3$ (A) and $(\text{GlcNAc})_4$ (B) by *SaHEX*.

The enzyme and substrate concentrations were $1.7 \mu\text{M}$ and 3.9 mM , respectively. The enzyme reaction was conducted in 20 mM sodium acetate buffer ($\text{pH } 5.0$) at 25°C .

Antifungal activity.

Aspergillus niger is a plant pathogen. It contains several classes of polysaccharides, including chitin in its cell walls. *A. niger* is also a common food contaminant, and causes black mold disease in certain vegetables and fruit.²⁹ Compared to the control—in which the *A. niger* hyphae were normal and healthy—treatment with *SaHEX* markedly affected the morphology of the hyphae. Close inspection of

the *SaHEX*-treated mycelium using a light microscope revealed cytoplasmic leakage, and swelling and bursting of the hyphal tips (Fig. 5B). However, we observed no such damage in the control experiment with the buffer solution (Fig. 5A). These changes seem to be due to degradation of the chitin and/or chitosan present in the cell walls, followed by changes to the membrane structure and mycelium permeability. In fact, when we evaluated the hydrolytic activi-

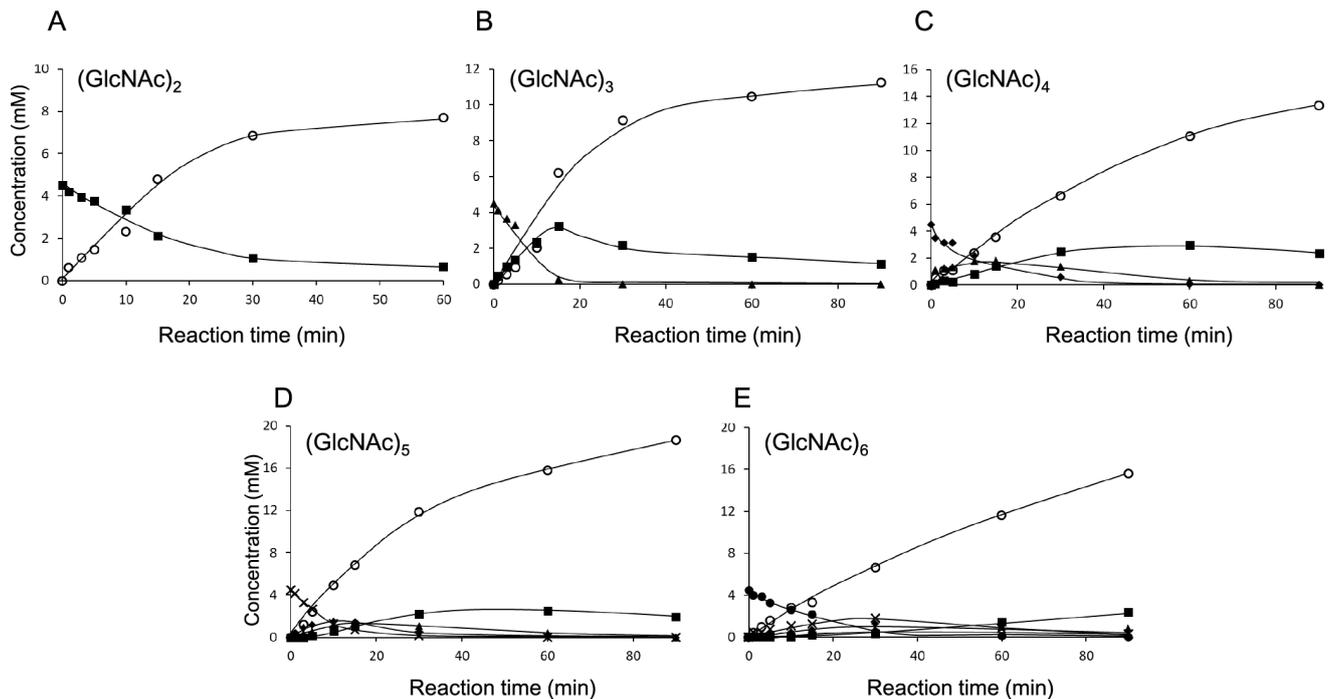


Fig. 4. Experimental time-courses of (GlcNAc) $_n$ ($n = 2-6$) degradation by *SaHEX*.

The enzyme and substrate concentrations were 1.28 μ M and 4.5 mM, respectively. The enzyme reaction was conducted in 20 mM sodium acetate buffer (pH 5.0) at 37 $^{\circ}$ C. The presented curves for individual (GlcNAc) $_n$ ($n = 1-6$) were obtained by visual estimation of the best fit to the experimental data points. Symbols: open circles, GlcNAc; squares, (GlcNAc) $_2$; triangles, (GlcNAc) $_3$; diamonds, (GlcNAc) $_4$; crosses, (GlcNAc) $_5$; closed circles, (GlcNAc) $_6$. (A) Substrate (GlcNAc) $_2$, (B) substrate (GlcNAc) $_3$, (C) substrate (GlcNAc) $_4$, (D) substrate (GlcNAc) $_5$, and (E) substrate (GlcNAc) $_6$.

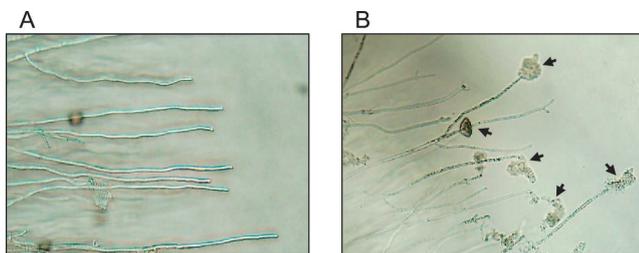


Fig. 5. Antifungal activity of *SaHEX* against *Aspergillus niger* on the culture medium.

Images showing the fungal hyphae treated with 20 mM sodium acetate buffer (pH 5.0) (A) and the fungal hyphae treated with the same buffer containing *SaHEX* (B). The black arrows indicate swollen and burst hyphal tips.

ty of *SaHEX* against the polymeric substrate glycol chitin, *SaHEX* increased the amounts of reducing sugars in the reaction mixture. These results indicate that *SaHEX* is indeed an antifungal protein that is active against fungal pathogens.

In conclusion, in the present study we demonstrated that *SaHEX* is an exo-acting enzyme that yields GlcNAc as the final product of (GlcNAc) $_n$ hydrolysis, and is also capable of hydrolyzing the hetero-disaccharide GlcNAc-GlcN. *SaHEX* also exhibited antifungal activity against *A. niger*, which was probably attributable to hydrolytic activity with regard to chitin in the hyphal tips. Therefore, *SaHEX* has potential for use in GlcNAc production and food preservation.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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