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Purification and Characterization of the Exo-β-D-Glucosaminidase from *Aspergillus flavus* IAM2044

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Abstract Chitosan-degrading activity induced by chitosan was found in culture filtrate of *Aspergillus flavus* IAM2044. *Aspergillus flavus* IAM2044 had a higher level of chitosanolytic activity when chitosan was used as a carbon source, and yeast extract and peptone were supplemented as nitrogen sources. One of the chitosan-degrading enzymes was purified to homogeneity by ammonium sulfate precipitation followed by cation-exchange and gel filtration chromatographies. The enzyme was monomeric, and its molecular mass was 45 kDa. The optimum pH and temperature of the enzyme were 5.0 and 50°C, respectively. The activity was stable in the pH range of 3.5 to 7.0 and at a temperature below 50°C. Reaction products analyzed by the viscosimetric assay and thin layer chromatography clearly indicated that the enzyme was an exo-type chitosanase, exo-β-D-glucosaminidase, that released GlcN from the nonreducing ends of the oligosaccharide chains.

Key words: *Aspergillus flavus*, chitosanase, exo-β-D-glucosaminidase

Chitin is one of the most abundant forms of biomass next to cellulose [8]. On the other hand, chitosan, a partially or fully deacetylated form of chitin, has been found only in the cell walls of limited groups of fungi in nature [3]. Recently, chitosan and its partially degraded oligosaccharides have become important natural products, because of their many potential applications in medicine and agriculture [2, 10, 11, 25].

Chitosanolytic enzymes have been found in a variety of microorganisms, including fungi and bacteria [1, 4, 7, 17, 18, 20, 21, 22]. In most cases, bacterial chitosanases (E.C. 3.2.1.132) are induced by the presence of chitosan, and play a role in the degradation and use of exogenous chitosan. In contrast, there have been only a few reports on fungal chitosanases, and their physiological role remains unclear, since chitosan is not utilized by fungi in most cases [1, 21]. Most purified chitosanases have been characterized as endo-type enzymes which cleave chitosans at random, and their reaction rates are highly dependent on the degree of acetylation (D.A.) of the chitosan. On the other hand, an exo-type chitosanase called exo-β-D-glucosaminidase, which releases glucosamine (GlcN) continuously from the nonreducing ends of the substrate, has so far been found in several microorganisms, including *Aspergillus oryzae* IAM2660, *A. fumigatus* KH-94, *Nocardia orientalis*, and *Trichoderma reesei* PC-3-7 [24, 14, 15]. A more detailed knowledge of the enzymatic difference of fungal chitosanases, including structural element, physiological role, and evolutionary origin, is needed for understanding their roles.

It was found that *A. flavus* IAM2044 secreted chitosanolytic enzymes when chitosan was present in the media. In this paper, the purification and characterization of the exo-β-D-glucosaminidase from *A. flavus* IAM2044 is described.

Materials and Methods

Materials

A. flavus IAM2044 was obtained from the Institute of Applied Microbiology (IAM). Various degrees of deacetylated chitosan (70% or 100%) were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Chitobiose, chitotriose, chitotetrose, chitopentose, chitohexose, and glycol chitosan were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chitin was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Glycol chitin was obtained from Seikagaku Kogyo Co., Ltd. Colloidal chitin was prepared by the method of Shimahara and Takiguchi [19]. Calcofluor white M2R and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade.

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Strain and Culture Conditions

The strain used in this study was *A. flavus* IAM2044. This strain was maintained on potato dextrose agar (Difco, USA) plates. The conidia were collected from mycelia by washing with sterilized water, and inoculated in Czapek-Dox (CD) medium (K_2HPO_4 1.0 g, KCl 0.5 g, $NaNO_3$ 2.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.02 g, soluble chitosan 0.5%, and deionized water 1,000 ml, pH 5.0) with 0.5% peptone and 0.5% yeast extract at a density of 1×10^6 spores ml^{-1}, and the resulting culture was incubated under the same conditions for an additional 3 days. Subsequently, this strain was spread onto CD-chitosan agar. Clear zones were observed around some colonies after incubation for several days at 28°C. After incubation at 28°C for several days, mycelia were collected by filtration and weighed after drying at 80°C overnight. For the large scale experiments, inoculum culture (1.0%) was added to a 3-l culture medium in a 5-l jar fermentor (Model KF-5L, Korea Fermentor Co., Ltd., Incheon, Korea) and the cultivation was carried out at 28°C for 4 days with vigorous shaking (200 rpm). After incubation, mycelia were removed by filtration, and the resulting culture filtrate was dialyzed against 50 mM sodium acetate buffer (pH 5.0) and used for the chitosanase assay.

Chitosanase Assay

A reaction mixture (1.0 ml) containing 1.0% (w/v) soluble chitosan (D.A. 30%) and an enzyme solution in 50 mM sodium acetate buffer, pH 5.0, was incubated for 15 min at 37°C. The reaction was terminated by immersing the test tube in boiling water for 5 min and the amount of reducing sugars liberated was determined by the method of Imoto and Yagishita [9], with glucosamine (GlcN) as standard. One unit of exo-β-D-glucosaminidase was defined as the amount of enzyme which liberated 1 μmol of reducing sugar as glucosamine per min.

Purification of Chitosanase

Unless otherwise indicated, all operations for the enzyme purification were carried out at 4°C. To the culture supernatant obtained from 920 ml culture containing 0.5% soluble chitosan (D.A. 30%) as the sole carbon source, enough ammonium sulfate was added to achieve 80% saturation. The resulting precipitate was collected by centrifugation and dissolved in 20 ml of 50 mM sodium acetate buffer (pH 5.0) (buffer A). The sample was dialyzed against buffer A and loaded onto a CM-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) column (2.7 by 23 cm) equilibrated with buffer A. Elution of the column with a linear gradient of buffer A from 0 to 1.0 M NaCl resulted in elution of adsorbed proteins with chitosanase activity in one distinct peak. The active fractions were pooled and concentrated by ultrafiltration (Amicon, Millipore, Bedford, U.S.A.). The sample was applied onto a Sephadex G-100 column (1.4 by 45 cm) equilibrated with buffer A at a flow rate of 15 mLh^{-1}. The resulting active fraction was concentrated by ultrafiltration (Amicon, U.S.A.), and used as the purified enzyme.

SDS-PAGE and Zymography

The purity of the enzyme was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli [13]. The polyacrylamide gels were stained with Coomassie brilliant blue R-250. After electrophoresis was completed, renaturation of the enzymes and detection of chitosanase activity in the gel containing 0.01% (v/v) soluble chitosan (D.A. 30%) in 50 mM sodium acetate buffer (pH 5.0) were performed by incubating the gel in 100 mM sodium acetate buffer containing 1.0% (v/v) Triton X-100 for 12 h at 37°C [23]. To identify protein bands with chitosanase activity, the gel was stained with 0.01% (w/v) Calcofluor white M2R in 500 mM Tris-HCl (pH 8.9) for 5 min. The gel was rinsed in distilled water for 1 h at room temperature to remove unadsorbed dye. Lytic zones were visualized under long-wave UV light and photographed.

Analytical Methods

Protein concentration was determined by the method of Bradford with bovine serum albumin as a standard [5]. The products of enzymatic hydrolysis of the GlcN oligomers were analyzed by thin layer chromatography by the method of Sakai *et al.* [18]. A viscometric chitosanase assay was performed according to the method of Ohtakara [16].

N-Terminal Amino Acid Sequencing

The N-terminal amino acid sequence analysis was performed by automated Edman degradation [6] by using a model 491 sequencer (Applied Biosystems, Foster City, U.S.A.).

N-Terminal Amino Acid Sequence Accession Number

This reported sequence has been assigned to the SWISS-PROT protein sequence database under the accession number P83488.

Results

Production of Chitosanolytic Enzyme by *A. flavus* IAM2044

The effect of various carbon sources on the production of chitosanolytic enzyme was investigated. The strain secreted a chitosanase into the medium containing chitosan as the carbon source, but did not secrete the enzyme into the medium containing Glc, GlcN, and GlcNAc (data not shown). The maximum chitosanase activity in the medium with chitosan (0.5%) reached 42.3 Uml^{-1} after 56 h incubation (data not shown). Furthermore, the chitosanase activity in

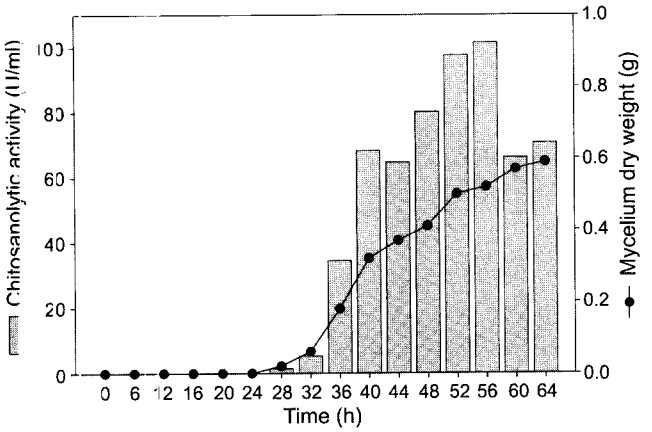

Fig. 1. Release of chitosanolytic activity during the growth of *A. flavus* IAM2044 in CD medium.
Cells were cultured in 200 ml of CD medium plus chitosan (0.5%), yeast extract (0.5%), and peptone (0.5%).

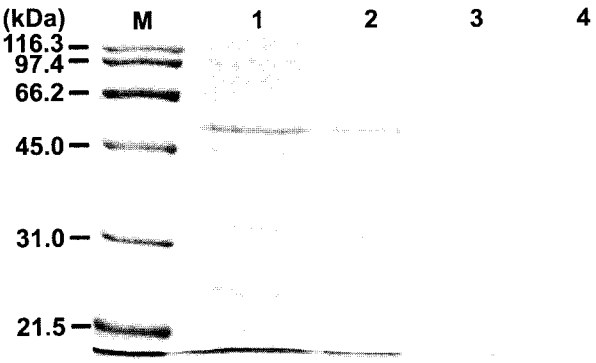

Fig. 2. SDS-PAGE analysis of chitosanase of the different stages purification.
Proteins from $(NH_4)_2SO_4$ precipitate (lane 1), dialysate (lane 2), the pool of active fractions from the ion-exchange column (lane 3), and the purified chitosanase (lane 4) were electrophoresed and stained with Coomassie brilliant blue R-250. Molecular mass markers (lane M) were broad-range protein markers (Bio-Rad).

the CD-chitosan medium plus yeast extract (0.5%) and peptone (0.5%) as the nitrogen source increased until it reached its maximum (101.5 U/ml) at 56 h of growth, and was 2.4-fold higher than that in the CD-chitosan medium, corresponding to the exponential period of cell growth (Fig. 1). Therefore, the culture fluid obtained under this culture condition was used as the source for purification of chitosanolytic enzyme.

Purification of Chitosanase

A summary of the specific activity and recovery of the enzyme during the purification procedure based on 920 ml of the culture filtrate is given in Table 1. The enzyme was purified about 9.4-fold with a yield of 1.8%. The purified enzyme showed a single protein band on 12% SDS-polyacrylamide gel electrophoresis. The molecular mass of the enzyme was estimated to be 45 kDa by SDS-PAGE (Fig. 2). A single protein band which migrated in the same region as the enzyme activity was also obtained by zymogram analysis on SDS-polyacrylamide gels containing soluble chitosan (D.A. 30%) (Fig. 3). The purified enzyme eluted from Sephadex G-100 gel filtration corresponded to the position of the protein with the molecular mass estimated by SDS-PAGE, indicating that it was monomeric. The N-terminal amino acid sequence of the purified protein was determined to be NH_2-LPTGPNNPTTLDNSSII.

Kinetic Properties of Chitosanase

Effects of pH and Temperature on Activity and Stability. To determine the pH dependence of chitosanase activity, 50 mM Na-acetate buffer (pH 3.0 to 6.0), 50 mM Na-phosphate buffer (pH 6.0 to 8.0), and 50 mM borate-NaOH buffer (pH 8.0 to 9.0) were used to prepare a range of pH values in the reaction mixtures. The enzyme showed a maximum activity at pH 5.0, when soluble chitosan was used as the substrate (Fig. 4). After 15 min of incubation at 37°C, more than 50% of its maximum activity remained in the range of pH 3.5 to 7.0 (Fig. 4). The optimum temperature of the enzyme was about 50°C (Fig. 5). The heat stability of the enzyme was measured by assaying the enzyme activity after incubation at various temperatures (30–90°C) for 15 min. Purified chitosanase was stable at below 50°C and became inactive at above 60°C (Fig. 5).

Substrate Specificity. Purified chitosanase was incubated with various substrates (0.25%), such as chitosan (D.A. 0%), chitosan (D.A. 30%), soluble chitosan (D.A. 30%), glycol chitosan, glycol chitin, colloidal chitin, and carboxymethyl cellulose (CMC) at pH 5.0 and 50°C for 15 min. The enzyme hydrolyzed effectively chitosan (D.A. 0%), chitosan (D.A. 30%), and soluble chitosan (D.A. 30%) as shown in Table 2. The degradation of soluble chitosan (D.A. 30%) was about 64.0% of the value of chitosan (D.A. 0%), but most of glycol chitosan, glycol chitin, colloidal chitin, and

Table 1. Purification of chitosanase from culture filtrate of *A. flavus* IAM2044.

Step	Total protein (mg)	Total activity[a] (U)	Specific activity (Umg^{-1})	Purification (-fold)	Recovery (%)
Culture filtrate	792.1	98,568.8	124.4	1	100
0–80% $(NH_4)_2SO_4$	274.1	62,400	227.7	1.8	63.3
CM-Sepharose	10.8	3,857.2	357.2	2.9	3.9
Sephadex G-100	1.5	1,749.9	1,166.6	9.4	1.8

[a] Soluble chitosan (D.A. 30%) was used as the substrate for the enzyme assay.

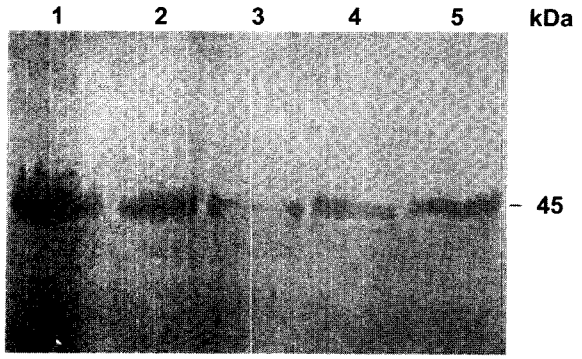

Fig. 3. Zymogram of chitosanase activity.
Protein samples from culture filtrate (lane 1), $(NH_4)_2SO_4$ precipitate (lane 2), dialysate (lane 3), the pool of fractions with enzymatic activity from the ion-exchange column (lane 4), and the purified chitosanase (lane 5). The gel was processed for renaturation, stained, and later processed for activity as described in Materials and Methods.

carboxymethyl cellulose (CMC) were not hydrolyzed by chitosanase.

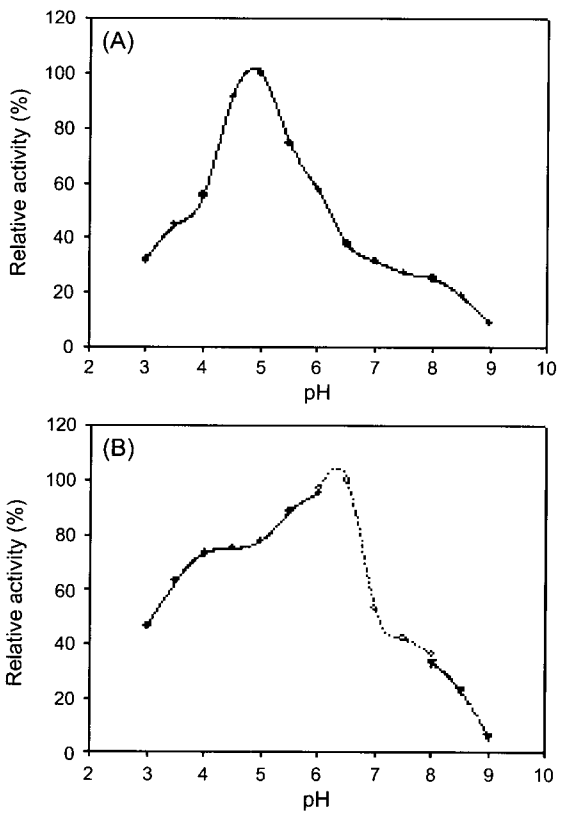

Fig. 4. Effect of pH on activity (A) and stability (B) of the purified chitosanase.
The chitosanase activity was assayed at 37°C for 15 min in 50 mM buffers with various pHs (3.0 to 9.0) and with chitosan (1.0%) as the substrate. The residual activities of the enzyme after incubation at 37°C for 15 min at various pHs between 3.0 and 9.0 were measured. Buffers used were 50 mM sodium acetate buffer (pH 3.0 to 6.0), 50 mM sodium phosphate buffer (pH 6.0 to 8.0), and 50 mM borate-NaOH buffer (pH 8.0 to 9.0).

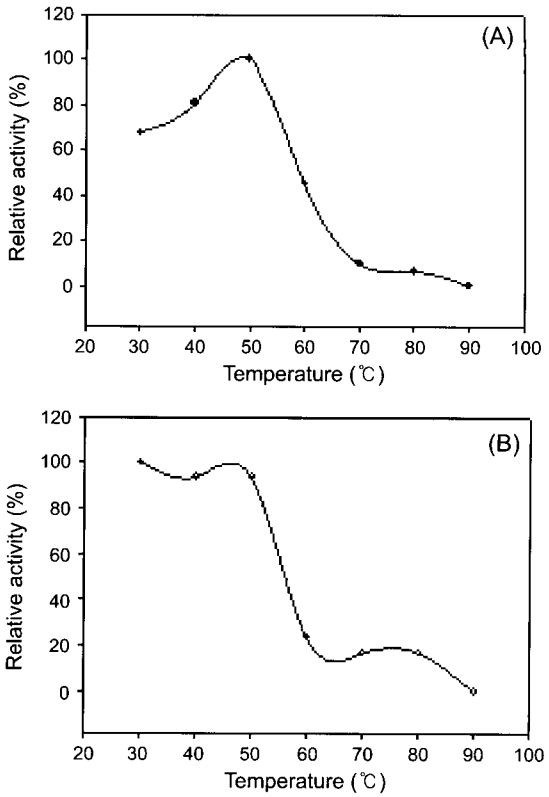

Fig. 5. Effects of temperature on activity (A) and stability (B) of the purified chitosanase.
The chitosanase activity was assayed at various temperatures (30–90°C). The heat stability of the enzyme was measured by assaying the enzyme activity after incubation at various temperatures (30–90°C) for 15 min.

Reduction in the Viscosity of Chitosan Solution. To investigate the cleavage pattern of soluble chitosan (D.A. 30%), viscometric assay of the enzyme reaction was carried out (Fig. 6). In the hydrolysis of soluble chitosan (D.A. 30%), the 45-kDa chitosanase did not reduce the viscosity of the reaction mixture, while the reaction time was increased. On the other hand, the crude from the dialysis step extensively decreased the viscosity in the initial phase of the reaction. This result of the viscosity of

Table 2. Substrate specificities of the chitosanase produced by *A. flavus* IAM2044.

Substrate[a]	Relative activity (%)[b]
Chitosan (D.A. 0%)	100
Chitosan (D.A. 30%)	69.6
Soluble chitosan	64
Glycol chitosan	5
Glycol chitin	<1
Colloidal chitin	<1
Carboxymethyl cellulose	<1

[a]Each reaction mixture contained substrate at a concentration of 0.25%.
[b]The amount of reducing sugar liberated after a 15 min reaction with 8.3 U of the purified chitosanase.

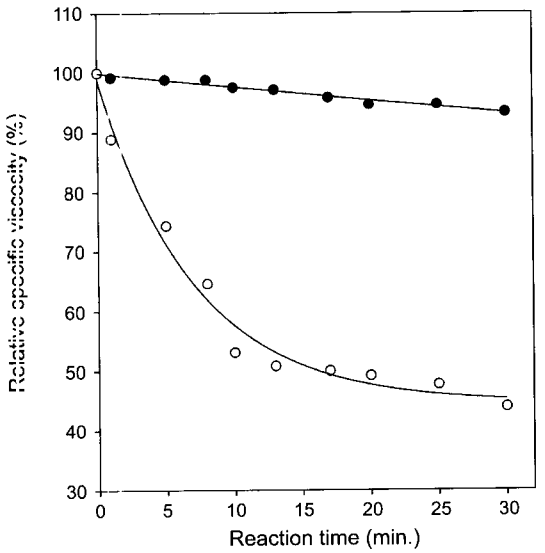

Fig. 6. Reduction in the viscosity of chitosan solution with chitosanase.
Reduction in viscosity was determined with an Ostwald viscosimeter. *Closed circles*, 20 U of the purified exo-β-D-glucosaminidase; *open circles*, crude enzyme was used.

the reaction mixture with the purified enzyme indicated that this enzyme hydrolyzed chitosan in an exo-type fashion. Furthermore, the decreased viscosity of the reaction mixture with the crude enzyme suggests that it possesses other endo-type chitosanolytic enzymes.

Analysis of the Reaction Products. The products of hydrolysis of $(GlcN)_{2-6}$ by the purified enzyme were analyzed by thin layer chromatography. Chitohexose, chitopentose, and chitotetrose were hydrolyzed to smaller GlcN oligosaccharides and GlcN. Chitotriose and chitobiose were hydrolyzed to glucosamine (Fig. 7). Furthermore, Fig. 8 shows the products of hydrolysis by the purified chitosanase, when

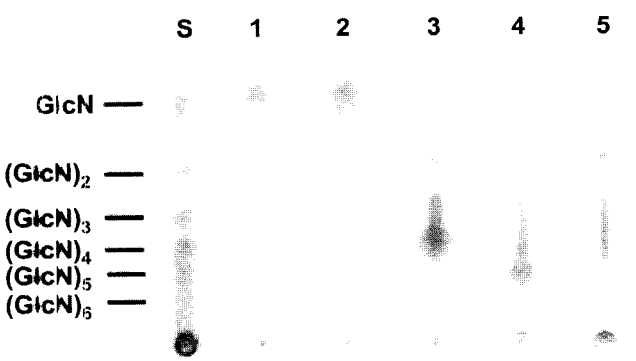

Fig. 7. Analysis of enzymatic hydrolysates by thin layer chromatography.
Enzymatic hydrolysis of $(GlcN)_{2-6}$ was performed in 50 mM Na-acetate buffer (pH 5.0) at 37°C for 15 min. The substrates used are chitobiose (lane 1), chitotriose (lane 2), chitotetriose (lane 3), chitopentaose (lane 4), and chitohexaose (lane 5). Lane S contained from glucosamine (GlcN) to chitohexaose $(GlcN)_6$.

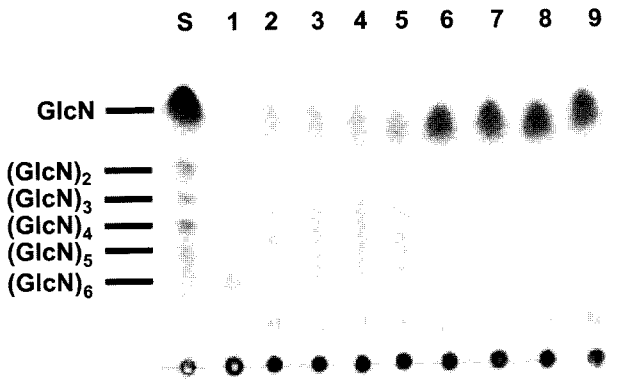

Fig. 8. Analysis of enzymatic hydrolysates by TLC.
Enzymatic hydrolysis of $GlcN_6$ was performed in 50 mM Na-acetate buffer (pH 5.0) at 37°C for various times. Lane S contained from glucosamine (GlcN) to chitohexaose $(GlcN)_6$; lane 1, unhydrolyzed substrate; lanes 2, 3, 4, 5, 6, 7, 8, and 9, hydrolysates obtained after 5 min, 15 min, 30 min, 1 h, 10 h, 15 h, 20 h, and 24 h of the reaction, respectively.

chitohexose was used as the substrate. Chitohexose appeared to be hydrolyzed to chitopentose and glucosamine at the initial stage of the reaction. The resulting GlcN oligosaccharides were further hydrolyzed to smaller GlcN oligosaccharides and GlcN on prolonged enzymatic reaction, and the final product was GlcN (Fig. 8, lanes 6–9). These results of the viscometric assay and the TLC analysis of the hydrolysates suggest that the purified enzyme from *A. flavus* IAM2044 may be an exo-β-D-glucosaminidase.

DISCUSSION

The purification and characterization of the exo-β-D-glucosaminidase from *A. flavus* IAM2044 is reported here. This is the first report that an exo-β-D-glucosaminidase with chitosan-degrading activity is induced in the presence of chitosan as a carbon source, although the production of the chitosan-degrading enzymes in *A. flavus* has already been reported by Zhang *et al.* [24]. In the present study, one of the chitosan-degrading enzymes was purified, characterized, and then determined to be exo-type chitosanase, called exo-β-D-glucosaminidase, by the viscometric assay and TLC analysis of the hydrolysate. Exo-β-D-glucosaminidase is a rare enzyme and the information about this enzyme is very important. Fungal chitosanolytic enzymes have been purified and characterized from *Penicillium islandicum*, *Mucor rouxii*, *Fusarium solani*, *T. reesei*, *A. oryzae*, and *A. fumigatus* [7, 1, 21, 15, 24]. Most of these enzymes are endo-type, and exo-type chitosanases have been reported in several cases, *A. oryzae* IAM2660, *N. orientalis*, *A. fumigatus* KH-94, and *T. reesei* PC-3-7 [24, 14, 15]. These enzymes have properties to indicate that they are the monomeric enzyme having a molecular mass of approximately 93, 97, 108, and 135 kDa, respectively,

but exo-β-D-glucosaminidase of *A. flavus* IAM2044 is 45 kDa in size. Furthermore, when compared to other exo-β-D-glucosaminidases, this enzyme showed extremely high specific activity as well as total enzyme activity in the culture filtrate. Also, its molecular mass was about half of the other exo-type enzymes. Most exo-enzymes do not hydrolyze chitin, cellulose, CMC, and glycol chitosan, showing a stringent substrate specificity. Exo-β-D-glucosaminidase from *A. flavus* IAM2044 hydrolyzed chitosan with a fully deacetylated chitosan (D.A. 0%) more efficiently than a low degree of deacetylation (D.A. 30%) (Table 2). This reaction velocity on the acetylation degree of the substrate is similar to that of the exo-type chitosanases from *A. oryzae* IAM2660, *N. orientalis*, *A. fumigatus* KH-94, and *T. reesei* PC-3-7 [24, 14, 15]. Furthermore, these exo-β-D-glucosaminidases released only GlcN residues from the nonreducing end of the chitosan polymer and cleaved GlcN-β(1→4)-GlcN and GlcN-β(1→4)-GlcNAc bonds, but not the GlcNAc-β(1→4)-X bond. It is possible that chitosanolytic enzymes are widely distributed in *Aspergillus* spp., and are composed of both endo- and exo-types.

A. flavus IAM2044 secreted more exo-β-D-glucosaminidase on CD-chitosan as a carbon source than with other carbon sources (data not shown). This result indicates that the production of chitosanolytic enzymes was inducible by chitosan. Furthermore, the chitosanase activity on CD-chitosan plus yeast extract (0.5%) and peptone (0.5%) as the nitrogen sources was 2.4-fold higher than that on CD-chitosan. This trait is highly desirable for large-scale production of exo-β-D-glucosaminidase for industrial use.

The N-terminal amino acid sequence of exo-β-D-glucosaminidase from *A. flavus* IAM2044 was determined to be LPTGPNNPTTLDNSSII and was compared with those of chitosanases deposited in the SWISS-PROT/TrEMBL, however, no homology to any known chitosanases was found. Therefore, this enzyme seems to be a novel exo-type enzyme, and this is the first report about the primary structure and is very useful. More detailed studies are required, because the physiological role of the *A. flavus* chitosanases, including exo-β-D-glucosaminidase, still remains unknown.

Acknowledgment

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