Purification and Characterization of β-N-acetylglucosaminidase from *Grifola frondosa*

Yi-Cheng Wang,[a] Te-Sheng Lien,[b] Nan-Yin Chen,[c] and Tai-Hao Hsu [a,*]

Using commercial API-ZYM screening kits, highly active α-glucosidase, β-glucosidase, and β-N-acetylglucosaminidase were found in *Grifola frondosa*, having potential for carbohydrate utilization. Of these, β-N-acetylglucosaminidase, which converts chitin to N-acetylglucosamine, was purified and characterized. The recovery was 24.5%, and the purified enzyme had a specific activity 0.67 U/mg protein. Chitinase activity was confirmed by zymogram analysis. The enzyme was also shown to be β-N-acetylglucosaminidase, as N-acetylglucosamine was the main hydrolysis product from colloidal chitin. Thus, the molecule was named NAG38, to indicate β-N-acetylglucosaminidase activity and a molecular weight of 38 kDa, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Enzymatic activity was optimal at pH 7.0 and 50 °C, with K_m and V_{max} values of 0.112 mM and 0.570 μmol/min/mg protein against p-nitrophenyl N-acetyl-β-D-glucosaminide. The bioactivity was inhibited by Hg^{2+}, Ag^{+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, and Mn^{2+}, with residual enzyme bioactivity only 11.1% after incubation in Hg^{2+}, but was not substantially inhibited by Ba^{2+}, K^{+}, and Na^{+}.

Keywords: Grifola frondosa; API-ZYM; N-acetylglucosamine; Chitinase; β-N-acetylglucosaminidase; Bioactivity

*Contact information: a: Department of Food Science and Biotechnology, Da-Yeh University, No. 168, University Rd., Dacun, Changhua 51591, Taiwan (R.O.C.); b: Department of Molecular Biology and Human Genetics, Tzu-Chi University, No. 701, Section 3, Zhong-Yang Road, Hualien 97004, Taiwan (R.O.C.); c: Department of Hospitality Management, Chung-Hwa University of Medical Technology, No.89, Wenhua 1st St., Rende Dist., Tainan 71703, Taiwan (R.O.C.); *Corresponding author: th0123@mail.dyu.edu.tw; Teyizo@gmail.com*

INTRODUCTION

Earlier, shrimp and crab shells were regarded as waste by seafood processing plants. However, the byproduct of chitin and its derivatives are gaining much attention due to their usages in various areas. The chitin is composed of 1,000 to 3,000 units of N-acetylglucosamine (GlcNAc) and is the second most abundant polysaccharide after cellulose (Tharanathan and Kittur 2003; Gaderer *et al.* 2017). Effective use of shrimp and crab shells can solve subsequent environmental issues, and the added value of the derivatives can also bring economic benefits.

Recently, GlcNAc was tested as a therapy for osteoarthritis (Shikhman *et al*. 2005), gastrointestinal inflammation (Salvatore *et al*. 2000), and as a contrast agent for imaging and diagnosing cancers (Rivlin and Navon 2016). N-acetylglucosamine can be prepared chemically or enzymatically, although chemical production from chitin is expensive and inefficient (yields less than 65%) and generates acidic waste from HCl and acetic anhydride (Chen *et al*. 2010). Hence, methods based on microbial enzymes are generally considered better because of higher yield and product purity, simplicity, and environmental

friendliness (Pereira *et al*. 1980).

β-N-acetylglucosaminidase (EC 3.2.1.52) cleaves N,N′-diacetylchitobiose or short-chain N-acetyl chitooligosaccharides to generate GlcNAc (Gooday 1990; Cohen-Kupiec and Chet 1998; Lee *et al*. 1999). Thus, the enzyme breaks polymers of N-acetylglucosamine (Cifali and Dias Filho 1999) and decomposes carbohydrate bonds in bacterial and fungal cell walls (Yamamoto *et al*. 1985), which contain chitin composed of acetamido glucose. Accordingly, the enzyme hydrolyzes chitin or murein during cell division to promote reproduction (Glasgow *et al*. 1977; Jones and Kosman 1980). Meanwhile, purified chitinolytic enzymes may enhance or simplify industrial applications.

In this study, β-N-acetylglucosaminidase from *Grifola frondosa* grown in a basic medium containing 3% glucose was purified and characterized. Enzyme bioactivity and stability across a range of pH and temperature were assessed, along with sensitivity to metal ions, reaction kinetics, and hydrolysis products.

EXPERIMENTAL

Strain, Culture, and Fermentation

G. frondosa (BCRC 36355) from the Bioresource Collection & Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan, was maintained on potato dextrose agar (PDA) slants and subcultured monthly by incubating at 25 °C and then stored in a refrigerator at 4 °C for further use. To obtain seed cultures, cells were transferred from slants onto a Petri dish containing PDA and cultured at 25 °C for 21 d. Five agar discs (5 mm in diameter) with *G. frondosa* colonies were punched out and inoculated in 100 mL of potato dextrose broth. This culture was then grown at 100 rpm and 25 °C for 14 d, inoculated at 10 vol% into 500 mL flasks with an initial pH of 5.5, and grown at 25 °C and 100 rpm for another 14 d, to evaluate the influence on β-N-acetylglucosaminidase activity. Fermentation media consisted of 30 g/L of glucose, 8 g/L of yeast extract, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.2 g/L of $MnSO_4$, and 0.5 g/L of K_2HPO_4.

Preparation of Colloidal Chitin

Chitin powder was partly dissolved in concentrated HCl to prepare a colloidal suspension that precipitates at 5 to 10 °C (Hsu and Lockwood 1975). Colloidal chitin is structurally more accessible and enables accurate measurement of chitinase activity (Hsu and Lockwood 1975).

Purification of β-N-acetylglucosaminidase

Preparation of crude enzyme

Mycelium biomass was collected from the fermentation culture by centrifugation at 2280 *g* for 10 min, washed with 20-mM phosphate buffer (pH 6.5), centrifuged at 4 °C and 11,428 *g* for 5 min, resuspended in 20-mM phosphate buffer (pH 6.5) at a 1:10 ratio of biomass wet weight to buffer, lysed in a tissue grinder, and centrifuged. The resulting supernatant was precipitated by adding ammonium sulfate to 20% to 80% saturation. The precipitate was separated by centrifugation, redissolved in 5 mL of 50-mM Tris-HCl (pH 7.8), and dialyzed against the same buffer at 4 °C for 48 h.

Ion exchange chromatography

The dialysate was loaded onto a DEAE-sepharose CL-6B column (1.6 cm × 20 cm)

(Sigma-Aldrich, St. Louis, MO, USA) pre-equilibrated with 50-mM Tris-HCl (pH 7.8). Adsorbed proteins were eluted at 0.5 mL/min over 0-M to 0.5-M NaCl in Tris-HCl (pH 7.8) (Cruz *et al*. 2002; Iranzo *et al*. 2002). Fractions were collected and assayed for protein content (OD_{280}) and chitinase activity. Active fractions were then pooled, freeze-dried to a constant weight, and redissolved in 50-mM Tris-HCl (pH 7.4) with 0.15-M NaCl for gel filtration chromatography.

Gel filtration chromatography

Sephacryl S-100 HR (Sigma-Aldrich) was packed in a separator tube (1.0 cm × 100.0 cm) and equilibrated with 50-mM Tris-HCl (pH 7.4) containing 0.15-M NaCl. The crude enzyme from the previous step was then eluted over this column at 0.1 mL/min in the same buffer (Wiwat *et al*. 1999). As before, fractions were collected and assayed for protein content (OD_{280}) and chitinase activity. Finally, active fractions were pooled and stored at -20 °C until use.

Characterization of β-N-acetylglucosaminidase

The enzyme was shown to be β-N-acetylglucosaminidase, as N-acetylglucosamine was the main hydrolysis product from colloidal chitin. Thus, the molecule was named NAG38, to indicate β-N-acetylglucosaminidase activity and a molecular weight of 38 kDa.

Optimal pH and pH stability

The NAG38 activity was measured in McIlvaine buffer (pH 3.0 to 8.0) or Tris-HCl (pH 7.0 to 10.0) against 0.36 mM p-nitrophenyl N-acetyl-β-D-glucosaminide (p-NP-(GlcNAc)). To assess pH stability, the enzyme was pre-incubated for 1 h at 40 °C in the same buffers and the residual activity was examined in standard conditions.

Optimal temperature and thermal stability

The NAG38 was assayed for 30 min at 10 to 80 °C in McIlvaine buffer (pH 7.0). To assess thermal stability, aliquots were pre-incubated for 1 h at 10 to 80 °C in 50-mM citrate (pH 4.5) and assayed for residual activity in standard conditions.

Effect of metal ions

The NAG38 was pre-incubated for 1 h at 40 °C in 0.1-M McIlvaine buffer (pH 7.0) with 10-mM cations and assayed for residual activity.

Kinetic parameters

Kinetic parameters were determined at 40 °C in 0.1-M McIlvaine buffer (pH 7.0) with 0-mM to 1.44-mM p-NP-(GlcNAc). The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) were determined by Lineweaver-Burk double reciprocal plot.

Hydrolysis patterns

Colloidal chitin (1% w/v) was digested with 1 U/mL of NAG38 for 1 h at 40 °C in 0.1-M McIlvaine buffer (pH 7.0). Reactions were then centrifuged at 11,428 *g* for 10 min, and the resulting supernatant was analyzed by high-performance liquid chromatography.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram analysis

The molecular mass of the NAG38 was estimated by SDS-PAGE according to a

previous report (Laemmli 1970). The β-N-acetylglucosaminidase activity was detected by zymogram analysis, *i.e*., electrophoresis on a 12% polyacrylamide gel containing 0.01% glycol chitin (Trudel and Asselin 1989). Gels were incubated for 2 h at 37 °C in 0.1-M sodium acetate (pH 5.0) containing 1% Triton X-100 (Sigma-Aldrich) and stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich) (Guo *et al.* 2004).

Analytical Methods

API-ZYM enzyme screening

Mycelia were collected by air filtration, mixed with glass beads, and lysed on an oscillator. The protein content in the resulting supernatant was quantified spectrophotometrically (U-2000, Hitachi, Tokyo, Japan) and adjusted to 1 mg/mL. Samples (65 µL) were dispensed into cupules in the API-ZYM strips, incubated for 4 h at 37 °C, and reacted with one drop each of API reagents A and B (Biomerieux, Craponne, France). The intensity of the color reaction that developed within 5 min was scored from 0 to 5 according to the API-ZYM color reaction chart.

β-N-acetylglucosaminidase assay against p-NP-(GlcNAc)

The β-N-acetylglucosaminidase activity was determined against p-NP-(GlcNAc) according to an earlier study (Frändberg and Schnürer 1994). Reactions consisted of 150 µL of p-NP-(GlcNAc) (0.72 mM), 30 µL of the enzyme, and 150 µL of 0.1-M sodium phosphate buffer (pH 7.0) and were terminated with 30 µL of 0.1-M NaOH after 1 h at 40 °C. Released p-nitrophenol was quantified by absorbance at 405 nm (U-2000, Hitachi). In this assay, 1 U of β-N-acetylglucosaminidase activity was the amount of enzyme required to release 1 µmol of p-nitrophenol per min at 40 °C and pH 7.0.

Protein quantification

Protein concentration was measured by absorbance at 280 nm (U-2000, Hitachi) during chromatographic separation and subsequently by Bradford assay (Bradford 1976).

Analysis of chitin hydrolysates

Hydrolyzed chitin was analyzed by high-performance liquid chromatography (Hitachi L-7100, Tokyo, Japan) at 1 mL/min on a LiChrospher 100 NH_2 column (5 µm, 4 mm × 250 mm, Merck, Darmstadt, Germany). The mobile phase was 70/30 (v/v) acetonitrile/water, and elution was followed at 205 nm on a diode array detector (Hitachi L-4500, Tokyo, Japan) (Chang *et al*. 2000).

RESULTS AND DISCUSSION

Enzyme Profiles of *G. frondosa* Strains

Intracellular enzymes in *G. frondosa* strains were screened on API-ZYM commercial kits, as shown in Fig. 1 and Table 1. As shown in Fig. 2, the six strains could be distinguished from each other and *Cordyceps militaris* based on the presence or absence of 6 enzymes (out of 19 tested), including esterase lipase (C8) (No. 4), cystine arylamidase (No. 8), α-chymotrypsin (No. 10), β-galactosidase (No. 14), β-glucuronidase (No. 15), and α-glucosidase (No. 16). In particular, α-glucosidase (No. 16) activity was different between positive and negative strains and between strains with strong and weak activities, highlighting its potential value as a marker.

Fig. 1. Intracellular enzymes from *Grifola frondosa* strains and *Cordyceps militaris*, as scored on API-ZYM commercial kits. Numbers below cupules represent enzymes tested, as listed in Table 1. Negative reactions are colorless or very pale yellow, as is the control reaction (No. 1), while positive reactions are violet for Nos. 2, 3, 4, 5, 11, 13, 14, 16, 17, 19, and 20; orange for Nos. 6, 7, 8, 9, and 10; blue for Nos. 12 and 15; and brown for No. 18.

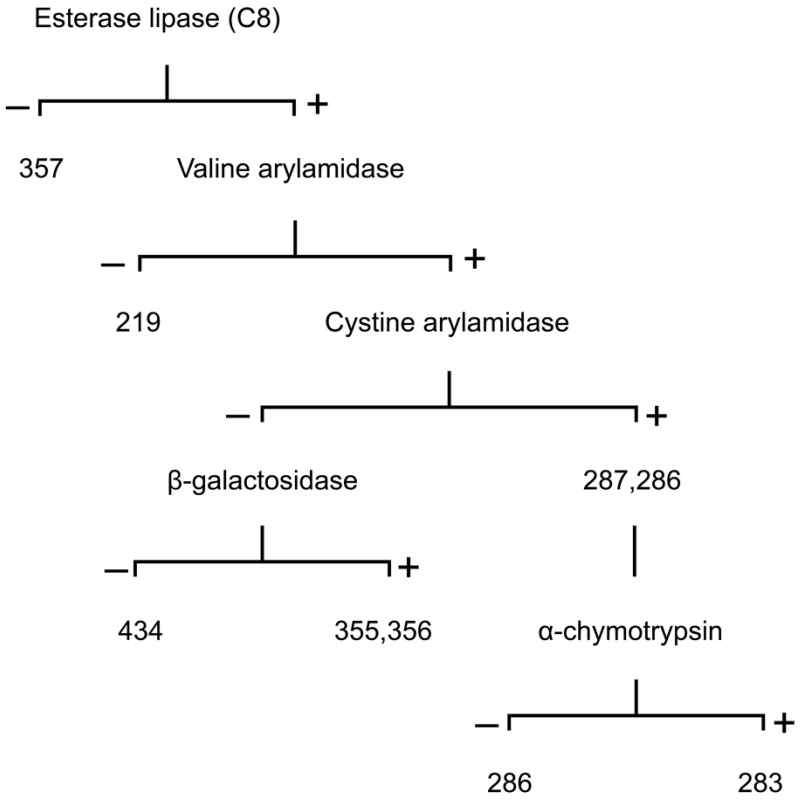

Fig. 2. Classification of *Grifola frondosa* strains and *Cordyceps militaris* based on intracellular enzymes: (283) strain BCRC 36283, (286) strain BCRC 36286, (355) strain BCRC 36355, (356) strain BCRC 36356, (357) strain BCRC 36357, (434) strain BCRC 36434, and (219) strain BCRC 32219

Table 1. Intracellular Enzymes in *Grifola frondosa* [a] and *Cordyceps militaris* [b] as Scored by API-ZYM Color Reactions [c]

BCRC Strain	Enzyme Tested [d]																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
36283	0	4	1.5	1.5	0	4	3	1	0	1.5	5	5	2.5	1	0.5	1.5	3	4	1.5	0
36286	0	1	2	1	0	4	3	1.5	0	0	4	5	3	1	1	3	3	2	1	0
36355	0	2	2	2	0	4	3	0	0	0	4	5	3	2	1	4	4	3	3	0
36356	0	2	2	1	0	4	3	0	0	0	4	5	3	2	0.5	3.5	3.5	3	3	0
36357	0	3	1	0	0	5	4	0	0	0	5	5	3	1	0	3	4	3	3	0
36434	0	2	1	1.5	0	4	3	0	0	0	4	5	1	0	0.5	0	3	2	1.5	0
32219	0	4	1.5	1	0	3	0	0	5	0	4	4	1	3	0	0	2	3	2	0

[a] BCRC 36283, 36286, 36355, 36356, 36357, and 36434
[b] BCRC 32219
[c] Scores range from 0 to 5, which correspond to lack of color reaction and most intense color reaction, respectively.
[d] Enzymes tested: (1) control, (2) alkaline phosphatase, (3) esterase (C4), (4) esterase lipase (C8), (5) lipases (C14), (6) leucine arylamidase, (7) valine arylamidase, (8) cystine arylamidase, (9) trypsin, (10) α-chymotrypsin, (11) acid phosphatase, (12) naphthol-AS-BI-phosphohydrolase, (13) α-galactosidase, (14) β-galactosidase, (15) β-glucuronidase, (16) α-glucosidase, (17) β-glucosidase, (18) N-acetyl-β-glucosaminidase, (19) α-mannosidase, and (20) α-fucosidase. Negative reactions are colorless or very pale yellow, while positive reactions are violet for Nos. 2, 3, 4, 5, 11, 13, 14, 16, 17, 19, and 20; orange for Nos. 6, 7, 8, 9, and 10; blue for Nos. 12 and 15; and brown for No. 18.

High activity (color reaction scoring at least 4) was observed for alkaline phosphatase (No. 2), leucine arylamidase (No. 6), valine arylamidase (No. 7), acid phosphatase (No. 11), naphthol-AS-BI-phosphohydrolase (No. 12), α-glucosidase (No. 16), β-glucosidase (No. 17), and N-acetyl-β-glucosaminidase (No. 18). Of these, α-glucosidase (No. 16), β-glucosidase (No. 17), and N-acetyl-β-glucosaminidase (No. 18) are involved in carbohydrate utilization. N-acetyl-β-glucosaminidase (No. 18) also hydrolyzes chitin or murein in cell walls during cell division or thalli autolysis. Indeed, the enzyme is one of several that decompose chitin into GlcNAc. Accordingly, N-acetyl-β-glucosaminidase regulates the production of extracellular and intracellular polysaccharides. Therefore, the yield of mycelia can be increased by inhibiting the activity of this enzyme. Further, by enhancing the enzyme activity, the hyphae can be broken and the yield of intracellular polysaccharides can be increased. The enzyme can also be used for specific matrix decomposition, *e.g*., applying it on chitin-related waste to generate high-value metabolites. According to Table 1, the N-acetyl-*β*-glucosaminidase secreted by *G. frondosa* BCRC 36355 has the highest activity, although it still requires further purification.

Purification of a Chitinase from *G. frondosa*

Ion exchange chromatography

The dialysate was separated on DEAE-sepharose CL-6B, as shown in Fig. 3. Tubes 43 to 51 had β-N-acetylglucosaminidase activity and thus were pooled and freeze-dried to a constant weight.

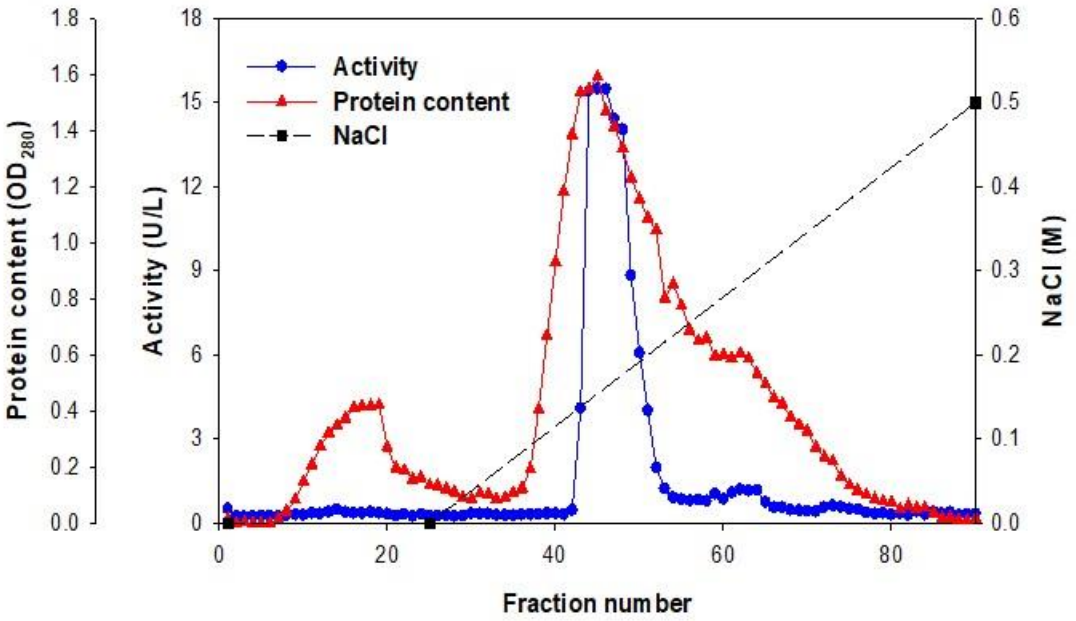

Fig. 3. Ion exchange chromatogram of *Grifola frondosa* crude enzyme loaded on DEAE-sepharose CL-6B

Gel filtration chromatography

The freeze-dried extract was redissolved in 50-mM Tris-HCl with 0.15-M NaCl and loaded onto a Sephacryl S-100 HR column, from which a peak (tubes 29 to 33) with β-N-acetylglucosaminidase activity was collected, as shown in Fig. 4. These fractions were pooled and stored at -20 °C.

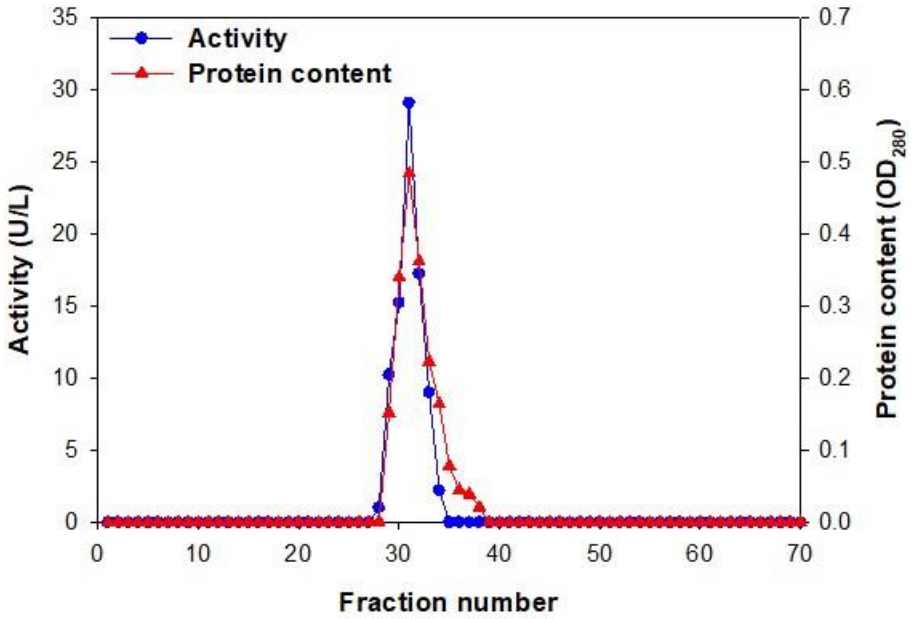

Fig. 4. Gel filtration of fractions 43 to 51 in Fig. 3 on Sephacryl S-100 HR

Zymogram analysis

Samples collected at each step were analyzed by SDS-PAGE to determine molecular weight and by zymogram analysis to confirm chitinase activity. Notably, separation on DEAE-Sepharose CL-6B enriched a 38-kDa protein that hydrolyzes glycol chitin in gel to produce a clearance zone, as shown in Fig. 5. However, there were other proteins, approximately 50 kDa, that were without chitinase activity. These contaminants were subsequently removed on Sephacryl S-100 HR, obviating the need for further purification. As fractions collected at each step were tested against p-NP-(GlcNAc), as shown in Table 2 along with protein content (OD_{280}), the purified 38-kDa protein was confirmed as β-N-acetylglucosaminidase, characterized as such, and named NAG38. The specific activity of the crude enzyme was 0.18 U/mg protein, while that of purified β-N-acetylglucosaminidase was 0.67 U/mg protein, for a recovery percentage of 24.5% and purification fold of 3.72.

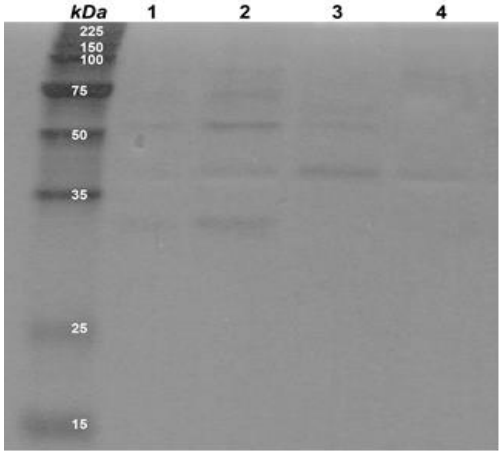

Fig. 5. Zymogram analysis: (lane 1) extract, (lane 2) crude enzyme, (lane 3) after purification on DEAE-Sepharose CL-6B, and (lane 4) after purification on Sephacryl S-100 HR

Table 2. Purification of β-*N*-acetylglucosaminidase from *Grifola frondosa*

Step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg protein)	Yield (%)	Purification (fold)
Extract	15.21	82.97	0.18	100	1.00
Crude enzyme	11.02	52.41	0.21	72.45	1.17
DEAE Sepharose CL-6B	6.13	14.93	0.41	40.30	2.28
Sephacryl S-100 HR	3.72	5.52	0.67	24.46	3.72

Characterization of Purified B-N-acetylglucosaminidase

Optimal pH and pH stability

The NAG38 was assayed against 0.36 mM p-NP-(GlcNAc) at pH 3.0 to 10.0 (Fig. 6). The activity was highest at pH 7.0, with residual activity higher than 50% at pH 6.0 and pH 8.0 but lower than 40% at all other pH values. Therefore, the optimal pH for NAG38 (at or near pH 7.0) was similar to that of the 95-kDa β-N-acetylglucosaminidase from *Mucor hiemalis* f. *hiemalis* (pH 6.0 to 6.5) (Kadowaki *et al*. 1990). In contrast, the optimal pH values for the 135-kDa β-N-acetylglucosaminidase from *Sclerotinia fructigena*, the 58.9-kDa enzyme from *Streptomyces cerradoensis*, and the 93-kDa enzyme from *Trichoderma reesei* PC-3-7 were reported to be 5.5, 5.5, and 4.0, respectively (Reyes and

Byrde 1973; Nogawa *et al*. 1998; Sobrinho *et al*. 2005), most of which are weakly acidic. In any case, the production of *G. frondosa* polysaccharides can potentially be controlled by pH. For example, the pH of the fermentation liquor sometimes decreases to less than 5.0 in the late stages and thus can probably be increased to stimulate the release of intracellular polysaccharides.

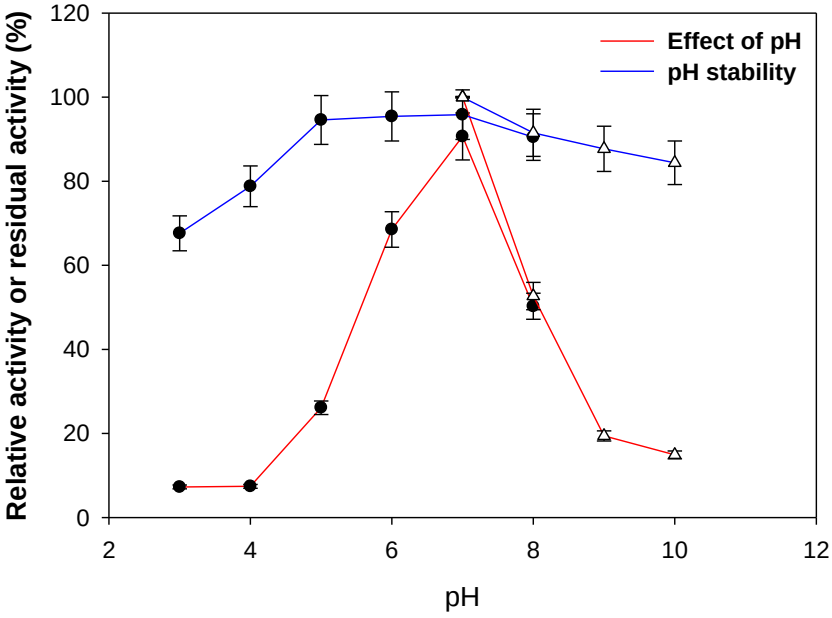

Fig. 6. Effect of pH on and pH stability of β-N-acetylglucosaminidase from *Grifola frondosa* cultivated in glucose medium. Circles (●) and triangles (△) denote reactions in McIlvaine buffer (pH 3.0 to 8.0) and Tris-HCl buffer (pH 7.0 to 10.0), respectively.

As shown in Fig. 6, the NAG38 incubated for 1 h at pH 4.0 to 10.0 retained approximately 80% activity when subsequently assayed against 0.36 mM p-NP-(GlcNAc) at pH 7.0. Similarly, the 93-kDa β-N-acetylglucosaminidase from *T. reesei* PC-3-7 retains more than 60% activity after 30 min at pH 6.0 to 9.0 (Nogawa *et al*. 1998), while the *Sclerotinia fructigena* β-N-acetylglucosaminidase retains only 40% activity after 30 min at pH 3.0 but is highly stable at pH 4.0 to 10.0 (Reyes and Byrde 1973). Therefore, NAG38 appeared to be stable at pH 4.0 to 10.0, which corresponds to the optimal culture pH for *G. frondosa*. Indeed, *G. frondosa* hyphae grow mainly at pH 4.0 to 10.0, presumably because β-N-acetylglucosaminidase is required to generate new hyphae.

Optimal temperature and thermal stability

At pH 7.0, NAG38 hydrolyzed 0.36-mM p-NP-(GlcNAc) between 10 and 80°C, as shown in Fig. 7, although the residual activity was higher than 80% only at 40 to 60°C. Activity peaked at 50°C, the apparent optimal temperature, but decreased to less than 20% at 80°C. This behavior was similar to that of β-N-acetylglucosaminidases secreted from other fungi. For example, activity is also optimal at 50 °C for the 60-kDa enzyme from *Streptomyces thermoviolaceus* OPC-520, the 93-kDa enzyme in *T. reesei* PC-3-7, the 58.9-kDa enzyme from *Streptomyces cerradoensis*, and the 135-kDa enzyme in *Aspergillus oryzae* IAM2660 (Reyes and Byrde 1973; Nogawa *et al*. 1998; Zhang *et al*. 2000; Kubota *et al*. 2004; Sobrinho *et al*. 2005).

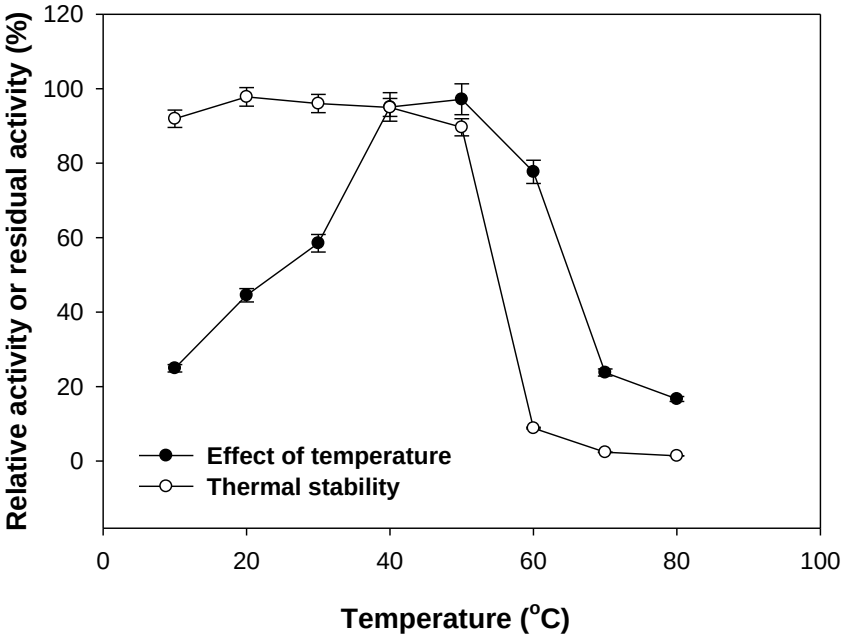

Fig. 7. Effect of temperature on and thermal stability of β-N-acetylglucosaminidase from *Grifola frondosa*

Figure 7 also shows that NAG38 retained 90% residual activity when incubated for 1 h at 10 to 50 °C and subsequently assayed against 0.36-mM *p*-NP-(GlcNAc) at pH 7.0. However, incubation for 1 h at temperatures of at least 60 °C diminished residual activity to less than 10 %. Similarly, the 135-kDa β-N-acetylglucosaminidase secreted from *A. oryzae* IAM2660 retains 70% residual activity after 20 min at 60 °C (Zhang *et al.* 2000), while the 60-kDa enzyme in *Streptomyces thermoviolaceus* OPC-520 maintains 95% residual activity after 1 h at 45 °C but less than 5% after preincubation at 50 °C (Kubota *et al.* 2004). However, the 58.9-kDa enzyme from *Streptomyces cerradoensis* retains less than 12% residual activity after 30 min at 55 °C (Sobrinho *et al*. 2005). Collectively, the data indicated that NAG38 exhibited higher thermal stability than β-N-acetylglucosaminidases secreted from other fungi.

Kinetic parameters

The activity of NAG38 at pH 7.0 against 0-mM to 1.44-mM p-NP-(GlcNAc) is shown in Fig. 8. The reaction was first-order at less than 0.112 mM, above which reaction rates did not further increase, indicating zero-order reaction at higher substrate concentrations. Based on double reciprocal plots of the enzyme reaction rate with substrate concentration, the K_m against p-NP-(GlcNAc) was 0.112 mM, with a maximum reaction rate (V_{max}) of 0.570 μmol/ (min · mg protein). The 135-kDa β-N-acetylglucosaminidase from *Sclerotinia fructigena* hydrolyzes the same substrate with K_m and V_{max} values of 2.0 mM and 45.0 μmol/h, respectively (Reyes and Byrde 1973). For the 149-kDa enzyme from *Aspergillus niger* and the 58.9-kDa enzyme from *Streptomyces cerradoensis*, the K_m values against p-NP-(GlcNAc) are 0.27 mM and 0.13 mM, respectively (Jones and Kosman 1980; Sobrinho *et al*. 2005). Therefore, NAG38 had a better affinity for p-NP-(GlcNAc) than β-N-acetylglucosaminidases secreted from other fungi.

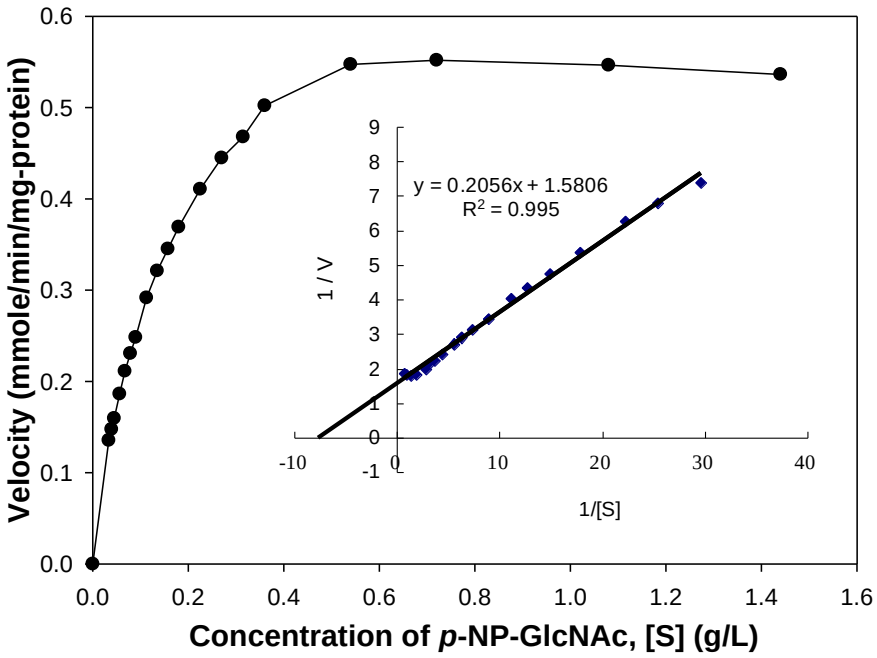

Fig. 8. K_m and V_{max} for p-NP-(GlcNAc) of β-N-acetylglucosaminidase from *Grifola frondosa*. Inset: Lineweaver-Burk double reciprocal plot with linear $R^2 = 0.995$

Table 3. Effect of Metal Ions on the Activity of β-N-acetylglucosaminidase from *Grifola frondosa*

Ion (10 mM)	Residual Activity (%)
None	100 ± 0.02
Ba^{2+}	101.7 ± 0.12
Hg^{2+}	11.1 ± 0.14
Ag^{+}	34.7 ± 0.23
K^{+}	104.6 ± 0.02
Mg^{2+}	80.5 ± 0.08
Zn^{2+}	11.3 ± 0.21
Ca^{2+}	71.5 ± 0.02
Mn^{2+}	46.6 ± 0.11
Na^{+}	110 ± 0.03

Effect of metal ions

As shown in Table 3, incubation for 1 h at 40 °C in Hg^{2+}, Ag^{+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, and Mn^{2+} inhibited NAG38 activity in subsequent assays against 0.36-mM *p*-NP-(GlcNAc) at pH 7.0. For example, Hg^{2+} reduced the residual activity to only 11.1%, although the enzyme appeared to be insensitive to Ba^{2+}, K^{+}, and Na^{+}. Similarly, the 58.9-kDa β-N-acetylglucosaminidase from *Streptomyces cerradoensis* loses all activity after 2 min in 10-mM Hg^{2+} but not in Mg^{2+}, Zn^{2+}, and Ca^{2+} (Sobrinho *et al*. 2005). Further, the 95-kDa β-N-acetylglucosaminidase in *Mucor hiemalis* f. *hiemalis* loses all activity after 10 min in 1-mM Hg^{2+} and Ag^{+}, although Mg^{2+}, Zn^{2+}, Fe^{2+}, and Mn^{2+} are not inhibitory (Kadowaki *et al*. 1990). Therefore, β-N-acetylglucosaminidases in different species appear to be sensitive to different metal ions.

Hydrolysis pattern

The NAG38 was reacted in McIlvaine buffer (pH 5.5) against 1.0% colloidal chitin under optimal conditions, and products were analyzed by high-performance liquid chromatography (Fig. 9). By comparison to N-acetylchitooligosaccharide standards, the hydrolysate contained GlcNAc, confirming that the enzyme was β-N-acetylglucosaminidase. Remarkably, the hydrolysis product was highly pure, so NAG38 can probably be used to produce GlcNAc from chitin without the need for subsequent purification, thereby reducing production costs.

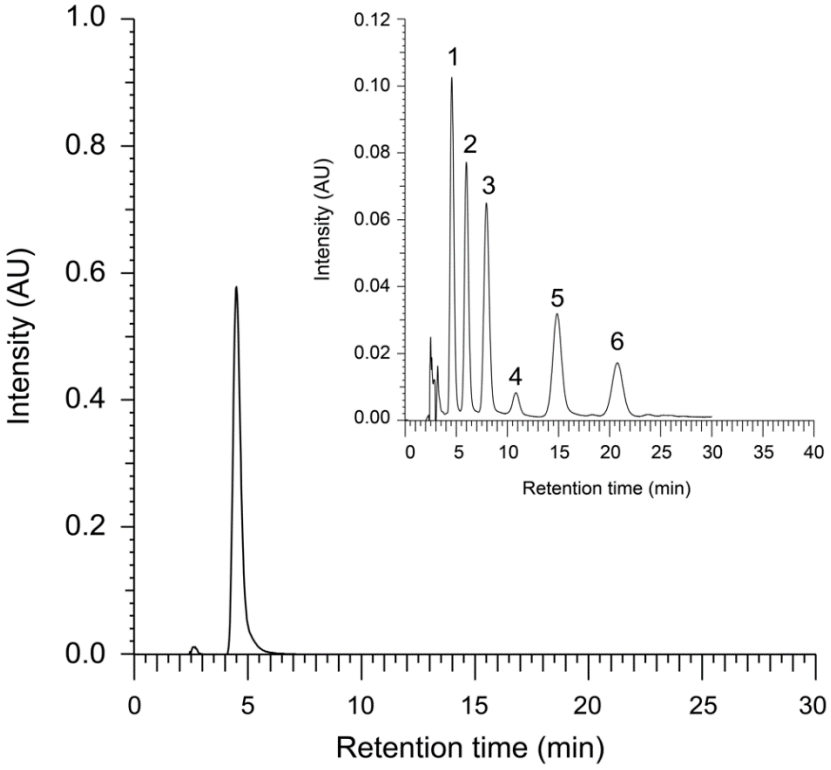

Fig. 9. Chromatogram of chitin hydrolysates generated by β-N-acetylglucosaminidase from *Grifola frondosa*

CONCLUSIONS

1. The purification of a 38-kDa enzyme, which generates GlcNAc from chitin at high purity, was described in this work. The enzyme was found to be stable over wide ranges of pH and temperature, but it was sensitive to various cations, especially Hg^{2+}.
2. *G. frondosa* strains can be distinguished based on the intracellular enzymes in mycelia, as surveyed by API-ZYM commercial kits. Enzymes related to carbohydrate utilization, including α-glucosidase, β-glucosidase, and N-acetyl-β-glucosaminidase, were highly active in the strains tested.
3. *G. frondosa* was cultured in basal media, and intracellular β-N-acetylglucosaminidase was purified by standard methods after seven days. The specific activity of the purified enzyme was 0.67 U/mg protein, with a recovery percentage of 24.5% and a purification fold of 3.68.

4. A 38-kDa enzyme that generates GlcNAc from chitin, with high purity was obtained. Hence, the enzyme was named NAG38.
5. NAG38 was stable over wide ranges of pH and temperature, but it was sensitive to various cations, especially Hg^{2+}.

ACKNOWLEDGMENTS

The authors thank Te-Sheng Lien for the kind help in the purification of this enzyme.

REFERENCES CITED

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Anal. Biochem.* 72(1-2), 248-254. DOI: 10.1016/0003-2697(76)90527-3

Chang, K. L. B., Lee, J., and Fu, W.-R. (2000). "HPLC analysis of N-acetyl-chito-oligosaccharides during the acid hydrolysis of chitin," *J. Food Drug Anal*. 8(2), 75-83. DOI: 10.38212/2224-6614.2837

Chen, J.-K., Shen, C.-R., and Liu, C.-L. (2010). "N-acetylglucosamine: Production and applications," *Mar. Drugs* 8(9), 2493-2516. DOI: 10.3390/md8092493

Cifali, A. P., and Dias Filho, B. P. (1999). "Purification and partial characterization of N-acetyl-b-D-glucosaminidase from *Tritrichomonas foetus*," *Parasitol. Res*. 85(4), 256-262. DOI: 10.1007/s004360050544

Cohen-Kupiec, R., and Chet, I. (1998). "The molecular biology of chitin digestion," *Curr. Opin. Biotech*. 9(3), 270-277. DOI: 10.1016/S0958-1669(98)80058-X

Cruz, M. A. L., Gomes, V. M., Fernandes, K. V. S., Machado, O. L. T., and Xavier-Filho, J. (2002). "Identification and partial characterization of a chitinase and a β-1,3-glucanase from *Copernicia cerifera* wax," *Plant Physiol. Bioch*. 40(1), 11-16. DOI: 10.1016/S0981-9428(01)01340-7

Frändberg, E., and Schnürer, J. (1994). "Evaluation of a chromogenic chito-oligosaccharide analogue, p-nitrophenyl-β-D-N,N'-diacetylchitobiose, for the measurement of the chitinolytic activity of bacteria," *J. Appl. Bacteriol*. 76(3), 259-263. DOI: 10.1111/j.1365-2672.1994.tb01625.x

Gaderer, R., Seidl-Seiboth, V., and Kappel, L. (2017). "Chitin and N-acetylglucosamine metabolism in fungi – A complex machinery harnessed for the design of chitin-based high value products," *Curr. Biotechnol.* 6, 178-193. DOI: 10.2174/2211550105666160330205801

Glasgow, L. R., Paulson, J. C., and Hill, R. L. (1977). "Systematic purification of five glycosidases from *Streptococcus* (*Diplococcus*) *pneumoniae*," *J. Biol. Chem*. 252(23), 8615-8623.

Gooday, G. W. (1990). "The ecology of chitin degradation," in: *Advances in Microbial Ecology: Volume 11*, K. C. Marshall (ed.), Plenum Press, New York, NY, USA, pp. 387-430. DOI: 10.1007/978-1-4684-7612-5_10

Guo, S.-H., Chen, J.-K., Lee, W.-C. (2004). "Purification and characterization of extracellular chitinase from *Aeromonas schubertii*," *Enzyme Microb. Tech*. 35(6-7),

550-556. DOI: 10.1016/j.enzmictec.2004.08.025

Hsu, S. C., and Lockwood, J. L. (1975). "Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil," *Appl. Microbiol*. 29(3), 422-426.

Iranzo, M., Aguado, C., Pallotti, C., Cañizares, J. V., and Mormeneo, S. (2002). "The use of trypsin to solubilize wall proteins from *Candida albicans* led to the identification of chitinase 2 as an enzyme covalently linked to the yeast wall structure," *Res. Microbiol*. 153(4), 227-232. DOI: 10.1016/S0923-2508(02)01307-4

Jones, C. S., and Kosman, D. J. (1980). "Purification, properties, kinetics, and mechanism of beta-N-acetylglucosamidase from *Aspergillus niger*," *J. Biol. Chem*. 255(24), 11861-11869.

Kadowaki, S., Yamamoto, K., Fujisaki, M., Izumi, K., Tochikura, T., and Yokoyama, T. (1990). "Purification and characterization of a novel fungal endo-β-N-acetylglucosaminidase acting on complex oligosaccharides of glycoproteins," *Agr. Biol. Chem. Tokyo* 54(1), 97-106. DOI: 10.1271/bbb1961.54.97

Kubota, T., Miyamoto, K., Yasuda, M., Inamori, Y., and Tsujibo, H. (2004). "Molecular characterization of an intracellular β-N-acetylglucosaminidase involved in the chitin degradation system of *Streptomyces thermoviolaceus* OPC-520," *Biosci. Biotech. Bioch*. 68(6), 1306-1314. DOI: 10.1271/bbb.68.1306

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature* 227, 680-685. DOI: 10.1038/227680a0

Lee, E.-A., Pan, C.-H., Son, J.-M., and Kim, S.-I. (1999). "Isolation and characterization of basic exochitinase from leaf extract of *Rehmannia glutinosa*," *Biosci. Biotech. Bioch*. 63(10), 1781-1783. DOI: 10.1271/bbb.63.1781

Nogawa, M., Takahashi, H., Kashiwagi, A., Ohshima, K., Okada, H., and Morikawa, Y. (1998). "Purification and characterization of exo-β-D-glucosaminidase from a cellulolytic fungus, *Trichoderma reesei* PC-3-7," *Appl. Environ. Microb*. 64(3), 890-5. DOI: 10.1128/AEM.64.3.890-895.1998

Pereira, M. E., Loures, M. A., Villalta, F., and Andrade, A. F. (1980). "Lectin receptors as markers for *Trypanosoma cruzi*. Developmental stages and a study of the interaction of wheat germ agglutinin with sialic acid residues on epimastigote cells," *J. Exp. Med*. 152(5), 1375-1392. DOI: 10.1084/jem.152.5.1375

Reyes, F., and Byrde, R. J. W. (1973). "Partial purification and properties of a β-N-acetylglucosaminidase from the fungus *Sclerotinia fructigena*," *Biochem. J*. 131(2), 381-388. DOI: 10.1042/bj1310381

Rivlin, M., and Navon, G. (2016). "Glucosamine and N-acetyl glucosamine as new CEST MRI agents for molecular imaging of tumors," *Sci. Rep.-UK* 6. DOI: 10.1038/srep32648

Salvatore, S., Heuschkel, R., Tomlin, S., Davies, S. E., Edwards, S., Walker-Smith J. A., French, I., and Murch, S. H. (2000). "A pilot study of N-acetyl glucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease," *Aliment. Pharm. Ther*. 14(12), 1567-1579. DOI: 10.1046/j.1365-2036.2000.00883.x

Shikhman, A. R., Amiel, D., D'Lima, D., Hwang, S.-B., Hu, C., Xu, A., Hashimoto, S., Kobayashi, K., Sasho, T., and Lotz, M. K. (2005). "Chondroprotective activity of N-acetylglucosamine in rabbits with experimental osteoarthritis," *Ann. Rheum. Dis*. 64(1), 89-94. DOI: 10.1136/ard.2003.019406

Sobrinho, I. d. S. J., Bataus, L. A. M., Maitan, V. R., and Ulhoa, C. J. (2005). "Purification and properties of an N-acetylglucosaminidase from *Streptomyces*

cerradoensis," *Biotechnol. Lett*. 27, 1273-1276. DOI: 10.1007/s10529-005-0218-2

Tharanathan, R. N., and Kittur, F. S. (2003). "Chitin-the undisputed biomolecule of great potential," *Crit. Rev. Food Sci. Nutr*. 43: 61-87. DOI: 10.1080/10408690390826455

Trudel, J., and Asselin, A. (1989). "Detection of chitinase activity after polyacrylamide gel electrophoresis," *Anal. Biochem*. 178(2), 362-366. DOI: 10.1016/0003-2697(89)90653-2. DOI: 10.1016/0003-2697(89)90653-2

Wiwat, C., Siwayaprahm, P., and Bhumiratana, A. (1999). "Purification and characterization of chitinase from *Bacillus circulans* No. 4.1," *Curr. Microbiol*. 39(3), 134-140. DOI: 10.1007/s002849900434

Yamamoto, K., Lee, K. M., Kumagai, H., and Tochikura, T. (1985). "Purification and characterization of β-N-acetylhexosaminidase from *Penicillium oxalicum*," *Agr. Biol. Chem. Tokyo* 49(3), 611-619. DOI: 10.1080/00021369.1985.10866789

Zhang, X.-Y., Dai, A.-L., Zhang, X.-K., Kuroiwa, K., Kodaira, R., Shimosaka, M., and Okazaki, M. (2000). "Purification and characterization of chitosanase and exo-β-D-glucosaminidase from a koji mold, *Aspergillus oryzae* IAM2660," *Biosci. Biotech. Bioch*. 64(9), 1896-1902. DOI: 10.1271/bbb.64.1896

Article submitted: January 14, 2021; Peer review completed: May 1, 2021; Revised version received and accepted: September 4, 2021; Published: September 10, 2021.
DOI: 10.15376/biores.16.4.7234-7248