Characterization of New Multifunctional GH20 β -*N*-Acetylglucosaminidase From *Chitinibacter* sp. GC72 and Its Application in Converting Chitin Into *N*-Acetyl Glucosamine

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Abstract

Background

N-Acetyl glucosamine (GlcNAc), possesses many specific bioactivities and has been widely used in biomedical, food, and chemical industries. Alternatively, enzymatic hydrolysis of chitin into GlcNAc using chitinolytic enzymes was shown to be a more attractive approach in recent years, because of the green process and the excellent bioactivity of product. Therefore, it is of economic and environmental value to realize the efficient production of GlcNAc from abundant chitin resources.

Results

In this study, a gene encoding β -*N*-acetylglucosaminidase, designated NAGaseA, was cloned from *Chitinibacter* sp. GC72 and subsequently functional expressed in *Escherichia coli* BL21 (DE3). NAGaseA contains a glycoside hydrolase family 20 catalytic domain that shows low identity with the corresponding domain of the well-characterized NAGases. The recombinant NAGaseA had a molecular mass of 92 kDa. Biochemical characterization of the purified NAGaseA revealed that the optimal reaction condition was at 40°C and pH 6.5, and exhibited great pH stability in the range of pH 6.5–9.5. The $V_{\max}$, K_m , K_{cat} , and K_{cat}/K_m of NAGaseA toward *p*NP-GlcNAc were 3333.33 $\mu\text{mol min}^{-1} \text{L}^{-1}$, 39.99 μM , 4667.07 s^{-1} , and 116.71 $\text{mL } \mu\text{mol}^{-1} \text{s}^{-1}$, respectively. Analysis of the hydrolysis products of *N*-acetyl chitinoligosaccharides (*N*-Acetyl COSs) indicated that NAGaseA capable of converting *N*-Acetyl COSs((GlcNAc)₂–(GlcNAc)₆) into GlcNAc with hydrolysis ability order: (GlcNAc)₂ > (GlcNAc)₃ > (GlcNAc)₄ > (GlcNAc)₅ > (GlcNAc)₆. Moreover, NAGaseA could generate (GlcNAc)₃–(GlcNAc)₆ from (GlcNAc)₂–(GlcNAc)₅, respectively. These results showed that NAGaseA is a multi-functional NAGase with transglycosylation activity. In addition, significantly synergistic action was observed between NAGaseA and other sources of chitinases during hydrolysis of colloid chitin. Finally, 0.759 g/L, 0.481 g/L and 0.986 g/L of GlcNAc with purity of 96% were obtained using different chitinase combinations, resulting in 1.6–2.69 fold increase, respectively.

Conclusions

The hydrolytic properties and good environmental adaptations indicate that NAGaseA has great potential in the bioconversion of chitin waste and behaved as an excellent candidate in GlcNAc producing.

Background

Chitin is the second most abundant polysaccharides on earth after cellulose, it mainly derived from fungal cell walls, insect exoskeletons, as well as the crab and shrimp shells. An estimated 10^{10} – 10^{11} tons of chitin are produced per year [1]. However, 35–45% of chitin biomass is discarded as waste due to lack

of efficient refinery protocols, which leads to waste of resource and severe environmental problems [2, 3]. *N*-Acetyl glucosamine (GlcNAc), the monomeric unit of chitin, possesses many specific bioactivities and has been widely used in biomedical, food, and chemical industries [4–6]. Therefore, it is of economic and environmental value to realize the efficient production of GlcNAc from abundant chitin resources [7].

Commercial GlcNAc was often produced via acid hydrolysis of chitin. However, this protocol is difficult to directly obtain GlcNAc owing to the deacetylation of *N*-acetyl group of products [8]. In this case, chitin is first hydrolyzed to GlcN, and then chemical acetylated to form GlcNAc. This multi-step process not only results in low yield, high cost, and poor biological activity of products, but also leads to numerous environmental issues [9, 10]. Alternatively, enzymatic hydrolysis of chitin into GlcNAc using chitinolytic enzymes was shown to be a more attractive approach in recent years, because of the green process and the excellent bioactivity of product [11].

Chitinolytic enzymes is complex enzyme system with a good synergistic effect, which could be classified into three types: endo-acting chitinases that cut randomly chitin chain to generate *N*-acetyl chitinoligosaccharides (*N*-acetyl COSs); progressive exo-acting chitinases that release GlcNAc dimer from the nonreducing or reducing end of chitin chains; NAGases that hydrolyze *N*-acetyl COSs or GlcNAc dimer into GlcNAc[12–14]. Among, NAGase plays a key role in control of the ratio and yield of GlcNAc during the hydrolysis process of chitin. Thus, it is of great significance to excavate NAGase with high activity for the efficiently converting *N*-acetyl COSs into GlcNAc.

In our previous study, chitinolytic enzymes derived from bacterium *Chitinibacter* sp. GC72 isolated from pond mud in Nanjing were capable of hydrolyzing chitin into GlcNAc as the sole product [15]. Moreover, only one gene encoding NAGase, named NAGaseA, was found in strain GC72 via the complete genome sequencing and analysis [16]. In this study, the NAGaseA gene was cloned from the genome of strain GC72 and heterologous expressed in *Escherichia coli* (BL21). The enzymatic properties and hydrolysis mode of the recombinant NAGaseA were investigated. Furthermore, the synergetic effect between NAGaseA and various chitinases in converting chitin to produce GlcNAc were also studied. This study provided a possible application in the enzymatic production of GlcNAc.

Results And Discussion

Cloning of the NAGaseA gene and sequence analysis.

Based on the gene function prediction of the complete genome of *Chitinibacter* sp. GC72, ORF 159 was annotated as a potential β -N-acetylglucosaminidase (NAGaseA) gene. The total length of NAGaseA gene is 2, 535 bp, encoding 844 amino acids. After PCR, the same nucleic acid sequence was obtained, which indicated that NAGaseA gene was successfully cloned. Besides, the predicted molecular weight and theoretical pI of NAGaseA were 92.4 kDa and 5.24, respectively.

According to result of BLASTP analysis of the amino acid sequence, NAGaseA belonged to glycoside hydrolase (GH) family 20 (GH20) and shared the highest identity of 94.43% with the putative GH20

NAGase from *Chitinibacter fontanus* (WP_180317904), followed by 88.27% with GH20 NAGase from *Chitinibacter* sp. ZOR0017 (WP_047394852). However, the relative enzymatic characterization of these proteins has not been reported. Among the studied NAGases, NAGaseA displayed the highest identity (68.84%) with GH20 NAGase from *Aeromonas* sp. 10S-24 (accession no. BAA92145) [24], followed by 67.65% with GH20 NAGase from *C. meiyuanensis* (accession no. WP_148716590) [20], 32.83% with GH20 NAGase from *Serratia marcescens* (PDB 1QBA) [25], 30.79% with GH20 NAGase from *Vibrio harveyi* (PDB 6EZR) [26], 27.05% with GH20 NAGase from *Microbacterium* sp. HJ5 (PDB 7BWG) [27]. A phylogenetic tree of NAGaseA with some putative and verified GH20 family NAGases was further constructed base on their sequence similarities. The results suggested that NAGaseA shared low sequence similarity with most experimentally characterized GH20 NAGases (Fig. S1).

The result of multiple alignment of NAGaseA with other GH20 NAGases was shown in Fig. S2. The typical acidic pairs D512-E513 in NAGaseA are completely aligned with many other functionally characterized GH20 NAGases, which probably functioned as the catalytic residues. In addition, other highly conserved residues among NAGase species including R319, H426, V467, Q468, W562, W600, Y625, D627, L628, Y639, W641, W698, and E700 were also observed, which may play an important role in binding the GlcNAc ligand [28]. Furthermore, the consensus H-X-G-G motif before the catalytic residue in NAGaseA is highly conserved among the catalytic domain of GH20 NAGases. Based on the analysis of secondary structure, NAGaseA possesses 20 α -helices and 30 β -sheets with the typical $(\beta/\alpha)_8$ barrel fold in the GH20 catalytic domain, which is highly consistent with various GH20 NAGases from different sources [29].

The structure feature of NAGaseA was shown in Fig. 1 (A). The predicted protein structure consisted of four domains as follows: domain I (CHB_HEX domain of residues 2-153); domain II (Glyco_hydro_20b domain of residues 174-287); domain III (Glyco_hydro_20 domain residues 308-726, the catalytic domain containing a TIM barrel fold); domain IV (CHB_HEX_C domain of residues 758-841). As presented in Fig. 1 (B), the model of NAGaseA was predicted on the basis of the crystal structure of GH20 NAGase from *Serratia marcescens* (PDB 1QBA) with a protein identity of 34.22% [25]. The active pocket formed by R319, H426, V467, Q468, D512, E513, W562, W600, Y625, D627, L628, Y639, W641, W698, and E700 were labeled in the 3D structure model of NAGaseA (Fig. 1 (C)).

Expression of NAGaseA gene and purification of recombinant NAGaseA

The gene encoding NAGaseA was successfully expressed as soluble protein in *E. coli* BL21(DE3). The SDS-PAGE analysis (Fig. 2) showed that a single target protein band was obtained with a molecular weight of ~ 92 kDa after Ni-NTA resin affinity purification, which was consistent with the 92,379 Da calculated from the amino acid sequence containing the His6-tag. This is different from that of some GH20 NAGases from *Microbacterium* sp. HJ5 (55.9 kDa) [27], *Paenibacillus* sp. (57.5 kDa), *V. harveyi* 650 (73 kDa) [26] and *S. thermoviolaceus* (60 kDa) [31]. However, the M_w of NAGaseA is similar with the previously reported GH20 NAGase from *C. meiyuanensis* with a molecular mass of 92,571Da [32]. The specific activity of recombinant NAGaseA exhibited a 1.39-fold increase from 270.17 U/mg to 373.29 U/mg with a protein recovery of 78.6% yield after purification (Table S1).

Effects of temperature and pH on the enzymatic activity and stability of purified recombinant NAGaseA

The temperature and pH profiles of recombinant NAGaseA were investigated in Fig 3. As shown in Fig. 3(A), the recombinant NAGaseA displayed the optimal temperature at 40°C, which was consisted with NAGase from *C. Meiyuanensis* SYBC-H1 (40°C) [32], but different from NAGases from *S. marcescens* (52°C) [33], *Microbacterium* sp. HJ5 (45°C) [27], *Streptomyces* sp. F-3 (60°C) [34], and *P. hydrolytica* S66 (50°C) [35]. As for the thermostability profile, the activity dropped rapidly after incubation at temperatures above 40°C, suggesting the poor thermostability of NAGaseA, which were similar with that of GH20 GlcNAGases from *C. meiyuanensis* [32], *Microbacterium* sp. HJ5 [27] and *Aeromonas* sp. 10S-24[24].

Regarding the effect of pH, NAGaseA exhibited the optimum pH at 6.5 (Figure 3 (B)). The optimal pH value of NAGaseA was higher than some reported NAGases, such as NAGase from *C. meiyuanensis* (5.4) [18], *Salmonella enterica* (4.0) [36] and *Lactobacillus casei* (5.0) [33]. In addition, NGAaseA retained excellent activity after incubation under the corresponding buffers of pH 6.5–9.5, indicating that NAGaseA possessed a good pH stability compared to other reported NAGases [37-39].

Effects of metal ions on activity of recombinant NAGaseA

Many reports have shown that metal ions affected the enzymatic activity. Thus, the effects of various metal ions on NAGaseA activity were also investigated. As shown in Table 1, the enzyme retained approximately 96% of its initial activity after incubation in 10 mM EDTA, suggesting that EDTA did not inhibit the enzymatic activity and NAGaseA is non-metal dependent. Cu²⁺ showed greatly inhibition effect on activity of NAGaseA, which was similar with that of NAGases from *A. caviae* [40] and *C. meiyuanensis* [20]. Besides, NAGaseA activity was partially inhibited by Fe³⁺ and Co²⁺, NAGases from *R. miehei* and *Streptomyces alfalfa* shared the same profile as reported [13, 41].

Table 1. Effects of metal ions on the activity of NAGaseA

Metal ions	Chemicals	Concentration (mM)	Relative activity (%)
No addition	-	0	100
Cu ²⁺	CuCl ₂	10	24.27 ± 2.42
Fe ³⁺	FeCl ₃	10	45.19 ± 2.25
Co ²⁺	CoCl ₂	10	78.55 ± 5.49
Ni ²⁺	NiCl ₂	10	92.95 ± 7.43
Ca ²⁺	CaCl ₂	10	94.01 ± 1.88
Al ³⁺	AlCl ₃ ·6H ₂ O	10	91.97 ± 3.67
Mg ²⁺	MgCl ₂	10	90.19 ± 6.31
Zn ²⁺	ZnCl ₂	10	93.07 ± 4.65
Mn ²⁺	MnCl ₂	10	98.98 ± 2.96
EDTA	EDTA	10	95.71 ± 4.78

Substrate specificity of NAGaseA

The substrate specificity of NAGaseA was measured using standard assay conditions. As depicted in Table 2, NAGaseA exhibited the highest specific activity toward *p*NP-GlcNAc, with specific activity of 333.33 U/mg. Among (GlcNAc)₂₋₆, NAGaseA showed the highest activity toward (GlcNAc)₂, followed by (GlcNAc)₃, (GlcNAc)₄, (GlcNAc)₅ and (GlcNAc)₆, which showed that the specific activity toward *N*-acetyl COSs decreased with increasing degree of polymerization [42]. Besides, little activity (0.0037 U/mg) was detected using colloid chitin as substrate, which was agreed with other reported GH20 NAGases that capable of degrading chitin to some extent without the addition of other chitinases [27]. Moreover, no activity was observed when chitosan, chitin power, CMC was used as the substrates. These results indicated that NAGaseA possessed the typical NAGase activity with strict substrate specificity.

In addition, the kinetic parameters for NAGaseA were also measured with *p*NP-GlcNAc as the substrate. The results showed that the V_{max} , K_m , K_{cat} and K_{cat}/K_m for NAGaseA were 3333.33 $\mu\text{mol min}^{-1} \text{L}^{-1}$, 39.99 μM , 4667.07 s^{-1} , 116.71 $\text{mL } \mu\text{mol}^{-1} \text{s}^{-1}$, respectively.

Table 2. Substrate specificity of NAGaseA

Substrates	Specific activity (U/mg of protein)
Colloidal chitin	0.0037 ± 0.00047
Chitosan	0
Chitin power	0
<i>p</i> NP-GlcNAc	333.33±19.21
(GlcNAc) ₂	201.68 ± 11.69
(GlcNAc) ₃	152.84 ± 7.18
(GlcNAc) ₄	81.34 ± 5.49
(GlcNAc) ₅	55.52 ± 2.11
(GlcNAc) ₆	23.59 ± 1.13

Hydrolysis mechanism of NAGaseA toward colloid chitin and *N*-acetyl COSs

The hydrolysis patterns of colloid chitin and *N*-acetyl COSs by NAGaseA were measured (Fig. 4). As shown in Fig. 4(A), GlcNAc was the sole product hydrolyzed by colloid chitin, with its concentration raised as hydrolysis time increased. In the hydrolysis process of NAGaseA, (GlcNAc)₂ was converted to GlcNAc as the sole product (Figure 4 (B)), (GlcNAc)₃ to (GlcNAc)₂ and GlcNAc (Figure 4 (C)), (GlcNAc)₄ to (GlcNAc)₃, (GlcNAc)₂ and GlcNAc (Fig.4 (D)), (GlcNAc)₅ to (GlcNAc)₄, (GlcNAc)₃, (GlcNAc)₂ and GlcNAc (Fig.4 (E)), and (GlcNAc)₆ was converted to (GlcNAc)₅, (GlcNAc)₄, (GlcNAc)₃, (GlcNAc)₂ and GlcNAc (Fig.4 (F)) at the initial incubation within 5 min. Furthermore, NAGaseA could hydrolyze (GlcNAc)₂–(GlcNAc)₆ into pure GlcNAc after an incubation time of 15 min to 180 min, respectively. The overall rates of hydrolysis were in the order: (GlcNAc)₂ > (GlcNAc)₃ > (GlcNAc)₄ > (GlcNAc)₅ > (GlcNAc)₆, which was in accordance with the results of substrate specificity. These results indicated that NAGaseA is a typical exo-NAGase.

In addition, minor (GlcNAc)₃, (GlcNAc)₄, (GlcNAc)₅, (GlcNAc)₆, were also produced from (GlcNAc)₂, (GlcNAc)₃, (GlcNAc)₄, and (GlcNAc)₅ in short reaction times. These results indicated that NAGaseA capable of producing higher *N*-acetyl COSs ((GlcNAc)₃–(GlcNAc)₆) from (GlcNAc)₂–(GlcNAc)₅, which exhibited trans glycosylation activity. Our previous study also reported that *Cm*NAGase from *Chitinolyticbacter meiyuanensis* SYBC-H1 could produce higher *N*-acetyl COSs (GlcNAc)₃–(GlcNAc)₇ from (GlcNAc)₂–(GlcNAc)₆, respectively [32]. However, unlike *Cm*NAGase, no new peak presumed as (GlcNAc)₇ generated when using (GlcNAc)₆ as the substrate, which could be attributed to the lower reverse hydrolysis activity of NAGaseA.

Synergistic action between NAGaseA and chitinases on chitin degradation

To investigate the potential application of NAGaseA in GlcNAc production, the synergistic action between NAGaseA and other chitinases on chitin degradation was studied. As illustrated in Fig 5, the released reducing sugar concentrations from cooperation of NAGaseA with purified chitinase chiA, crude enzyme from *C. meiyuanensis* SYBC-H1 and crude enzyme from *Chitinibacter* sp. GC72 were 0.759 g/L, 0.481 g/L and 0.986 g/L, which were 1.6-, 2.36-, and 2.69- fold that of concentration of the two enzyme accumulated, respectively. Among, NAGaseA behaved the best improve efficiency with the crude enzyme from GC72, which could be attributed to the better synergistic effect with other chitinases from *Chitinibacter* sp. GC72. Zhou et al reported a combination of commercial chitinase CtnSg and NAGase rHJ5Nag used for chitin degradation, with an improvement rate of 2.02- fold [27]. Chenyin Lv et. al also investigated the synergistic action between commercial chitinase SgCtn and NAGase SaHEX, which obtained higher production of reducing sugars than the single enzyme for SgCtn (4.3-fold) and SaHEX (8.1-fold) [13]. In our study, NAGaseA can not only combine with purified chitinase but also crude chitinases in the production of GlcNAc from chitin. Moreover, it was worth noted that GlcNAc purity of 96% was obtained and little other *N*-acetyl COSs were detected in the final reaction mixture, indicating that NAGaseA has great potential in the production of GlcNAc in the multi-enzyme combination system.

Conclusions

In this study, gene encoding a GH20 family β -N-acetylglucosaminidase NAGaseA from chitinolytic bacterium *Chitinibacter* sp. GC72 was cloned and functionally expressed. The domain structure prediction showed that NAGaseA contains GH20 family catalytic domain and exhibited low similarity with reported GH20 NAGases. Analysis from the HPLC revealed that NAGaseA was a multi-functional NAGase exhibited the exo-acting activity and trans glycosylation activity. Furthermore, NAGaseA also behaved excellent synergistic performance with other chitinases during degradation of colloidal chitin and high purity of GlcNAc was obtained as the final product. These results indicated that NAGaseA has great potential in the bioconversion of chitin waste and behaved as an excellent candidate in GlcNAc producing.

Methods And Materials

Chemicals, strains, and plasmids

Chitin powder, 4-methylumbelliferyl *N*-acetyl glucosaminide (4-MU-GlcNAc), and *p*NP-acetyl galactosaminide (*p*NP-GlcNAc) were purchased from Aladdin reagent Co.,Ltd (Shanghai, China). *N*-acetyl chitooligosaccharides (*N*-acetyl-COSs) standard ranging from dimer to hexamer were purchased from Qingdao BZ Oligo Biotech Co., LTD. (Qingdao, China). The molecular reagents were purchased from Takara Bio Inc. (Dalian, China). All chemicals used in this study were of analytical grade or higher purity. Colloidal chitin was prepared from chitin powder according to the methods described by Gao et al [15].

Escherichia coli Trans1-T1, BL21(DE3) and the expression vector pET-28a (+) plasmid were purchased from Novagen. The strain *Chitinibacter* sp. GC72 (CCTCC M 2014113) and *Chitinolyticbacter*

meiyuanensis SYBC-H1 (ATCC BAA-2140) used in this study were isolated and stored in our laboratory [15, 17].

Culture conditions

Strain GC72 and SYBC-H1 were cultured according to our previous study [15, 17]. *E. coli* strains were routinely cultivated aerobically at 37°C in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) or agar plates containing 50 µg/mL kanamycin.

Molecular cloning and sequence analysis

The genomic DNA of strain GC72 was extracted using a bacteria genome extraction Kit (TIANGEN, China) and was used as the PCR template. Two primers used to amplify the NAGaseA were synthesized by Genscript Biotech (Nanjing, China) and the sequences were as followed: Forward primer 5'-GTGCCGCGCGGCAGCCATATGAACAAGCCAGCAGGT -3'; Reserve primer 5'-GTGGTGGTGGTGCTCGAGCACCGCAACCAACCCGGCT-3'. The PCR system and conditions were as follows: 94°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. The NAGaseA gene generated from PCR and the plasmid pET-28a (+) were double digested with *Nde*I and *Xho*I, followed by a ligation using the ClonExpress™ II/One Step Cloning Kit (Vazyme, China). The recombinant plasmid was transformed into *E. coli* Trans-T1 competent cells and sequenced by Genscript Biotech (Nanjing, China).

Nucleotide and amino acid sequences were analyzed using Snap Gene™ 1.1.3 software (<http://www.snapgene.com/>) and the ExPASy protparam tool (<http://web.expasy.org/protparam/>). The DNA and protein sequence alignments were performed via the NCBI server with the programs BLASTN and BLASTP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), respectively. Phylogenetic trees were inferred using neighbor-joining algorithm in MEGA 7. The conserved domains and the GH family classification were identified via the website (<http://prosite.expasy.org/scanprosite/>). Signal peptide was predicted in the SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>). Protein homologous sequences alignment was carried out using ClustalX 2.1 software and ESPript 3.0 (<http://esprict.ibcp.fr/ESPript/cgi-bin/ESPript.cgi>). The structure of NAGaseA was predicted with RaptorX (<http://raptorx.uchicago.edu/StructPredV2/predict/>).

Expression and purification of recombinant NAGaseA

The recombinant plasmid pET-28a (+) harboring NAGaseA gene was transformed into *E. coli* BL21(DE3), incubated in LB liquid medium (containing 50 µg/mL kanamycin), and then cultured at 37°C with shaking at 200 rpm. When the optical density (OD₆₀₀) of the culture medium was approximately 0.6, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM for protein induction, and the culture was incubated overnight at 18°C with shaking at 200 rpm.

Cultures were harvested by centrifugation at $6000 \times g$ and 4°C for 10 min, after which the pellet was gently resuspended in binding buffer A (50 mM phosphate buffer, 500 mM NaCl, 50 mM imidazole, pH 7.4) and lysed by JY92-IIN ultrasonication (Ningbo Xinzhi Biotechnology, Ltd., Ningbo, China). The cell debris was removed by centrifugation at $12000 \times g$ for 10 min at 4°C and the supernatant was retained as crude enzyme. The recombinant NAGaseA were purified using a fast protein liquid chromatography 448 (FPLC) system (GE AKTA Pure 150; General Electric Co., Iowa, America with a Ni- 449 nitrilotriacetic acid affinity chromatography (Ni-NTA) column (His Trap™ FF 5 mL). The supernatant was filtered with a $0.22 \mu\text{m}$ membrane before loaded onto a Ni Sepharose column. The NAGaseA protein was eluted with buffer B (50 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4) under a flow rate of 3 mL/min. The eluted protein was collected, concentrated, exchanged with 20 mM phosphate buffer (pH 7.0) via ultrafiltration and stored at 4°C prior to use [18].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to identify the target protein, and the protein concentration was determined using the Bradford method [19].

Enzyme assay and substrate specificity of recombinant NAGaseA

NAGaseA activity was assayed using *p*NP-GlcNAc as substrate. 1 mL reaction mixture containing 50 μL *p*NP-GlcNAc (10 mM) and 10 μL of purified NAGaseA in 50 mM PBS buffer (pH 7.0). The mixture was incubated at 40°C for 10 min and then 1 mL NaOH (1 M) solution was added to terminate the reaction. The amount of *p*NP released was determined under the absorbance measured at 405 nm according to our previous reported [20]. One unit of NAGase activity was defined as the amount of enzyme required to release 1 μmol *p*NP per minute under the assay conditions.

Chitinase activity was measured using colloid chitin as the substrate. The 1 mL reaction mixture was performed with 0.2 mL of enzyme and 0.3 mL colloid chitin (10 g/L) in 50 mM phosphate buffer (pH 7.0). The reaction was conducted at 37°C for 30 min, and then 1 mL of 3, 5-dinitrosalicylic acid (DNS) was added to the mixture followed by boiling at 100°C for 5 min [21].

Enzymatic characterization of recombinant NAGaseA

The enzymatic characterization of recombinant NAGaseA was performed using *p*NP-GlcNAc as the substrate. To determine the optimum temperature of NAGaseA, the reaction was incubated under interval temperatures range from 30°C to 80°C in 50 mM PBS (pH 6.5). The thermostability of NAGaseA was determined by measuring the residual activity after being incubated at different temperatures ($30\text{--}60^{\circ}\text{C}$) for 30 min in 50 mM PBS (pH 6.5).

The optimal pH of NAGaseA was determined at 40°C under a pH range of 3.0–11.0 using different buffers (50 mM): citrate buffer (pH 3.0–6.0), phosphate buffer (pH 5.5–8.0), Tris-HCl buffer (pH 7.0–9.0), glycine-NaOH buffer (pH 8.5–11.0). To determine the pH stability, the recombinant enzyme was incubated in the above-mentioned buffers at 40°C for 30 min, and the residual activities were measured by the standard assay.

To determine the effect of different metal ions on NAGaseA activity, the purified enzyme was incubated with 10 mM EDTA at 40°C for 30 min, and then remove the EDTA using 50 mM PBS (pH 7.0) by ultrafiltration. After that, the metal-free NAGaseA was incubated with various metal salts containing Ca^{2+} (CaCl_2), Co^{2+} (CoCl_2), Mn^{2+} (MnCl_2), Cu^{2+} (CuCl_2), Fe^{3+} (FeCl_3), Mg^{2+} (MgCl_2), Zn^{2+} (ZnCl_2), Al^{3+} ($\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$), Ni^{2+} (NiCl_2) at a final concentration of 10 mM for 30 min. The residual activities were measured using *p*NP-GlcNAc at 40°C in 50 mM PBS (pH 7.0) for 30 min, and the residual activity of NAGaseA without metal ions incubation was used as the control (100%).

The kinetics parameters were determined by measuring the enzyme activity toward *p*NP-GlcNAc at 40°C in 50 mM PBS (pH 6.5) for 10 min using different concentrations of substrate (50 μM –2500 μM) as the substrate. The values of V_{max} , K_{m} , and K_{cat} were estimated by linear regression from double-reciprocal plots according to the method of Lineweaver [22].

Hydrolytic pattern of recombinant NAGaseA

The reaction mixtures containing purified NAGaseA (60 ng) and various substrates ((GlcNAc)₂–(GlcNAc)₅) at a final concentration of 10 g/L were incubated at 40°C at various time intervals. In each case, the supernatant after hydrolysis was diluted with 50% acetonitrile and centrifuged at 8000 × *g* for 10 min to remove the protein. The hydrolysis products were analyzed by Agilent 1260 series HPLC system according to our previous study [20].

Cooperative interaction analysis of NAGaseA with other chitinases

The fermentation broth of strain GC72 and SYBC-H1 were centrifuged at 12000×*g* for 15 min at 4°C, and the supernatant were collected as crude enzyme prior to use. Exochitinase ChiA from *Serratia proteamaculans* (stored in our laboratory) was cloned, expressed and purified as previous reported [23].

The cooperative interaction between NAGaseA and other sources of chitinases derived from strain *Serratia proteamaculans* (recombinant ChiA), strain SYBC (crude enzyme), and strain GC72 (crude enzyme) were determined using colloid chitin as the substrate. The reaction mixture contained colloidal chitin with final concentration of 10 g/L and either NAGaseA (4.8 U/mL reaction system), ChiA (6.1 U/mL reaction system), SYBC chitinase (2.8 U/mL reaction system) and GC72 chitinase (5.2 U/mL reaction system) or both enzymes and was incubated for 30 min. The amount of reducing sugars released were measured using DNS method and HPLC mentioned above.

Declarations

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Figures

Figure 1

The domain and structure prediction of NAGaseA. (A) The conserved domain of NAGaseA, the image was generated using IBS Illustrator [30]. (B) The prediction of the 3D structure of NAGaseA, (C) The active sites of NAGaseA.

Figure 2

SDS-PAGE analysis of the expression and purification of recombinant NAGaseA. The amount of the loaded protein is 10 μ g. Lane M, protein molecular mass marker; lane 1, crude enzyme deposit of NAGaseA; lane 2, crude enzyme preparation of NAGaseA; lane 3, NAGaseA purified by His6-tag affinity chromatography.

Figure 3

Effect of pH and temperature on the activity and stability of NAGaseA. (A) Optimal temperature and temperature stability of the recombinant NAGaseA. The temperature optimum was determined at different temperatures (30–80 °C) in 50 mM phosphate buffer (pH 6.5). To determine the temperature stability, the residual activity was measured in 50 mM phosphate buffer (pH 6.5) after incubation for 30 min at different temperatures. (B) Optimal pH and pH stability of the recombinant NAGaseA. The optimal pH was determined in 50 mM solutions of various buffers within the pH range 3–11. To determine pH stability, the enzyme was incubated at 40 °C for 30 min with various pH buffers, and the residual activities were measured. (● Citrate buffer (pH 3.0-6.0); ◻ Phosphate buffer (pH (6.0-8.0); ▲ Tris-HCl buffer (pH 7.0-9.0); ■ Glycine-NaOH buffer (pH 8.5-10.5))

Figure 4

Hydrolysis analysis of NAGaseA toward colloid chitin and *N*-Acetyl COSs. (A) Colloid chitin and (B)-(F) (GlcNAc)₂₋₆ were performed in PBS (pH 6.5) at 40 °C contained 60 ng NAGaseA and 10g/L various substrate. Numbers 1–6 represent GlcNAc to (GlcNAc)₆.

Figure 5

Production GlcNAc from chitin using cocktail enzyme. (A) Effect of cooperative interaction between NAGaseA with chitinases on the hydrolysis of chitin. 1: cooperation of NAGaseA with purified chitinase chiA; 2: cooperation of NAGaseA with crude enzyme from *C. meiyuanensis* SYBC-H1; 3: cooperation of NAGaseA with crude enzyme from *Chitinibacter* sp. GC72. (B) HPLC analysis of the final products of reaction mixture.

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