

Molecular cloning, expression and characterization of a novel gene β -*N*-acetylglucosaminidase from *Bombyx mori*

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ABSTRACT

Previously, we have reported that a gene encoding *Bombyxmori* β -*N*-acetylglucosaminidase 2 (BmGlcNAcase2) has been identified differentially expressed in the midgut of *Bombyxmori* strain NB resistant to nucleopolyhedrovirus (BmNPV), strain 306 susceptible to NPV and a near isogenic line BC₉ with similar genetic background to 306 but resistant to NPV by two-dimensional gel electrophoresis (2-DE). To get more knowledge about the relationship between β -*N*-acetylglucosaminidase and the resistance of NPV, in this study, the 1542 bp open reading frame of a putative *bombyxmori* β -*N*-acetylglucosaminidase 2 gene (BmGlcNAcase2) was amplified from a pool of *bombyxmoric*DNAs and inserted into the prokaryotic expression plasmid pET-30a(+). Western blotting analysis showed that BmGlcNAcase2 was expressed in hemolymph, ovary, testis, fat body, trachea, midgut and silk gland of fifth instar larvae respectively. Immunofluorescence analysis indicated that BmGlcNAcase2 was mainly located to the cytoplasm or some structure in cytoplasm.

Keywords: *Bombyx mori*; β -*N*-acetylglucosaminidase2; Western Bolt; Sub-Cellular Localization

1. INTRODUCTION

Sericulture is an important component of ariculture in China, which has a history of over 5,000 years in raising silkworms (*Bombyxmori*L.). It is also widely practiced in the world, such as in Japan, the former Soviet Union and Brazil. Silkworm viral diseases are major diseases causing great loss in sericulture, and *Bombyxmori*nucleopolyhedrovirus (BmNPV) is one of the most disastrous. Therefore, it is a subject of intensive research to control silkworm NPV disease. The key to the sericulture is to develop pathogen-resistant silkworm strains [1]. Recently, comparative gene expression techniques, such as differ-

ential display, cDNA microarray assay and two-dimensional gel electrophoresis have become routine to examine changes in gene expression. Such methodologies provide useful approaches to identify differently expressed transcripts, because many genes can be examined simultaneously. To date, although a few BmNPV resistant genes have been reported, such as serine protease [2] and Bmlipase-1 [3], knowledge on the molecular mechanisms of *Bombyx mori* against this virus remains very limited.

β -*N*-acetylglucosaminidase(GlcNAcase) are widely distributed in various organisms. These enzymes catalyze the hydrolysis of an O-glycosidic bond in nonreducing terminal *N*-acetylglucosamine(GlcNAc) residues in an oligosaccharide chain. In insects, GlcNAcase plays an important role in the degradation of various oligosaccharides and glycoconjugates [4-6], a putative *bombyxmori* β -*N*-acetylglucosaminidase 2 showed broad substrate specificity, and cleaved terminal *N*-acetylglucosamine residues from the α -3 and α -6 branches of a bi-antennary *N*-glycan substrate, and also hydrolyzed chitotriose to chitobiose [7]. In our previous study, by comparison the proteomes of the resistant *Bombyx mori* strain NB, the susceptible strain 306 and the near isogenic line BC₉ strain by two-dimensional gel electrophoresis (2-DE), many differential protein spots have been obtained, one of which was identified as *bombyxmori* β -*N*-acetylglucosaminidase 2 by mass .We speculate that the excessive expression of β -*N*-acetylglucosaminidase in hemolymph of the resistant silkworm can probably disturb the *N*-linked glycans of GP64 protein on the cell membrane which is an essential process for initiating second infections, and thus reduce the reproduction of infectious viruses [8,9]. In this paper,the open reading frame (ORF) of BmGlcNAcase2 was cloned and the recombinant enzyme was expressed in *E. coli*. The amino acid sequence of recombinant BmGlcNAcase2 was verified by mass spectroscopic analysis. Western blotting analysis showed that there is no obvious difference of BmGlcNAcase2 expression in hemolymph, fat body, trachea, ovary, mid-

gut, silk gland and testis of fifth instar larvae. The sub-cellular localization study through immunofluorescence analysis indicated for the first time that the BmGlcNAcase2 was located to cytoplasm or some structure in cytoplasm. The obtained results would facilitate further studies to elucidate molecular mechanisms of *Bombyx-mori* against BmNPV infection.

2. MATERIALS AND METHODS

2.1. Insect, Cell and Virus

B. mori strain C108 (standard strain of silkworm) was maintained in our laboratory. All larvae were reared with fresh mulberry leaves at 27°C under a 12 h light/12 h dark photoperiod.

The BmN cell line was maintained at 27°C in TC-100 insect medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, USA) using standard techniques.

The expression vector pET-30a(+) and *E. coli* strains BL21 (DE3) were obtained from Novagen (CA, USA). All primers, RNase-free DNaseI, EX Taq polymerase, restriction enzymes, T4 DNA ligase and DNA the sub-cloning vector pMD18-T were purchased from TaKaRa (Dalian, China). Chemicals are all from sigma (MO, USA) or a domestic provider in China if not stated otherwise.

2.2. Cloning of the Bmglcnacase2 Gene

The BmGlcNAcase2 specific primers, forward primer (5'-CATGCCATGGCACCAGGGACCCGAATAT-3') with a NcoI site (underlined), and reverse primer (5'-ATAAGAATGCGGCCGCCTAAGCGCCTAGGCAGA-3') with an NotI site (underlined) were designed to amplify the ORF of the putative BmGlcNAcase2 gene (GenBank accession no. [AB286958](#)). The PCR reaction was carried out with 30 amplification cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 90 s) in a Gene Amp 2400 System thermocycler. The PCR product was ligated into pMD 18-T vector using T4 DNA ligase and then transformed into *E. coli* TG1. A fragment between NcoI and NotI containing the BmGlcNAcase2 gene was excised from the recombinant plasmid. The purified fragment was subcloned into the pET-30a(+) expression vector and transformed into *E. coli* BL21 (DE3). DNA sequencing confirmed that the *BmGlcNAcase2* gene was correctly fused to the N-terminal 6 × His-tag.

2.3. Expression and Purification of Recombinant Protein

To express recombinant protein, a freshly transformed colony was cultured in LB medium supplemented with kanamycin (50 µg/ml) at 37°C overnight. This overnight culture was inoculated into fresh LB medium and cul-

tured at 37°C with vigorous shaking. When OD₆₀₀ reach 0.5, the expression of BmGlcNAcase2 was induced with IPTG (final concentration 0.1-1 mM during optimization) and further cultured at 37°C for another 8 hours and 16°C for another 12 hours respectively. Cells were harvested by centrifugation (4500 g, 4°C, 15 min) and SDS-PAGE analysis on 15% gel was performed to estimate the expression level of BmGlcNAcase2. The cell pellet was resuspended in buffer A (50 mM sodium phosphate, 300 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, pH 8.0), then the suspension was lysed by sonication. The lysate was clarified by centrifugation (16,000 g, 4°C, 25 min). The supernatant was loaded onto a Ni-NTA affinity column (Qiagen). Purification conditions were standardized by optimizing pH, the concentration of salt and imidazole. After washing the captured column with 20 mM and 40 mM imidazole, the fusion protein was eluted with 250 mM imidazole. The eluted protein was dialyzed against buffer B (50 mM sodium phosphate, 150 mM NaCl, pH 7.5) at 4°C.

2.4. Mass Spectrometry

The specific bands corresponding to BmGlcNAcase2 were excised manually from the gel with a sterile scalpel and digested with trypsin according to Li's [10] method. The digested samples were analyzed by an ultraflex MALDI-TOF-TOF (BRUKER, GERMANY). Peptide mass fingerprinting (PMF) was performed by comparing the masses of peptides to NCBI protein database using the MASCOT search engine (<http://www.matrixscience.com>).

2.5. Antibody Production and Western Blot Analysis

The antibody was prepared by standard techniques [11]. Briefly, purified BmGlcNAcase2 protein (about 2 mg) was injected subcutaneously to immunize New Zealand white rabbits in complete Freund's adjuvant, followed by two booster injections in incomplete Freund's adjuvant within a gap of 2 weeks before exsanguinations. Then, the polyclonal rabbit antibody against 6 × His-BmGlcNAcase2 was obtained and used for immunoassay.

After the SDS-PAGE (Bio-Rad Mini-Protean II, Hercules, CA) was finished, the proteins were transferred to a PVDF membrane with a Bio-Rad liquid transfer apparatus for Western blot. The rabbit anti-BmIDGF polyclonal antibodies (1:1,000 dilution) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (1:2,000 dilution) were used, and signals were detected by diaminobenzidine (DAB) (Sigma, USA). To detect if this protein expressed specially in tissues, total protein of each tissues was added to the lines of the

SDS-PAGE, and the added protein concentration of each tissues are 50 ug.

2.6. Immunofluorescence Microscopy

BmN cells seeded onto coverslips were washed with PBS, and fixed with 2 ml of 4% paraformaldehyde for 15 min. Then cells were washed three times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 15 min. After washing three times with PBS, cells were incubated with anti-BmGlcNAcase2 antibody (1:1000) as primary antibody, fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG antibody as secondary antibody (1:3000) (Qualex, Inc), and nuclei were stained with DAPI (Roche), then examined with a confocal laser scanning microscope (Zeiss lsm 5 live).

3. RESULTS AND DISCUSSION

3.1. Cloning of the BmGlcNAcase2 Gene

The recombinant BmGlcNAcase2 protein was expressed in *E. coli* BL21 (DE3) harboring the expression vector pET-30a(+)-BmGlcNAcase2. The BmGlcNAcase 2 proteins formed inclusion bodies when host *E. coli* was cultivated at 37°C and induced with 1 mM IPTG. The expression of 6 His-tagged Bm122 was also confirmed by anti-6xHis monoclonal antibody (**Figure 1(b)**). The soluble BmGlcNAcase2 was purified by a Ni-NTA column (**Figure 1(a)**, lane 3).

3.2. Mass Spectrometry

To determine whether the amino acid sequence of recombinant BmGlcNAcase2 matches the one predicted from DNA sequencing results, the MALDI-TOF-TOF mass spectra of tryptic digest of recombinant BmGlcNAcase2 was characterized to identify the recombinant protein. 17 peptide fragments were identified. By comparing the masses of indentified peptides to the hypothetical tryptic peptides for proteins in non-redundant NCBI database using the MASCOT search engine, BmGlcNAcase2 was obviously identified with MOWSE score of 86. The identified 17 peptide fragments matched against the deduced amino acid sequence of BmGlcNAcase2 accounted for 41% peptide mass fingerprint sequence coverage (**Figure 2**).

3.3. Cellular Localization of BmGlcNAcase2 in BmN Cells

Confocal laser scanning fluorescence microscopy was utilized to determine the cellular localization of BmGlcNAcase2 protein in BmN cells. The result showed that the BmGlcNAcase2 protein was primarily located in the cytoplasm and was scarcely detectable in the nucleus, while no obvious fluorescence signal was observed in cells stained with pre-immune serum of rabbit as pri-

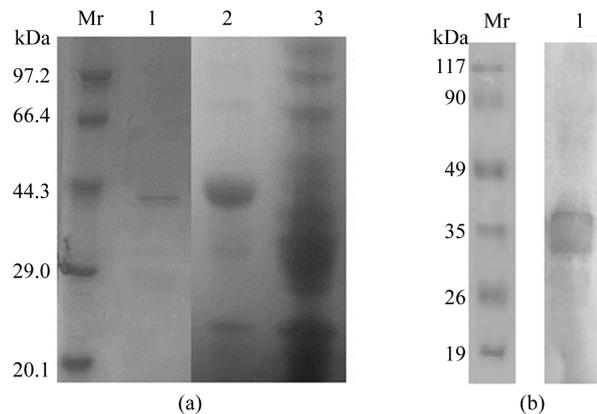


Figure 1. SDS-PAGE stained with coomassie blue and western blotting analysis of recombinant BmGlcNAcase2. (a) Lane1: recombinant BmGlcNAcase2 purified by Ni-NTA resin column; lane2: lysate of BL21 (DE3) harboring pET-30a(+)-BmGlcNAcase2 induced with 0.5 mM IPTG; lane3: lysate of host cell transformed with empty vector. (b) Lane1: western blotting analysis of recombinant BmGlcNAcase2 with anti-6His antibody.

mary antibody (**Figure 3**).

3.4. Western Blot Analysis

Antibody against BmGlcNAcase2 protein was used to perform western blot analysis *Bombyx mori*. The result showed that BmGlcNAcase2 was expressed in hemolymph, ovary, testis, fat body, trachea, midgut and silk gland of fifth instar larvae, and there was no obvious difference in expression of these tissues(**Figure 4**).

4. DISCUSSION

β -N-acetylglucosaminidase of *Bombyx mori* plays an important role in several physiological process, the enzyme is essential to hydrolyze chitooligosaccharides to their constitutive monomer and contribute to recycling GlcNAc pools for remodeling of the exoskeleton during metamorphosis, as indicated previously [12,13]. It was reported recently that *beta-N-acetylglucosaminidase* activity in BmNPV resistant strains NB and the near-isogenic line BC₉ was significantly higher than that in the BmNPV susceptible strain 306 [9] so it is interesting to find out the relationship between this enzyme and silkworm NPV disease.

In this report, we have successfully cloned, optimized the expression and purified β -N-acetylglucosaminidase of *Bombyx mori* in the *E. coli* strains BL21 (DE3) and we showed that the expression of BmGlcNAcase2 can be detected from almost all tissues(hemolymph, ovary, testis, fat body, trachea, midgut and silk gland) of the fifth instar larvae. Identifying the subcellular localization of protein is particularly helpful in the functional annotation of gene products. The result showed that BmGlcN-

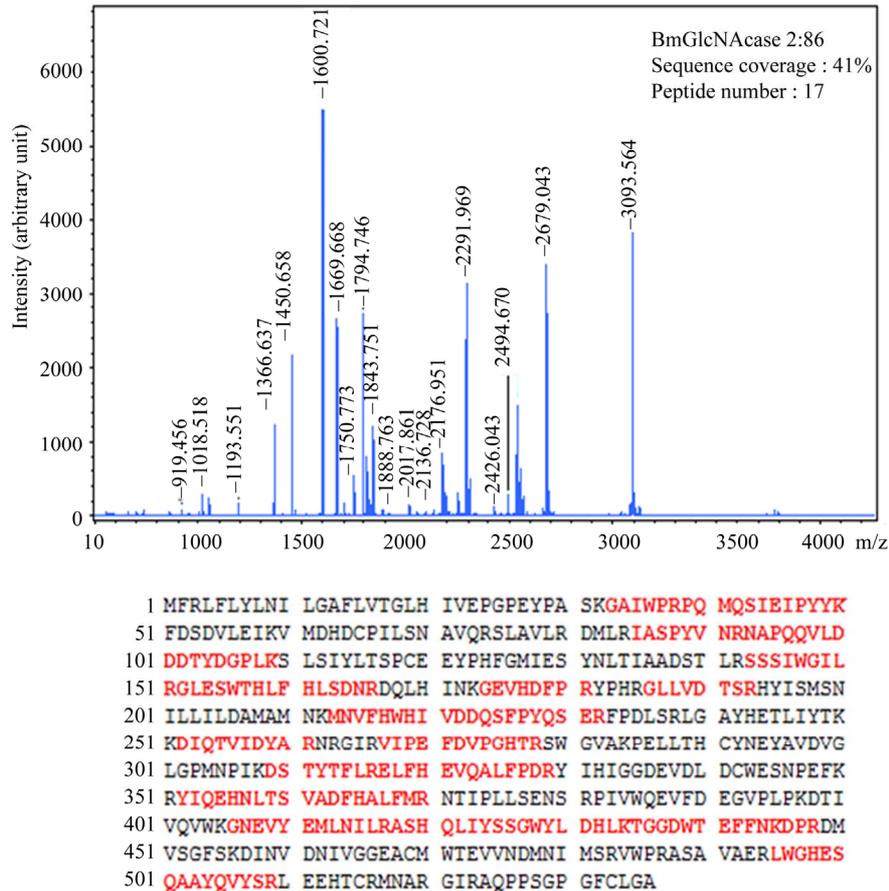


Figure 2. MALDI spectra of tryptic digest of recombinant BmGlcNAcase2. The identified protein, score, amino acid sequence coverage and the number of identified peptides are shown. Matched peptide sequences are showed in red.

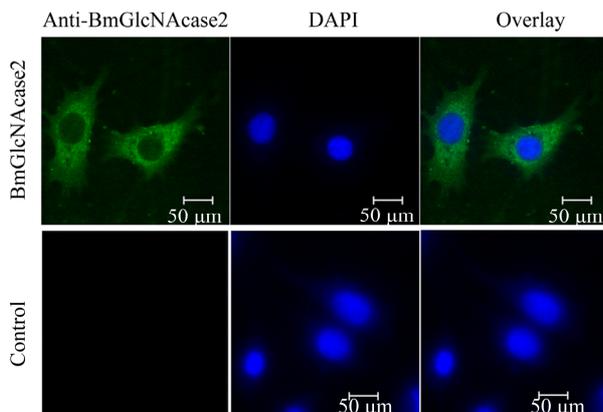


Figure 3. Subcellular localization of BmGlcNAcase2 in BmN cells treated with anti-BmGlcNAcase2 antibody, followed by treatment with FITC-conjugated goat anti-rabbit IgG, and examined in confocal laser microscope. From left to right: green fluorescence for BmGlcNAcase2, DAPI and the overlay images. For the control, pre-immune serum was used as the primary antibody. Nuclei were stained with DAPI (blue). Samples were observed under a confocal laser scanning microscope.

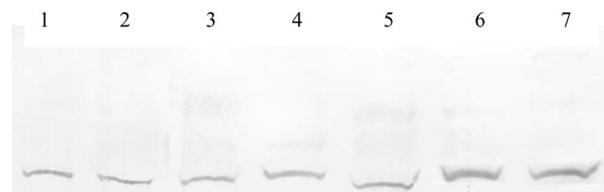


Figure 4. Expression profile of BmGlcNAcase2 in different tissues of *Bombyx mori*. Lanes 1 to 7 represented fat body, Malpighian tubule, midgut, ovary, hemocytes, testis, and silk gland.

Acase2 was a protein distributed mostly in cytoplasm firstly, which is consistent with prediction by computer tool of PLOC (<http://www.genome.jp/SIT/plocdir/>), whereas the precise biochemical function and the possible role of BmGlcNAcase2 in this process remain to be determined.

5. ACKNOWLEDGEMENTS

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