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Biochemical and Biophysical Research Communications 329 (2005) 331–336
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N-glycosylation is necessary for enzymatic activity of a beetle (*Apriona germari*) cellulase

Ya Dong Wei [a,1], Seong Jin Lee [a,1], Kwang Sik Lee [a], Zhong Zheng Gui [a], Hyung Joo Yoon [b], Iksoo Kim [b], Yeon Ho Je [c], Xijie Guo [d], Hung Dae Sohn [a], Byung Rae Jin [a,*]

[a] *Department of Applied Biotechnology, College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Republic of Korea*
[b] *Department of Agricultural Biology, National Institute of Agricultural Science and Technology, Suwon 441-100, Republic of Korea*
[c] *School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Republic of Korea*
[d] *Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, China*

Received 17 January 2005

Abstract

We previously reported that the β-1,4-endoglucanase (EGase) belonging to glycoside hydrolase family 45 cloned from the mulberry longicorn beetle, *Apriona germari* (Ag-EGase I), is composed of 237 amino acid residues and has a potential N-glycosylation site at 97–100 amino acid residues (NSTF). We here describe the N-glycosylation and its role for enzymatic activity of the Ag-EGase I. The N-glycosylation of Ag-EGase I was revealed by the treatment of tunicamycin to the recombinant virus-infected insect Sf9 cells and by endoglycosidase F to the purified recombinant Ag-EGase I, demonstrating that the carbohydrate moieties are not necessary for secretion but essential for Ag-EGase I enzyme activity. To further elucidate the functional role of the N-glycosylation in Ag-EGase I, we have assayed the cellulase enzyme activity in Thr99Gln mutant. Lack of N-glycosylation in Ag-EGase I showed no substantial enzyme activity. This result demonstrates that N-glycosylation at site 97–100 amino acid residues (NSTF) is essential for enzyme activity.

Keywords: *Apriona germari*; Baculovirus; Beetle; Cellulase; N-glycosylation; Endoglucanase; Enzyme; Insect cells

Hydrolysis of cellulose is enzymatically completed through the synergistic action of three types of enzymes such as endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and cellobiases (EC 3.2.1.21) [1,2]. Along with an extensive interest in cellulose as a major source for renewable energy and raw materials, the cellulolytic enzyme also has been subjected to investigation.

The cellulose is a major food source for many insect species, especially for xylohagous and/or phytophagous insects, and the presence of cellulolytic enzyme may be advantageous for insects to increase available energy/nutriments obtained from food sources. Recent works on the origin of the cellulases showed that the enzymes are produced from the insect itself [3–7], from symbiotic organisms harboring in the insect gut [8], or both [3,8,9].

Although the enzymatic activities of the endogenous cellulase in digestive enzymes have previously been reported without isolating the corresponding gene in some xylophagous insects [10–12], biochemical characterization of cellulase of insect origin is difficult due to the low amounts of enzyme detected in their native tissues. Gene cloning of cellulase of insect origin and its expression in a gene expression system constitute a major advance in obtaining high amounts of recombinant proteins. In insects, cellulolytic enzyme genes from three

* Corresponding author. Fax: +82 51 200 7594.
E-mail address: brjin@daunet.donga.ac.kr (B.R. Jin).
[1] These authors contributed equally to this paper.

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.01.131

beetles [6,7,13] and a few termites [3–5] have been isolated. Of three cellulase genes of beetle origin, a cellulase gene was cloned from the mulberry longicorn beetle, *Apriona germari*, in our research team [7]. We have first reported the functional expression of cellulase of insect origin in baculovirus-infected insect cells. The recombinant *A. germari* cellulase was purified and characterized with respect to enzymatic properties such as activity and stability [7]. Insect cells are capable of performing many of the processes required for the formation of active recombinant proteins. Therefore, the gene expression of insect origin in baculovirus-infected insect cells has a major advantage in producing homogeneous functional proteins as like in vivo system.

Insect cells have post-translational events such as glycosylation [14–17], proteolytic processing [18], phosphorylation [19,20], etc. Of these post-translational events, glycosylation is especially important for the biological activity and stability of synthesized proteins [21–24]. Even if these processes exhibit several differences between mammalian and insect cells, many biologically active recombinant glycoproteins have been produced from insect cells [17,25–28].

The role of glycosylation in enzyme activity of the fungal cellulase has been investigated. Because most of the fungal cellulases are glycoproteins the modification of the native protein by glycoprotein has been reported to play an important role in synthesis, secretion, and stability of cellulases [29]. A study on fungal cellulase has yielded contrasting result that N-glycosylation had no effect on enzymatic activity [30]. However, the role of glycosylation in enzyme activity of the cellulase of insect origin has not been reported yet.

The beetle *A. germari* cellulase has one potential N-glycosylation site at 97–100 amino acid residues (NSTF) [7]. In the beetle cellulase of *Phaedon cochleariae*, three potential N-glycosylation sites are found in the sequences [6], and the potential N-glycosylation site in *A. germari* cellulase matches, at least at one site (Asn97-Ser98-Thr99), to that of *P. cochleariae* cellulase. In the present study, the N-glycosylation and its role for enzymatic activity in *A. germari* cellulase, β-1,4-endoglucanase (Ag-EGase I), were evaluated. The role of N-glycosylation in Ag-EGase I was examined using the N-glycosylation inhibitor, tunicamycin, and endoglycosidase F that cleaves N-linked glycans. In addition, the potential N-glycosylation site of Ag-EGase I was mutated to determine the role of N-glycosylation in cellulase enzyme activity. We conclude that N-glycosylation in Ag-EGase I is essential for enzymatic activity.

Materials and methods

Cell culture and virus. Insect Sf9 cells [31] were maintained at 27 °C in TC100 medium (Gibco-BRL Life Technologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum (FBS; Gibco-BRL Life Technologies) as described by standard methods [32]. Wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant AcNPV expressing Ag-EGase I (AcNPV-AgEGase I) were prepared as described in our previous study [7]. The virus was propagated in Sf9 cells and the titer was expressed as plaque forming units (PFU) per ml [32].

Enzyme and antibody. The purified recombinant Ag-EGase I and polyclonal antiserum against recombinant Ag-EGase I were prepared as described in our previous study [7].

SDS–polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. Insect Sf9 cells were mock-infected or infected with the recombinant AcNPV in a 35-mm diameter dish (1×10^6 cells) at a multiplicity of infection (MOI) of 5 PFU per cell. After incubation at 27 °C, cells were harvested at 1, 2, and 3 days post-inoculation (p.i.). For SDS–PAGE [33] of cell lysates, uninfected Sf9 cells and cells infected with virus were washed twice with PBS, mixed with protein sample buffer, and boiled. For SDS–PAGE of culture supernatant, the supernatant from cells infected with recombinant AcNPV was concentrated 10-fold using a membrane (Amicon) with a 10 kDa cut-off. The total cellular lysates and concentrated culture supernatants were subjected to 10% SDS–PAGE. For Western blot analysis, 10% SDS–PAGE was performed as described above. Proteins of cellular lysates and culture supernatant were blotted to nitrocellulose membrane (Sigma, 0.45 μm of pore size) [34]. The blotting was performed in transfer buffer (25 mM Tris and 192 mM glycine in 20% methanol) at 30 V overnight at 4 °C. After blotting, the membrane was blocked by incubation in 1% BSA solution for 2 h at room temperature. The blocked membrane was incubated with antiserum solution (1:1000 v/v) for 1 h at room temperature and washed in TBST (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, and 0.05% Tween 20). Subsequently, the membrane was incubated with goat anti-mouse IgG horseradish peroxidase conjugate (1:10,000 v/v, Sigma) for 30 min at room temperature. After repeated washing, the immunoreactive bands were visualized with ECL Western Blotting Detection System (Amersham Pharmacia Biotech.).

Determination of enzyme activity. Enzyme activity was determined by incubating the samples in acetate buffer (0.05 M, pH 6.0) containing 1%(w/v) carboxymethyl cellulose (CMC; Sigma) for 1 h at 50 °C and assaying the reducing sugars released for dinitrosalicylic (DNS) reagent method [7,35]. One unit of the enzyme was defined as the activity producing 1 μmol of reducing sugars in glucose equivalents per minute. In addition, CMC plate assay for Ag-EGase I activity was treated on the CMC plate [1%(w/v) CMC, 1.5%(w/v) agarose, and 50 mM acetate buffer (pH 5.0)] at 27 °C for 10 h, stained with 0.1% Congo red (Sigma), and the activity was measured by yellow halo zone [7,36].

Tunicamycin treatment. The addition of N-linked carbohydrate by infected insect cells was verified by culture in the presence of tunicamycin (5 μg/ml, Sigma) to prevent the addition of N-linked carbohydrate [14,15]. Sf9 cells were infected with the recombinant virus AcNPV-AgEGase I in a 35-mm diameter dish (1×10^6 cells) and incubated for 2 h at 27 °C. The supernatants were replaced with 5 ml of supplemented TC100 medium containing 5 μg tunicamycin per ml medium. After incubation 27 °C, total cellular lysates and culture supernatants were harvested from infected cells at 1, 2, and 3 days p.i. Total cellular lysates were subjected to 10% SDS–PAGE and Western blot analysis. Culture supernatants were analyzed by Western blot analysis and assayed for enzyme activity.

Endoglycosidase F treatment. The purified recombinant Ag-EGase I was treated with endoglycosidase F (Sigma) under favorable condition for endoglycosidase F activity (0.05 U/μg recombinant Ag-EGase I, 200 mM NaAc, pH 5.0, and 20 mM EDTA). After incubation for 24 h at 37 °C, the endoglycosidase-treated and untreated samples were prepared for electrophoresis by addition of sample buffer and analyzed

on 10% SDS–PAGE. Enzyme activity for endoglycosidase-treated and untreated samples was determined.

Site-directed mutagenesis. Site-directed mutagenesis was used to generate the mutated protein based on PCR method [37]. The consensus sequence of N-glycosylation, Asn-X-Ser/Thr, was mutated by creating codons for Gln (CAA) instead of Thr (ACA). Mutant primers were as follows (underline indicating the mutated site): forward primer for mutant (Thr99Gln): 5′-GTTAACTCCCAATTCGCTCTTGGA-3′; reverse primer for mutant: 5′-CTGCAGTTAATAATTGCATCC AGTAAT-3′. The DNA fragment was amplified, one by the paired primers, forward primer and reverse primer, which wild-type cDNA of pBacPAK9-AgCell [7] was used as template. After a 35-cycle amplification (94 °C for 30 s; 48 °C for 40 s; and 72 °C for 2 min), the PCR products were purified with AccuPrep PCR Purification Kit (Bioneer, Korea). The purified PCR products were digested with *Hinc*II and *Pst*I, and inserted into pBacPAK9-AgCell digested previously with the same enzymes to yield pBacPAK-AgCell^{-N}. The mutant cDNA was sequenced in both directions on an automated sequencer (Model 310 Genetic Analyzer; Perkin–Elmer Applied Biosystems, Foster City, CA). The recombinant AcNPV expressing mutated Ag-EGase I (AcNPV-AgEGase I^{-N}) was constructed as described in our previous study [7].

Results and discussion

N-glycosylation of Ag-EGase I

We have previously cloned and expressed a novel β-1,4-endoglucanase cDNA belonging to GHF 45 from the mulberry longicorn beetle, *A. germari* [7]. The Ag-EGase I is composed of 237 amino acid residues and has a potential N-glycosylation site at 97–100 amino acid residues (NSTF). In order to assess whether the expected addition of an N-linked carbohydrate moiety in the potential N-glycosylation site at 97–100 amino acid residues (NSTF) is being accomplished or not, recombinant baculovirus (AcNPV-AgEGase I)-infected cells [7] were incubated with tunicamycin, which is a specific inhibitor against the addition of N-linked oligosaccharides, and the total cellular lysates were subjected to SDS–PAGE (Fig. 1A) and Western blot analysis (Fig. 1B), following culture supernatants were subjected to Western blot analysis (Fig. 2A) and enzyme activity assay (Fig. 2B).

Fig. 1 shows an apparent shift in the molecular weight of the recombinant Ag-EGase I in tunicamycin-treated Sf9 cells (see lanes 4, 5, and 6). The results of SDS–PAGE and Western blot analysis indicated the presence of 29 kDa band and 27 kDa band corresponding to the N-glycosylated and non-glycosylated recombinant Ag-EGase I, respectively.

The effect of tunicamycin on the secretion of the recombinant Ag-EGase I into the culture medium was determined by Western blot analysis (Fig. 2A). The result showed that the recombinant carbohydrate-deficient Ag-EGase I was secreted into the culture supernatant, but the level of which was relatively low in the Sf9 cells treated with tunicamycin than those untreated ones. It

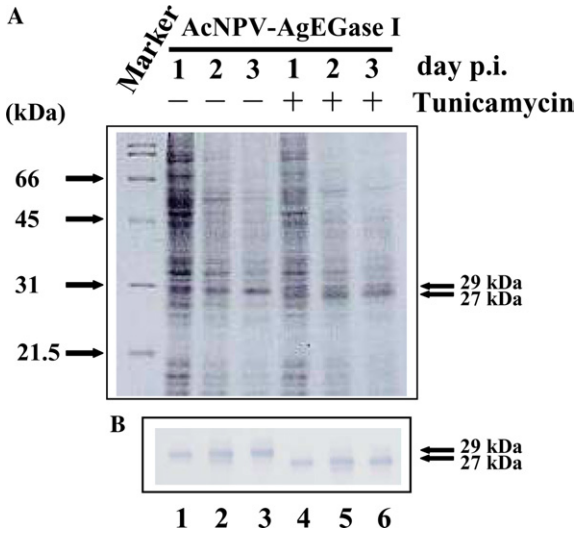

Fig. 1. N-glycosylation of the Ag-EGase I in baculovirus-infected insect cells. Sf9 cells were infected with recombinant AcNPV (AcNPV-AgEGase I) at an MOI of 5 PFU per cell. Cells were cultured without (lanes 1, 2, and 3) or with (lanes 4, 5, and 6) tunicamycin (5 μg/ml). Total cellular lysates were collected at 1 (lanes 1 and 4), 2 (lanes 2 and 5), and 3 (lanes 3 and 6) days p.i., and then subjected to 10% SDS–PAGE (A) and Western blot analysis (B). The N-glycosylated (29 kDa) and non-glycosylated (27 kDa) Ag-EGase I polypeptides are, respectively, indicated by arrows.

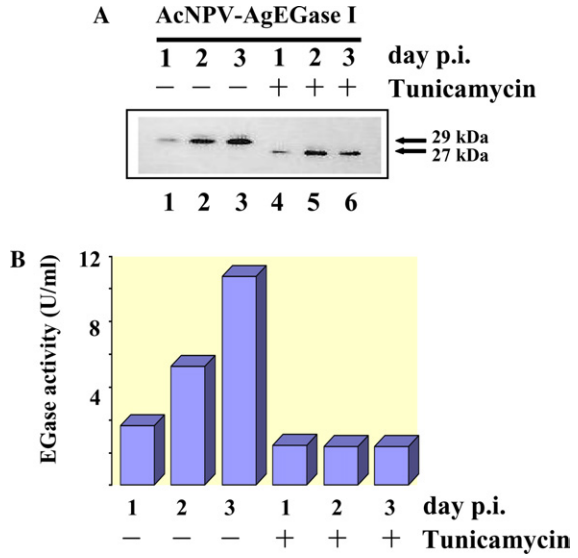

Fig. 2. Secretion and enzyme activity of the N-glycosylated and non-glycosylated Ag-EGase I. (A) Western blot analysis of the N-glycosylated and non-glycosylated Ag-EGase I secreted into medium. The recombinant AcNPV-infected Sf9 cells were cultured without (lanes 1, 2, and 3) or with (lanes 4, 5, and 6) tunicamycin (5 μg/ml). Culture supernatants were collected at 1 (lanes 1 and 4), 2 (lanes 2 and 5), and 3 (lanes 3 and 6) days p.i., 10-fold concentrated, and then subjected to 10% SDS–PAGE and Western blot analysis. The N-glycosylated (29 kDa) and non-glycosylated (27 kDa) Ag-EGase I polypeptides are indicated by arrows. (B) Enzyme activity assay of the glycosylated and non-glycosylated Ag-EGase I. The recombinant AcNPV-infected cells were cultured without (–) or with (+) tunicamycin (5 μg/ml). Culture supernatants were collected at 1, 2, and 3 days p.i., and then assayed for Ag-EGase I activity by DNS reagent method.

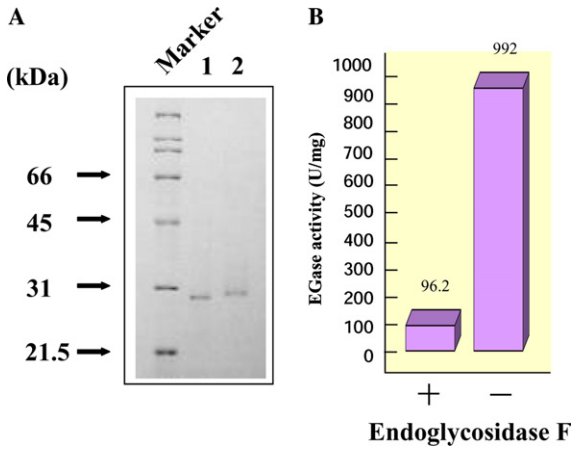

Fig. 3. Deglycosylation of the purified recombinant Ag-EGase I. (A) Deglycosylation of the purified recombinant Ag-EGase I by endoglycosidase F treatment. The purified recombinant Ag-EGase I was treated (lane 1) or untreated (lane 2) with endoglycosidase F and the samples were analyzed by 10% SDS–PAGE. (B) Enzyme activity assay of the glycosylated and deglycosylated recombinant Ag-EGase I. The purified recombinant Ag-EGase I was treated (+) or untreated (−) with endoglycosidase F and then assayed for Ag-EGase I activity by DNS reagent method.

may be due to the effect of the major N-glycosylated viral protein gp64, a structural protein of the membrane of the budded virus, on tunicamycin treatment [14,15]. It is notable in this regard that the carbohydrate moieties are not necessary for Ag-EGase I secretion.

The significance of N-glycosylation was further tested by measuring the enzymatic activity (Fig. 2B). The N-glycosylated recombinant Ag-EGase I showed the enzyme activity pattern corresponding to the culture time-dependent increase, whereas the non-glycosylated recombinant Ag-EGase I did not, indicating an essential role of the carbohydrate moieties for Ag-EGase I activity.

N-glycosylation of the purified recombinant Ag-EGase I was also demonstrated by treatment of endoglycosidase F, which cleaves at N-linked glycans. Fig. 3A shows an apparent decrease of the molecular weight in the purified recombinant Ag-EGase I (lane 1) after endoglycosidase F treatment, indicating that Ag-EGase I is apparently N-glycosylated.

For the additional evidence of the effect of N-glycosylation in EGase I activity, the purified recombinant Ag-EGase I [7] was deglycosylated by endoglycosidase F and assayed for enzymatic activity (Fig. 3B). The result showed that the enzyme activity of the deglycosylated recombinant Ag-EGase I is dramatically low compared to the N-glycosylated recombinant Ag-EGase I. The weak enzyme activity in the deglycosylated recombinant Ag-EGase I may have possibly stemmed from contamination of the non-deglycosylated recombinant Ag-EGase I.

Mutation of N-glycosylation site of Ag-EGase I

To further elucidate the functional role of the N-glycosylation site, 97–100 (NSTF), in Ag-EGase I, one mutant, Thr99Gln, was constructed by site-directed mutagenesis assay (Fig. 4A), and then the mutated cDNA was expressed in baculovirus-mediated insect cells. The SDS–PAGE analysis (Fig. 4B) showed that the mutant (Thr99Gln) was expressed as the mutated Ag-EGase I, which shows an apparent decrease of the molecular weight.

The effect of N-glycosylation on the secretion of the mutated Ag-EGase I into the culture medium was determined by Western blot analysis (Fig. 4C). It can be pointed out that the mutated Ag-EGase I was still secreted into culture medium, although the secretion of mutant was slightly downregulated as compared with wild-type, implying that the N-glycosylation site, 97–100 (NSTF), was important, albeit not essential, for the secretion.

To evaluate the effect of the N-glycosylation on the cellulase enzyme activity, furthermore, the mutated Ag-EGase I expressed by mutant (Thr99Glu) was assayed (Fig. 4D). The enzyme assay revealed that, like tunicamycin and endoglycosidase F treatments, the mutated Ag-EGase I showed no substantial enzyme activity. This result demonstrates that N-glycosylation at site 97–100 amino acid residues (NSTF) is necessary for enzyme activity.

It is estimated that 90% proteins with the sequence Asn-X-Ser/Thr are glycosylated [38]. Generally, the N-linked glycoproteins could facilitate protein folding, protect proteins from proteolysis, and guide protein trafficking; meanwhile, the glycans of secreted proteins are important for secretion and biological activity [39–44]. A human secreted glycoprotein, hPAP21, showed that N-glycosylation is required for efficient secretion [45]. Lack of N-glycosylation in Ag-EGase I showed no substantial enzyme activity, demonstrating that N-glycosylation is necessary for enzyme activity of Ag-EGase I. The role of N-glycosylation sites and their glycosylation has been studied in several fungal cellulases, and it seems that the role varies from protein to protein. As for other glycoproteins it has been shown that N-linked glycans are required for enzymatic activity of fungal cellulases [29]. However, in other fungal cellulase N-glycosylation had no effect on enzymatic activity [30,46].

In conclusion, we found that the N-glycosylation in EGase, at least in the beetle *A. germeri*, of insect origin is essential for enzymatic activity. We are now interested in the functional role of carbohydrate moieties in an EGase of insect origin, which may help to further our understanding of insect cellulase.

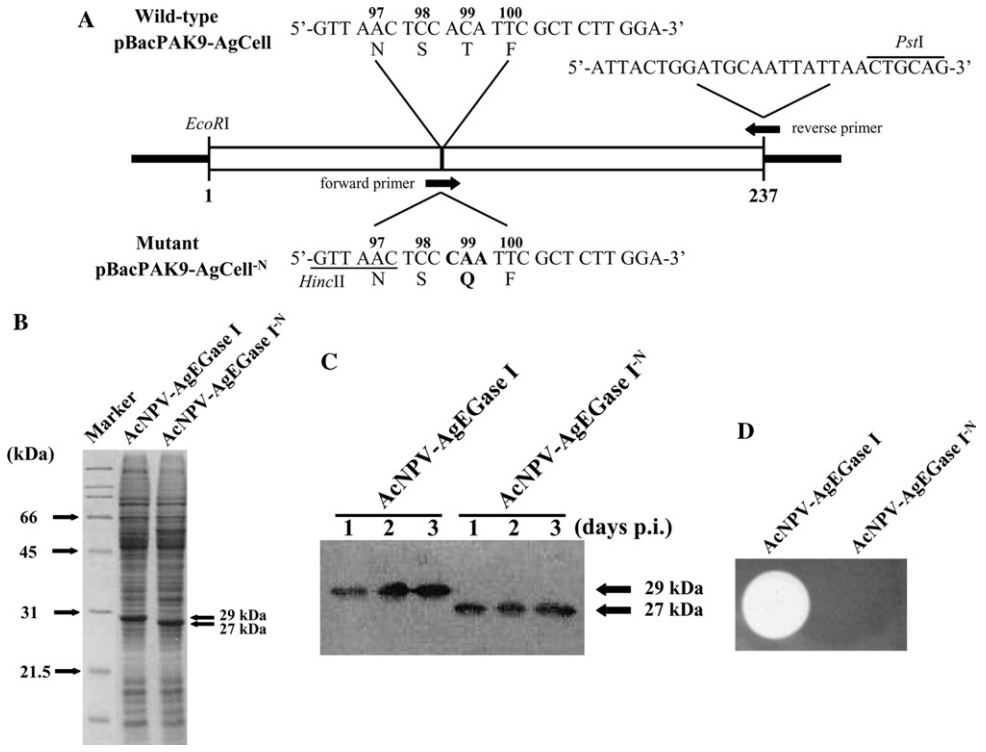

Fig. 4. Mutation of N-glycosylation site of Ag-EGase I. (A) Schematic structure of wild-type Ag-EGase I and its mutant. The consensus sequence of N-glycosylation, Asn-X-Ser/Thr, was mutated by creating codons for Gln (CAA) instead of Thr (ACA). The numbers in schematic structure indicate the position of amino acid residues in Ag-EGase I. (B) SDS–PAGE analysis of wild-type Ag-EGase I and mutated Ag-EGase I. Sf9 cells were infected with recombinant AcNPVs expressing wild-type Ag-EGase I (AcNPV-AgEGase I) or mutated Ag-EGase I (AcNPV-AgEGase I^{-N}) at an MOI of 5 PFU per cell. Total cellular lysates were collected at 3 days p.i. and then subjected to 10% SDS–PAGE. The wild-type (29 kDa) and mutated (27 kDa) Ag-EGase I polypeptides are indicated by arrows. (C) Western blot analysis of the wild-type and mutated Ag-EGase I secreted into medium. Sf9 cells were infected with recombinant AcNPVs expressing wild-type Ag-EGase I (AcNPV-AgEGase I) or mutated Ag-EGase I (AcNPV-AgEGase I^{-N}) at an MOI of 5 PFU per cell. Culture supernatants were collected at 1 (lanes 1 and 4), 2 (lanes 2 and 5), and 3 (lanes 3 and 6) days p.i., 10-fold concentrated, and then subjected to 10% SDS–PAGE and Western blot analysis. The wild-type (29 kDa) and mutated (27 kDa) Ag-EGase I polypeptides are indicated by arrows. (D) Enzyme activity assay of the wild-type and mutated Ag-EGase I. Sf9 cells were infected with recombinant AcNPVs expressing wild-type Ag-EGase I (AcNPV-AgEGase I) or mutated Ag-EGase I (AcNPV-AgEGase I^{-N}) at an MOI of 5 PFU per cell. Culture supernatants were collected at 3 days p.i., 10-fold concentrated, and then assayed for Ag-EGase I activity by yellow halo zone.

Acknowledgments

This paper was supported by Dong-A University Research Fund. We thank Dr. Hirofumi Watanabe (National Institute of Agrobiological Sciences, Tsukuba 305-8634, Japan) for critical reading of the manuscript.

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