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N-linked glycosylation of a beetle (*Apriona germari*) cellulase Ag-EGase II is necessary for enzymatic activity

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Abstract

We previously reported that the β-1,4-endoglucanase (EGase) belonging to glycoside hydrolase family (GHF) 45 of the mulberry longicorn beetle, *Apriona germari* (Ag-EGase II), has three potential N-linked glycosylation sites; these sites are located at amino acid residues 56–59 (NKSG), 99–102 (NSTF), and 237–239 (NYSstop). In the present study, we analyze the functional role of these potential N-linked glycosylation sites. Tunicamycin treatment completely abolished the enzymatic activity of Ag-EGase II. To further elucidate the functional role of the N-linked glycosylation sites in Ag-EGase II, we have assayed the cellulase enzyme activity in Ser58Gln, Thr101Gln, or Ser239Gln mutants. Lack of N-linked glycosylation site at residues 99–102 (NSTF), the site of which is conserved in known beetle GHF 45 cellulases, showed loss of enzyme activity and reduced the molecular mass of the enzyme. In contrast, mutations in Ser58Gln or Ser239Gln affected neither the activity nor the apparent molecular mass of the enzyme, indicating that these sites did not lead to N-linked glycosylation. The present study demonstrates that N-linked glycosylation at residues 99–102 (NSTF), while not essential for secretion, is required for Ag-EGase II enzyme activity.

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

1. Introduction

Cellulose, the most abundant carbohydrate polymer on earth, is composed of repeating glucose units linked by β-1,4-glucosidic bonds and is mainly produced by terrestrial plants. Cellulose is used as a food source by a wide variety of organisms that possess endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and cellobiases (EC 3.2.1.21); together, these enzymes act in synergy to facilitate the complete cleavage of the cellulose β-1,4-glucosidic bonds (Henrissat et al., 1985; Beguin and Aubert, 1994). Cellulose is a major food source for many insect species, in particular, for the xylohagous and/or phytophagous insects. The presence of cellulolytic enzymes in insects may be advantageous to increase available energy/nutriments obtained from food sources. Recent studies on the origin of the cellulases have demonstrated that these enzymes may be produced from insect itself (Watanabe et al., 1998; Girard and Jouanin, 1999; Tokuda et al., 1999; Nakashima et al., 2002; Lee et al., 2004), from a symbiotic organism harbored in the insect gut (Ohtoko et al., 2000), or a combination of both (Breznak and Brune, 1994; Watanabe et al., 1998; Ohtoko et al., 2000).

In insects, cellulolytic enzyme genes have been isolated from three beetles (Girard and Jouanin, 1999; Sugimura et al., 2003; Lee et al., 2004) and a few termites (Watanabe et al., 1998; Tokuda et al., 1999; Nakashima et al., 2002). Known beetle cellulases belong to two glycosyl hydrolase families (GHFs):

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GHF 5 (*Psacothea hilaris*) and GHF 45 (*Phaedon cochleariae* and *Apriona germari*). Of the GHF 45 cellulase genes of beetle origin, two cellulase genes, Ag-EGase I (Lee et al., 2004) and Ag-EGase II (Lee et al., 2005), were cloned from the mulberry longicorn beetle, *A. germari*, and one cellulase gene from the phytophagous beetle, *P. cochleariae* (Girard and Jouanin, 1999). In addition, all the GHF 45 beetle cellulases have potential N-linked glycosylation sites. Ag-EGase I has one potential N-linked glycosylation site, located at Asn 97; this site is required for cellulase enzyme activity (Lee et al., 2005; Wei et al., 2005). This was the first report on the role of N-linked glycosylation in enzyme activity of an insect cellulase. Ag-EGase II (Wei et al., 2005) and *P. cochleariae* cellulase (Girard and Jouanin, 1999) have three potential N-linked glycosylation sites, respectively. Both Ag-EGase II and *P. cochleariae* cellulase contain two additional potential N-linked glycosylation sites rather than one site as in Ag-EGase I.

The role of N-linked glycosylation sites and their glycosylation has been studied in several cellulase enzymes and it appears that their roles vary from protein to protein. As for other cellulases, several fungal cellulases have been shown to require N-linked glycols for enzymatic activity (Bisaria and Mishra, 1989). However, not every fungal cellulase requires this modification (Wang and Gao, 2000). The role of the potential N-linked glycosylation site in cellulases of insect origin has only been evaluated in the beetle cellulase, Ag-EGase I (Wei et al., 2005).

In the present study, Ag-EGase II cDNA was expressed in baculovirus-infected insect cells. The role of N-linked glycosylation in Ag-EGase II was examined using the N-linked glycosylation inhibitor, tunicamycin and a series of mutants that lack the potential N-linked glycosylation sites. We show that Ag-EGase II is N-linked glycosylated at residues 99-102 (NSTF), and that N-linked glycosylation of Ag-EGase II is necessary for cellulase enzyme activity.

2. Materials and methods

2.1. Cell culture and virus

Insect Sf9 cells (Vaughn et al., 1977) 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 (O'Reilly et al., 1992). Wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant AcNPV expressing Ag-EGase II (AcNPV-AgEGase II) were prepared as previously described (Lee et al., 2005). The virus was propagated in Sf9 cells and the titer was expressed as plaque forming units (PFU) per ml (O'Reilly et al., 1992).

2.2. 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 (Laemmli, 1970) of cell lysates, uninfected Sf9 cells and cells infected with virus were washed twice with PBS and 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 pore size) (Towbin et al., 1979). 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 a 1% BSA solution for 2 h at room temperature. The blocked membrane was incubated with polyclonal antiserum against recombinant Ag-EGase II (1:1000 v/v) (Lee et al., 2005) at room temperature for 1 h and washed in TBST (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 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.).

2.3. Determination of enzyme activity

Enzyme activity was assayed by incubating the samples in acetate buffer (0.05 M, pH 6.0) containing 1% (w/v) carboxymethyl cellulose (CMC; Sigma) at 50 °C for 1 h and determined by the reducing sugars released for dinitrosalicylic (DNS) reagent method (Miller, 1959; Lee et al., 2005). One enzyme unit was defined as the activity producing 1 μmol of reducing sugars in glucose equivalents per minute. In addition, CMC plate assay for Ag-EGase II activity was treated on the CMC plate [1% (w/v) CMC, 1.5% (w/v) agarose, 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 (Beguin, 1983; Lee et al., 2005).

2.4. 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 (Hasemann and Capra, 1990; Jin et al., 1995). Sf9 cells were infected with the recombinant virus AcNPV-AgEGase II in a 35-mm diameter dish (1×10^6 cells) and incubated at 27 °C for 2 h. The supernatants were replaced with 5 ml of supplemented TC100 medium containing 5 μg tunicamycin per ml medium. After incubation at 27 °C, total