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N-glycan sialylation in a silkworm-baculovirus expression system

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A silkworm-baculovirus system is particularly effective for producing recombinant proteins, including glycoproteins. However, *N*-glycan structures in silkworm differ from those in mammals. Glycoproteins in silkworm are secreted as pauci-mannose type *N*-glycans without sialic acid or galactose residues. Sialic acid on *N*-glycans plays important roles in protein functions. Therefore, we developed pathways for galactosylation and sialylation in silkworm. Sialylated *N*-glycans on proteins were successfully produced in silkworm by co-expressing galactosyltransferase and sialyltransferase and providing an external supply of a sialylation-related substrate. α2,3/α2,6 Sialylation to *N*-glycans was controlled by changing the type of sialyltransferase expressed in silkworm. Furthermore, the co-expression of *N*-acetylglucosaminyltransferase II facilitated the formation of additional di-sialylated *N*-glycan structures. Our results provide new information on the control of N-glycosylation in silkworm.

[**Key words:** Baculovirus; Expression; *N*-glycan; Sialylation; Silkworm]

A silkworm-baculovirus protein expression system is suitable for producing a variety of soluble proteins with high efficiency. In particular, mammalian glycoproteins such as cytokines, membrane receptors, and enzymes have been expressed in silkworms (1–4).

However, *N*-glycan structures in silkworm differ from those in mammals. *N*-glycans are classified into high-mannose, complex, and hybrid types. Many serum proteins in humans and other mammals contain sialylated complex type (biantennary) *N*-glycans with *N*-acetylglucosamine (GlcNAc), galactose, and sialic acid (*N*-acetylneuraminic acid; Neu5Ac) added to the core mannose structure (5). In contrast, recombinant proteins produced in silkworm mainly have added pauci-mannose type *N*-glycans (6). Sialyl complex type *N*-glycans play important roles in many activities, including the stability (7–9), serum half-life, and organ specificity (10) of glycoproteins. Sialylated *N*-glycans in mammalian serum proteins have different linkage types (α2,3 and α2,6) and numbers of sialic acid molecules (mono-, di-, and tri-sialyl). Although the functional differences between the α2,3 and α2,6 sialyl linkage types are not fully understood, some reports suggest that there is an association with stability and receptor recognition (11,12). The functional differences resulting from the number of sialic acids are also unclear.

In mammalian expression systems, because they have inherent heterogeneous sialyl *N*-glycan structures, the strict control of the linkage types and number of sialic acid is difficult.

In silkworm, there has been success with the co-expression of mammalian *N*-acetylglucosaminyltransferase (GnT) II and β1,4-galactosyltransferase (β1,4GalT) and digalactosyl complex *N*-glycosylation (13). However, sialylation and the strict control of the linkage types and number of sialic acid are not yet possible.

It is not clear whether there is an essential enzyme activity or substrate for sialylation in the silkworm fat body, which is the main location for protein expression by baculovirus. There is an endogenous α2,6-sialyltransferase (α2,6SialT) in silkworm (14); however, the role and location of this enzyme are unclear. Furthermore, it is also not known whether CMP-sialic acid is present to act as a sugar donor in silkworm fat body.

We report the first successful approach to sialylation in silkworm using a simple method of supplying the sialic acid substrate from outside the silkworm. Our results show that the linkage and number of sialic acids can be controlled. Furthermore, our results contribute information to expand the understanding of the essential elements required for sialylation in silkworm.

MATERIALS AND METHODS

Cell line and silkworm strain BmN cells (15) were maintained at 27 °C in TC-100 medium containing 10% fetal bovine serum. Kinsyu-Showa silkworms were reared at 25 °C on an artificial diet (Sysmex, Kobe, Japan). Only fifth-instar larvae were used in experiments.

Preparation of baculoviruses containing introduced genes Each cDNA listed in Table 1 was amplified by PCR using the primer set shown in Table 1 and KOD Plus (Toyobo, Osaka, Japan). Each PCR reaction introduced a suitable restriction enzyme site (see Table 1) into the transfer vector for the production of the recombinant baculovirus (Sysmex; pM/pV series). Some genes had a tag sequence

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TABLE 1. Genes and primer sets for the baculovirus (BV).

Virus	Gene	Accession no.	Forward primer	Reverse primer	Purification tag
N-glycan related enzymes					
BV_hCSS	*Homo sapiens* CMP-*N*-acetylneuraminic acid synthase	NM_018686	(Kpn I) 5′-ggggtaccatggactcggtggagaaggggggc-3′	(Xba I) 5′-gctctagactattttggcatgaattattaac-3′	Dock tag (C-terminus)
BV_α2,6-hSialT	*Homo sapiens* ST6 β-galactosamide α2,6-sialyltransferase 1 (α2,6-hSialT)	NM_173216	(Sac I) 5′-catcgagctcatgattcacaccaacctgaag-3′	(Xba I) 5′-ctgtctagagcagtgaatggtccggaagccag-3′	Dock tag (C-terminus)
BV_α2,3-hSialT	Human ST3 beta-galactoside alpha-2,3-sialyltransferase 3 (α2,3-hSialT)	NM_174971	(Bgl II) 5′-ccaagatcttatgggactcttggtatttgtg-3′	(EcoRV) 5′-gggatatctcagatgccactgcttagatcag-3′	–
BV_β1,4-mGalT3	*Mus musculus* UDP-Gal: βGlcNAc β1,4-galactosyltransferase III (β1,4-mGalT3)	NM_020579	(EcoRV) 5′-gaggatatcatgttgcggaggctgctggag-3′	(Xba I) 5′-gactctagattagtgtgaggccacgggagctg-3′	–
BV_hGnT2	*Homo sapiens* α1,6-mannosyl-glycoprotein 2-β-*N*-acetylglucosaminyltransferase (hGnT2)	NM_002408	(Bgl II) 5′-ggagatctatgaggttccgcatctacaaacg-3′	(Xba I) 5′-gggatatcctgcagtcttctataactttacag-3′	His-tag (C-terminus)
Target glycoproteins					
BV_hIL18BP	*Homo sapiens* interleukin 18 binding protein (hIL18BP)	NM_001039659	(EcoRV) 5′-gatggtaccatgagacacaactggacacc-3′	(Xba I) 5′-ctggatatcacctgctgctgtggactg-3′	Strep II-tag (C-terminus)
BV_bIAP	Bovine intestine alkaline phosphatase type II (bIAP)	–	(Kpn I) 5′-tgcaaggcctcaatcctat-3′	(EcoRV) 5′-ggtctagaatcagacaagcctgcagggg-3′	Dock tag (C-terminus)

at the C-terminus (Strep-tag, FLAG-tag, His-tag, Dock tag (16); see Table 1). Restriction enzyme sites (5′Kpn I, 3′-Xba I) were added to the terminus of the bIAP (Bovine intestine alkaline phosphatase type II) gene (17), which was codon-optimized for silkworm and synthesized by Eurofins Genomics (Ebersberg, Germany). The sequence of the introduced gene in the expression vector was confirmed using a sequencer (ABI 3130 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). The ABv baculovirus (CPd strain; (18,19)) was used as the baculovirus strain, and the linearized viral DNA and constructed transfer vector plasmids were co-transfected into BmN cells and subjected to mono-cloning of the recombinant virus by limiting dilution (20).

Protein expression following baculovirus inoculation Fifth-instar silkworm larvae were inoculated with 50 μl baculovirus solution and the solution for co-infection was a mixture of various baculoviruses (each baculovirus at a final titer of 10^4–10^7 pfu) (18). Hemolymph was sampled at 6 d post-infection and ultracentrifuged at 100,000 ×*g* for 1 h. Supernatants were stored at −80 °C.

Administration of sialic acids For injection administration, the substrates sialic acid (NeuAc) and CMP-sialic acid (CMP-NeuAc) (both Nacalai Tesque, Kyoto, Japan) were diluted in Milli-Q water and filtered (0.45 μm) before administration percutaneously to fifth-instar silkworm larvae using a syringe (injection amount; 0.05–5 mg/50 μl). For oral administration, the substrate was added to an artificial diet fed to the larvae (0.1–1 g substrate/10 g diet fed).

Purification of proteins The Strep-tag fusion protein from the Strep-Tactin system (IBA GmbH, Goettingen, Germany) was purified with elution using elution buffer (20 mM Tris–HCl, 2.5 mM desthiobiotin, 150 mM NaCl, pH 7.4). The FLAG-tag fusion protein was bound to a M2 affinity gel (Sigma–Aldrich) and purified. All procedures were performed following the manufacturers' instructions. Elution buffer (20 mM Tris–HCl, 100 μg/ml FLAG peptide, 150 mM NaCl, pH 7.4) was used for elution. The Dock-tag fusion protein was purified as described previously (16). The target protein was bound to an affinity gel in buffer (1 mM $CaCl_2$, 25 mM Tris–HCl, 250 mM NaCl, pH 8.0) and eluted with elution buffer (25 mM Tris–HCl, 250 mM NaCl, 5 mM ethylene glycol tetraacetic acid (EGTA), pH 8.0).

Lectin blot (SNA, MAL, and RCA-120) Lectin blots were performed on the purified proteins prepared as described above. Protein concentrations were determined by absorbance at 280 nm in a Nanodrop spectrophotometer (ND-1000; Thermo Fisher Scientific, Wilmington, USA). *Sambucus nigra* agglutinin (SNA, J-Oil Mills, Tokyo, Japan), *Maackia amurensis* lectin (MAL, J-Oil mills), and *Ricinus communis* Agglutinin (RCA)-120 (Vector Laboratories, Burlingame, CA, USA) lectins were HRP-labeled using a HRP-NH_2 labeling kit (Dojindo Laboratories, Kumamoto, Japan). After quantification of the purified proteins, 1 μg of each protein was subjected to SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes using the semi-dry method. After blocking, the membranes were washed in TBS-T, treated with 5 μg/ml HRP-labeled lectin/1% BSA TBS-T, washed with TBS-T (0.05% Tween 20, 150 mM NaCl, 20 mM Tris–HCl, pH 7.4), and visualized using an ECL imaging system (GE Healthcare, Madison, WI, USA).

Enzyme activity assay *N*-Acetylglucosaminyltransferase I (GnT1) and II (GnT2) activities were assayed. Silkworm larvae on day 1 of the fifth-instar were inoculated with BV_hGnT2 or left uninoculated (normal silkworms) and reared at 25 °C. Fat bodies were excised from 5 silkworm larvae on day 6 of the fifth-instar, placed in 1 ml of homogenization buffer (250 mM sucrose, 5 mM imidazole-HCl pH 7.3), and homogenized using a pellet homogenizer. The cells were then disrupted using an ultrasonic crushing device (Advanced Digital Sonifier model 250DA; Branson Ultrasonics, Danbury, CT, USA) for sonication (intensity 20%, 20 s). The lysate was centrifuged at 7000 ×*g* using a high-speed centrifuge for 10 min. The supernatant was ultracentrifuged for 1 h at 105,000 ×*g*, and the precipitate was collected. Then, 10 μl of precipitate was added to 90 μl of buffer (250 mM sucrose, 5 mM imidazole-HCl pH 7.3, and 2% Triton X-100), and the mixture was dispersed by pipetting and solubilized on ice for 1 h. The mixture was then ultracentrifuged again at 105,000 ×*g* for 60 min, and the supernatant (microsomal fraction) was collected. To 6.5 μl of microsomal fraction, 93.5 μl of enzyme assay solution (25 mM HEPES, 1 mM $MnCl_2$, 200 nM PA-sugar chain, 5 mM 2-acetamidio-1,2-didoxynojirimycin 0.4% TritonX-100, complete protease inhibitor EDTA-free (Roche, Basel, Switzerland), 0.2 mM UDP-GlcNAc, pH 7.4) was added. GnT1 activity was measured using the 017 PA-sugar chain (Takara Bio, Kusatsu, Japan), and GnT2 activity was measured using the 100.2 PA-sugar chain (Glyence, Nagoya, Japan). As negative controls, buffers that lacked UDP-GlcNAc were used. Each enzyme reaction solution was incubated at 30 °C for 17 h and boiled at 98 °C for 3 min. The supernatant was collected after centrifugation at 12,000 ×*g* for 10 min. Supernatant components were separated using an HPLC system (LaChrome Elite, Hitachi High-Tec, Tokyo, Japan) with a Cosmosil 5C18-AR-II column (6 mm/250 mm) (Nacalai Tesque) and detected as described previously (21).

MALDI-TOF-MS and LC–MS analyses Affinity-purified proteins were used for *N*-glycan analysis. Glycopeptidase F (Takara Bio) and BlotGlyco reagent (Sumitomo Bakelite, Tokyo, Japan) were used to digest, label, and purify the *N*-glycan from purified proteins using the BlotGlyco method (22). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and liquid chromatography–mass spectrometry (LC–MS) of the labeled glycan sample, Identification of the MS peak and estimation of the peak area were conducted by

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