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Expression of Glycosylated Mucin-like Domain Using Baculovirus Expression System in Silkworm, *Bombyx mori*

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Baculovirus expression systems (BES) are widely used for recombinant protein production in lepidopteran cells or larvae. It is known that insect cells have a glycosylation pathway similar to that of mammalian cells. Thus, BES are possible to be used to produce recombinant proteins with higher eukaryotic modifications. In insect cells, the *N*-glycosylation pathway has been well-researched. However, the *O*-glycosylation pathway is poorly understood. In this study, we expressed a partial recombinant HsPRG4 (rPRG4) as a reporter for *O*-glycosylation and investigated whether BES in silkworm could produce the proteins with mucin-like clustered *O*-glycans. Analysis by SDS-PAGE and glycosidase digestion indicated that rPRG4 had Core 1 and Core 2 *O*-glycan chains. These results suggested that BES could produce the protein containing mucin-type *O*-glycosylation in *Bombyx mori*.

Key words: baculovirus expression system, *Bombyx mori*, *O*-glycosylation

INTRODUCTION

Baculovirus expression systems (BES) are valuable and popular tools for the production of recombinant proteins. Because insect cells have a posttranslational modifications similar to those of mammalian cells, such as glycosylation, phosphorylation, and protein folding, BES are used to produce proteins from higher eukaryotes, for example, canine interferons- α , human serine-threonine kinase 11, and human acidic and basic fibroblast growth factors (Liu *et al.*, 2005; Martinez *et al.*, 2005; Gouveia *et al.*, 2007). As regards the glycosylation in insect cells, the *N*-glycosylation pathway has been studied with a view to production of human-like *N*-glycosylated proteins, but there are few reports of *O*-glycosylation. In this study, to investigate whether the mucin-type *O*-glycosylation occurs in silkworm, *Bombyx mori*, we performed recombinant protein expression of a mucin-like protein as a target using BES in silkworm.

Mucins are heavily *O*-glycosylated glycoproteins found in secreted mucous and as transmembrane glycoproteins of the cell surface to serve as a last protective barrier against extracellular environmental factors like low pH or hydrolytic enzymes (Strouss *et al.*, 1992). Mucins have serine and threonine rich domains called variable number of tandem repeat (VNTR) regions. Mucins

may have hundreds of *O*-GalNAc glycans attached to serine or threonine residues in the VNTR regions and these abundant *O*-glycan chains may comprise 80% of the molecule by weight (Lan *et al.*, 1987). Four main *O*-glycan core structures are well known in mammals (Daniel, 2009). The most common *O*-GalNAc glycan is Gal β 1–3GalNAc α –Ser/Thr (Fig. 1). It is termed a Core 1 or T-antigen and forms many longer, more complex structures. Another common structure is Core 2, contains a branching N-acetylglucosamine attached to core 1 (Fig. 1). Core 1 and Core 2 *O*-GalNAc glycans are found in both glycoproteins and mucins from a variety of cells and tissues. Core 3 and Core 4 structures (Fig. 1) are found in a few tissues, such as colon (Podolsky, 1985), bronchi (Lamblin, 1984) and salivary glands (Wieruszkes *et al.*, 1987). In insects, Core 1 type mucin has been observed (E Tian *et al.*, 2008), but other type mucins are yet to be detected.

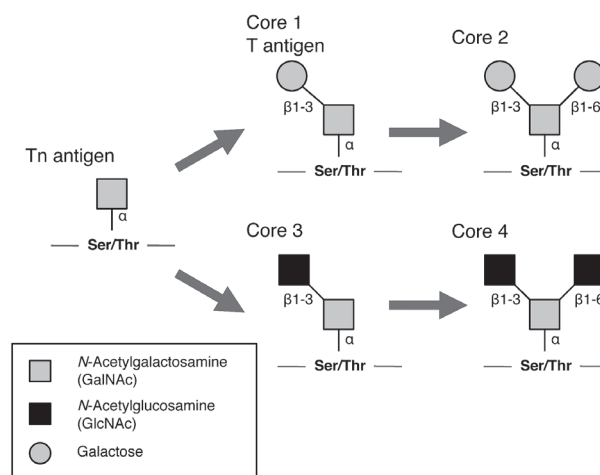


Fig. 1. Biosynthesis of *O*-GalNAc glycans core structures.

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To assess whether BES in silkworm was capable of producing the proteins with mucin-like clustered O-glycans, we constructed the reporter for O-glycosylation, which contains the portion of mucin-like repeat domain of PRG4 (proteoglycan 4). PRG4, also known as luricin, has been identified as megakaryocyte stimulating factor and articular cartilage superficial zone protein (Ikegawa *et al.*, 2000). PRG4 contains a large, central, mucin-like repeat domain supporting attachment of O-glycan chains (Jay GD, 2004). PRG4 has mainly Core 1 type O-glycans and low amounts of Core 2 type O-glycans (Estrella RP *et al.*, 2010). In this report, we show that BES can produce mucin glycoproteins.

MATERIALS AND METHODS

Cells and silkworm strain

The cell line PS140 (a laboratory stock of Dr. Imanishi, National Institute of Agrobiological Sciences) and the d17 silkworm strain (Kawakami *et al.*, 2008) used in this study were provided by the Institute of Genetic Resources, Graduate School of Agriculture, Kyushu University, Japan. The cell line was maintained in IPL-41 medium (Invitrogen, CA, USA) with 10% fetal bovine serum. The cells were grown at 27°C. Before cell transfection, the medium was replaced by COSMEDIUM 009 (CosmoBio Co, Tokyo, Japan).

Construction of the Gateway entry clone

To obtain the partial sequence of HsPRG4, we performed PCR with human liver cDNA template. The PCR reaction was carried out with two primers, PRG4-5NcoI (5'-GGGCCATGGCATGGAAAACACTTCCCATT-3'), PRG4-3XhoI (5'-CCCCTCGAGGGCTTCTTGGTGGTGGTGGT-3'). The 50- μ L PCR reaction contained 5 μ L of 10 \times KOD buffer, 5 μ L of 2 mM dNTPs, 1.5 μ L of each primer (10 μ M), 3 μ L of MgSO₄ (25 mM), 1 μ L of the template, and 1 μ L of KOD-Plus-Neo DNA polymerase (1.0 U/ μ L; Toyobo, Osaka, Japan). The amplification profile consisted of a heat denaturation as follows: 94°C for 2 min, and then 35 cycles at 94°C for 15 sec, 59°C for 30 sec, and 68°C for 1 min, and then 68°C for 5 min. The PCR product was digested with NcoI and XhoI was cloned into the NcoI / XhoI site of pENTR11 (Invitrogen). pENTR11 was modified to contain polyhistidine (8 \times His) tag. To confirm the inserted sequence, we did sequencing reactions with the ABI BigDye 3.1 Terminator Cycle Sequencing kit (Applied Biosystems, Warrington, UK) with primers, pENTR attL1 (5'-ACTCTTCCTGTTAGT-TAGTTACTTAAGCTC-3'), pENTR attL2 (5'-CAATG-TAACATCAGAGATTTTGAGACAC-3').

Generation of recombinant baculovirus

The DNA fragment of HsPRG4 was transposed to pDEST8 (Invitrogen) by means of Gateway LR clonase reaction (Invitrogen). The obtained pDEST8-PRG4-His8 transfer vector was transformed into *E. coli* BmDH10Bac (Motohashi *et al.*, 2005) and transposed to bacmid DNA mediated by Tn7 transposase (Park *et al.*, 2007). After the purification, the recombinant bacmid

DNA were transfected into the *B. mori* PS140 cells by lipofection method to generate recombinant baculoviruses. Three days after transfection, the culture medium was collected, and the infection was repeated twice for to prepare high-titer virus stock.

Expression and purification of recombinant protein

Larvae on day 3 of the fifth instar were carefully injected into a hemocoel with the recombinant HsPRG4-His8 baculovirus. Four days after infection, larval legs were cut and hemolymph was collected from each larva. The collected hemolymph was diluted with extraction solution (20 mM sodium phosphate, 0.5 M NaCl, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, Complete EDTA-free protease inhibitor cocktail [1 tablet/100 mL] (Roche Applied Science), 20 mM 1-phenyl-2-thiourea (pH 7.0) and centrifuged at 9,500 rpm for 20 min. The supernatant was filtrated through a 0.45- μ m membrane filter (Millipore, MA, USA). The recombinant protein was eluted with a linear solvent gradient (5, 20, 100, and 300 mM imidazole). Each fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the running gel was stained with Coomassie Brilliant Blue R-250.

Endoglycosidase digestions

To cleave O-glycan chains, purified rPRG4 was incubated in 1 \times Glycoprotein Denaturing Buffer (NEB) at 95°C for 10 min. The 20- μ L cleavage reaction contained 2 μ L of 10 \times G7 Reaction Buffer (NEB), 2 μ L of 10% NP40, 2 μ L of Neuraminidase (NEB), and 1 μ L of Endo- α -N-Acetylgalactosaminidase (NEB). The reaction mixture was incubated at 37°C for 4 h. The digested product was analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

Clustered mucin-type O-glycans exist on recombinant PRG4

To construct the partial recombinant PRG4 (rPRG4), we amplified the DNA fragment from a human liver cDNA library with the PRG4-5NcoI and PRG4-3XhoI primers. We had cloned a partial DNA fragment encoding N-terminal signal peptide, somatomedin B-like domains, heparin-binding domain, and a part of mucin-like repeat

Full-length HsPRG4



Recombinant PRG4



Fig. 2. Structure of recombinant PRG4 constructs. Domains indicated are: SP, signal peptide; SMB, somatomedin B-like; HEP, heparin-binding; HPX, hemopexin-like; 8xHis, C-terminal 8-histidine tag.

domain into pENTR11 (Fig. 2). To facilitate the protein purification, 8-histidine tag was added to C-terminus.

The recombinant baculovirus was produced by transfecting the bacmid DNA into PS140 cells, and the amplified P3 virus was injected into the hemocoels of silkworm larvae as described under MATERIALS AND METHODS. The hemolymph containing secreted rPRG4 was collected and purified by means of histidine affinity chromatography. The purified rPRG4 was analyzed by separation on SDS-PAGE (Fig. 3). After coomassie brilliant blue staining, the purified rPRG4 was detected at the fractions containing 20 mM and 100 mM imidazole. Although the molecular weight of rPRG4 without modification, calculated from the amino acid sequence, was to be 32.019 kDa, a smear band was appeared above 55 kDa. This result strongly suggested that clustered *O*-glycan chains exist on mucin-like repeat domain of rPRG4, and

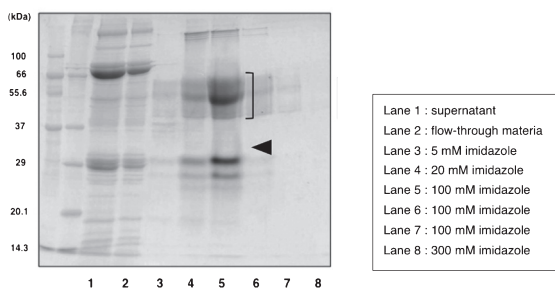


Fig. 3. Purification of rPRG4 detected by SDS-PAGE. Detection of His-tagged rPRG4 fractionated by affinity chromatography: lane 1, supernatant; lane 2, flow-through material; lane 3, eluate at 5 mM imidazole; lane 4, eluate at 20 mM imidazole; lane 5, 6, 7, eluate at 100 mM imidazole; lane 8, eluate at 300 mM imidazole. The black triangle indicates the position of rPRG4 without modification. The bracket indicates the position of *O*-glycosylated rPRG4.

that BES can produce mucin type *O*-glycan in silkworm.

The BES produced rPRG4 contains Core 2 type *O*-glycosylation

In order to confirm the addition of *O*-glycan chains, the purified rPRG4 (100 mM imidazole fraction; Fig. 3, lane 5) was digested with Neuraminidase and Endo- α -*N*-Acetylgalactosaminidase. Neuraminidase catalyzes the hydrolysis of α 2-3, α 2-6, and α 2-8 linked *N*-acetyl-neuraminic acid residues, which must be removed before the cleavage the *O*-glycan chains by Endo- α -*N*-Acetylgalactosaminidase. Endo- α -*N*-Acetylgalactosaminidase, also known as *O*-glycosidase, catalyzes the removal of Core 1 and Core 3 *O*-GalNAc glycan from glycoproteins (Koutsoulis *et al.*, 2008), but can not removes Core 2 *O*-glycan. The resulting product was analyzed by separation on SDS-PAGE. The digestion by the two glycosidases decreased the molecular size of rPRG4 markedly (Fig. 4). This result indicates that the rPRG4 has Core 1 and Core 2 type *O*-GalNAc glycans as well as native PRG4. Taken together, BES can produce the protein containing Core 2 type *O*-glycosylation in *Bombyx mori*. Interestingly, significant amount of deglycosylation-resistant species were

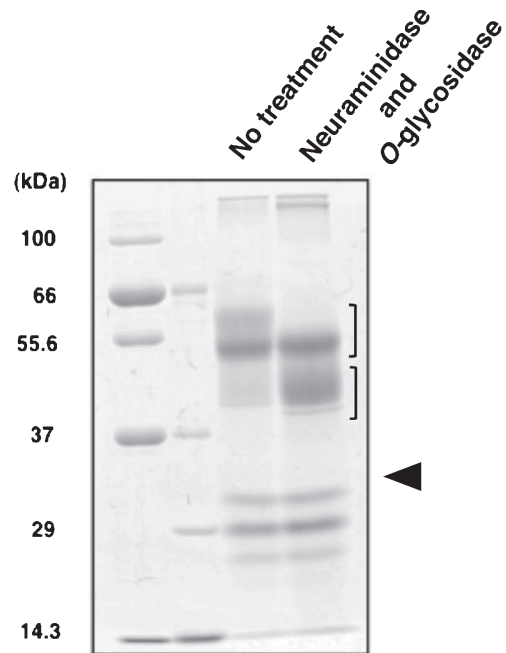


Fig. 4. *O*-Glycosidase digestion detected by SDS-PAGE. The black triangle indicates the position of rPRG4 without modification. Upper bracket indicates the position of *O*-glycosidase-resistant rPRG4. Under bracket indicates the position of deglycosylated rPRG4.

observed, suggesting that the rPRG4 produced using BES has a complicated modification other than Core 1 and Core 3 *O*-GalNAc glycan.

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