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Evaluation of N-glycosylation Property of Cultured *Antheraea pernyi* Cells in Comparison with Four Other Lepidopteran Cell Lines used in Baculovirus Expression Vector Systems

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Abstract N-glycosylation property of an *Antheraea pernyi* cell line, NISES-AnPe-428 (AnPe), used in a newly developed baculovirus expression vector (BEV) system of *A. pernyi* nucleopolyhedrovirus (AnpeNPV), was evaluated by investigating the structural characteristics of N-linked oligosaccharide on the recombinant glycoprotein, prothoracicotropic hormone (PTTH), and compared with those in four different lepidopteran cell lines, *Bombyx mori* (BmN4), *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (High5), and *Spilosoma imparilis* (FRI-Splm-1229; SpIm) used in BEV systems of *B. mori* NPV (BmNPV), *Autographa californica* NPV (AcNPV), and *Hyphantria cunea* NPV (HycuNPV), respectively. PNGaseF digestion and lectin blot analysis revealed that most recombinant PTTHs secreted from each cell line were N-glycosylated and bound with lectins GNA and AAA, which specifically bind to terminal α-linked mannose and α-linked fucose respectively. In contrast, there were no detectable bindings with RCA120, SNA and MAA which specifically bind to terminal β 1,4-linked galactose, α2,6-linked sialic acid and α2,3-linked sialic acid respectively. The results indicated that AnPe as well as the other lepidopteran cell lines efficiently added fucosylated N-glycans with terminal mannose on the PTTH and were incapable or extremely inefficient to form mammalian-like complex N-glycans containing galactose and/or sialic acid residues. In addition, N-glycans added by High5 were, in part, PNGaseF-resistant and reacted with anti-horseradish peroxidase antibody, suggesting that higher efficiency of α1,3-fucosylation in High5 than in other cell lines. PNGaseF digestion and N-terminal amino acid sequencing also revealed that variation in molecular mass of PTTH produced by each cell line was caused by not only heterogeneity of N-glycan structure but also differences in proteolytic processing.

Key words: N-glycosylation, *Antheraea pernyi*, baculovirus, insect cell, prothoracicotropic hormone

Introduction

Bacterial gene expression systems typically provide the high level production of recombinant proteins, but lack eukaryotic protein processing capabilities, and foreign gene products are often deposited as insoluble inclusion bodies. In contrast, mammalian gene expression systems have eukaryotic protein processing capabilities and can produce soluble recombinant proteins with structures similar to their natural counterparts, although there are several drawbacks such as low productivity, long production periods and high costs. Baculovirus expression vector (BEV) systems utilize protein processing capabilities of insect cells, which can perform some mammalian-like post-translational modifications, including glycosylation, and usually show productivity much higher than mammalian

expression systems. Therefore, BEV systems have been frequently used for production of recombinant mammalian glycoproteins since it was established (Smith *et al.*, 1983; Maeda *et al.*, 1985).

There are many examples that both insect and mammalian cells add N-linked oligosaccharides (N-glycans) as well as O-glycans at the same positions of polypeptides. However, differences in their precise structures, especially of N-glycans, have been noticed and extensively analyzed. In general, mammalian cells add complex or hybrid type N-glycans often possessing terminal sialic acids (Kornfeld and Kornfeld, 1985), which plays important roles for intracellular trafficking, biological function and biochemical stability of human glycoproteins (Fast *et al.*, 1993, Nagayama *et al.*, 1998, Janosi *et al.*, 1999), while insect cells add high mannose type or fucosylated paucimannose type N-glycans (Licari *et al.*, 1993, Kubelka *et al.*, 1994, Jarvis *et al.*, 1998, Hooker *et al.*, 1999), indicating that insect and mammalian N-glycosylation pathways are similar during the first several steps from the N-glycan addition occurring in ER to the removal of glucose and mannose residues occurring in ER and Golgi (Structure A to E in Fig. 1), but are different at the later steps as shown in Fig. 1. This generalized model of insect N-glycosylation pathway tells us that it is very difficult to produce recombinant glycoproteins with mammalian -like N-glycans in BEV systems. However, a few examples showed that structures of N-glycans varied among the insect cell lines and/or the type of glycoprotein, and even complex type N-glycans were identified on glycoproteins produced by BEV systems (Davidson *et al.*, 1990). Thus, we may expect the occurrence of insect cell lines possessing mammalian-like N-glycosylation properties and the establishment of novel BEV systems for the production of recombinant glycoprotein whose N-glycans have the same structures with those of human glycoproteins.

Recently, we have established a novel BEV system using the nucleopolyhedrovirus (AnpeNPV) and cell line (NISES-AnPe-428; AnPe) of *Antheraea pernyi* and demonstrated that the protein productivity of this new system is comparable to that of *Autographa californica* NPV (AcNPV)/Sf9 cell system (Huang *et al.*, 2001). In addition, a preliminary study on the N-glycosylation property suggested that AnPe might possess capability to form mammlian-like complex type N-glycans (Kobayashi, 2001). To confirm this possibility, we compared the N-glycosylation property of AnpeNPV/AnPe cell system with those of other BEV systems in this study by analyzing the N-glycan structure of a recombinant glycoprotein, *Bombyx mori* prothoracicotropic hormone (PTTH), by lectin blot analysis and PNGaseF digestion.

Materials and Methods

Bacteria, insect cell lines, plasmids and viral DNA

Competent *Escherichia coli* strain XL-1-Blue cells (Stratagene) were used for plasmid DNA transformations. Five lepidopteran insect cell lines, NISES -AnPe-428 (AnPe), BmN4, IPLB-SF9 (Sf9), BTI-TN5B1-4 (High5) and FRI-Splm-1229 (Splm), were maintained in both TC-100 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma) and Sf-900 II medium (Invitrogen) at 27°C.

The transfer vector plasmids, pApCH1 (Kobayashi *et al.*, 2001), pBM030 (Maeda, 1989), pAcLacZ (Huang *et al.*, 2001) and pHcMU1 (Takenaka *et al.*, 1999), were used for the construction of recombinant NPVs expressing the synthetic prothoracicotropic hormone (PTTH) gene of *Bombyx mori* (O'Reilly *et al.*, 1995) under the control of the polyhedrin promoters as described later. Viral DNA genomes of wild type AnpeNPV A (Wang *et al.*, 2000), *B. mori* nucleopolyhedrovirus (BmNPV) T3 (Maeda *et al.*, 1985) and *Hyphantria cunea* NPV (HycuNPV) A (Takenaka *et al.*, 1999) were prepared as described previously (Huang *et al.*, 2001). BaculoGold linearized DNA (Pharmingen) was used as the AcNPV DNA genome.

DNA manipulations

All plasmid DNA recombination techniques were essentially as described by Sambrook *et al.* (1989). Restriction enzymes and other DNA modifying enzymes were purchased from Takara Shuzo Co. Ltd.

Construction of recombinant NPVs expressing PHH gene

The synthetic *B. mori* PTTH gene cassette (0.5 kbp) was cleaved from the plasmid pPTTHMSig (3.4 kbp) (O'Reilly *et al.*, 1995) by digestion with *Xba*I and *Hin*dIII. After blunting reaction, the 0.5 kbp fragment was ligated with the transfer vectors pApCH1, pBM030 and pHcMU1, all of which were digested with *Sma*I. By these ligation reactions, recombinant transfer

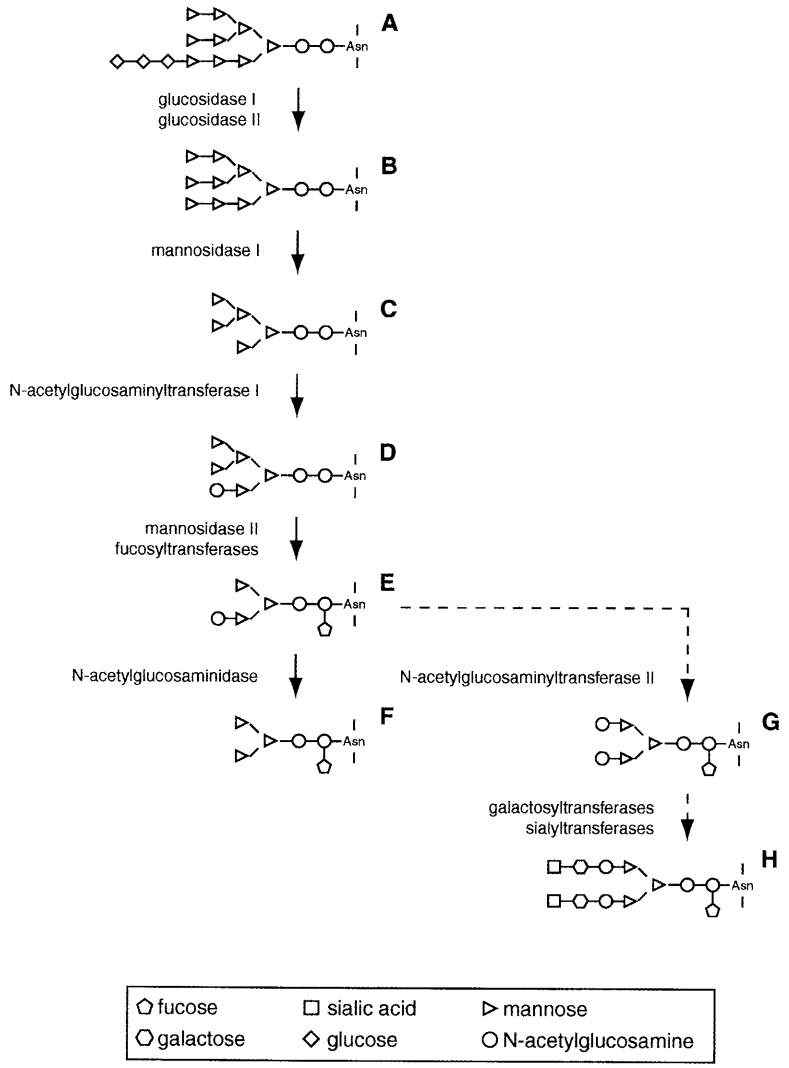
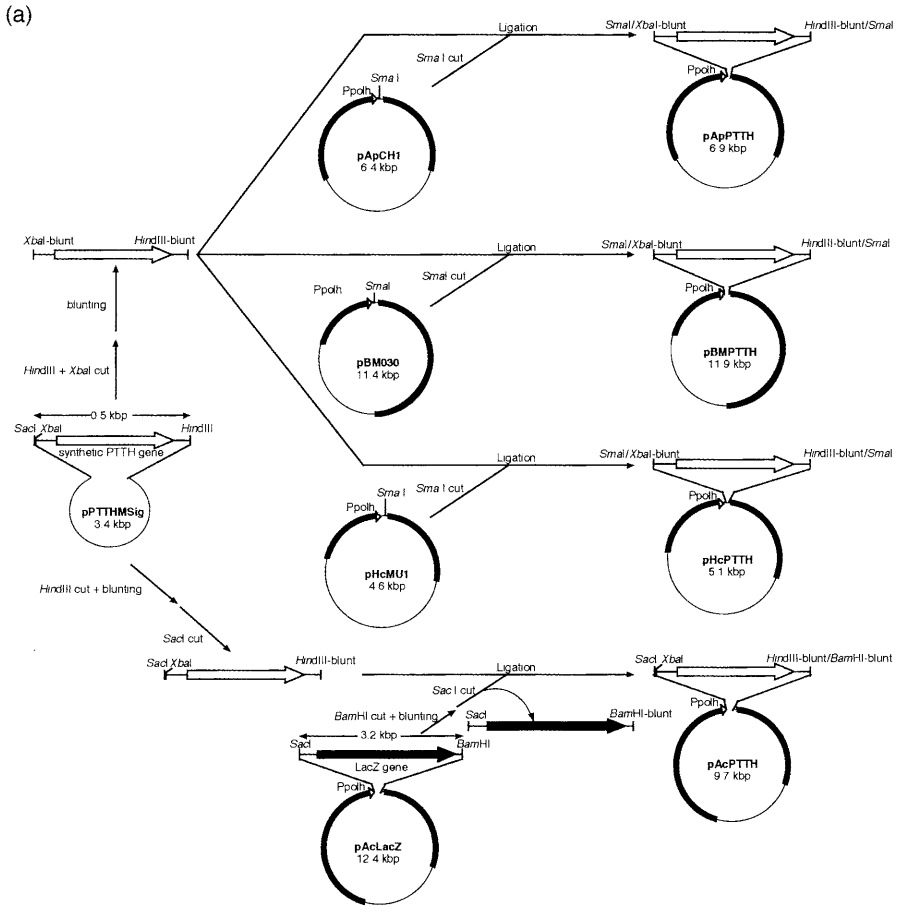


Fig. 1. Generalized N-glycosylation pathways in insect cells. Structure A assembled on the lipid carrier dolichol phosphate was transferred to the Asn residue. Trimming by glucosidases I and II converts A to B, then followed by removal of the outer four mannose residues by mannosidase I to form C. After removal of these mannose residues, N-acetylglucosaminyltransferase I converts C to D which becomes a substrate for mannosidase II and fucosyltransferases, resulting in the formation of E. The pathways from A to E are quite similar between insect and mammalian cells. Then, Golgi-associated N-acetylglucosaminidase and mannosidases convert most parts of E to fucosylated paucimannosidic N-glycans including F. Very few parts of E may be converted to G by N-acetylglucosaminyltransferase II and further extended into mammlian-like complex type N-glycans including H by galactosyltransferases and sialyltransferases.



(b)

Signal sequence of sarcotoxin IA

```
   1 atgaacttccaaaacatattcatattcgtggcgttaatattggcggtgttcgcgggacaa  60
   1  M  N  F  Q  N  I  F  I  F  V  A  L  I  L  A  V  F  A  G  Q   20

  61 tctcaggcggaaacattcaagttgaaaaccaagctattccggatccaccttgcacttgc 120
  21  S  Q  A  G  N  I  Q  V  E  N  Q  A  I  P  D  P  P  C  T  C   40
                   a                 b

 121 aaatacaagaaagaaatagaagacttgggcgaaaactctgttccacgcttcattgaaacc 180
  41  K  Y  K  K  E  I  E  D  L  G  E  N  S  V  P  R  F  I  E  T   60
          N-glycosylation site
 181 agaaactgtaataaaacacaacagccgacttgtcgacccccctacatttgcaaagaaagt 240
  61  R  N  C  N  K  T  Q  Q  P  T  C  R  P  P  Y  I  C  K  E  S   80

 241 ttatacagtataactatttaaaaagaagggaaactaaatcgcaggagtctctcgagata 300
  81  L  Y  S  I  T  I  L  K  R  R  E  T  K  S  Q  E  S  L  E  I  100

 301 ccgaatgaattgaaatatcgatgggtggcggaatctcaccccgtcagcgtggcgtgtttg 360
 101  P  N  E  L  K  Y  R  W  V  A  E  S  H  P  V  S  V  A  C  L  120

 361 tgtacaagagactaccaactacgatataataataattaa 399
 121  C  T  R  D  Y  Q  L  R  Y  N  N  N  *  132
```

Fig. 2. Schematic illustrations of recombinant transfer vector plasmids, pApPTTH, pBMPTTH, pHcPTTH and pAcPTTH, used for the construction of recombinant NPVs expressing synthetic PTTH gene (a) and nucleotide and deduced amino acid sequences of the synthetic PTTH gene (b). The signal sequence of sarcotoxin IA and N-glycosylation site are boxed. N-terminal amino acid sequences, a (G-N-I-Q-V- and ?-N-I-Q-V-) and b (N-Q-A-I-P), of deglycosylated PTTHs are underlined.

vectors, pApPTTH, pBMPTTH and pHcPTTH, were obtained (Fig.2a). Another 0.5 kbp PTTH fragment prepared from pPTTHMSig by *Hin*dIII digestion followed by blunting reaction and further digestion with *Sac*I was ligated with pAcLacZ which was digested with *Bam*HI, blunted and further digested with *Sac*I to remove LacZ gene. The resulting transfer vector was designated as pAcPTTH.

To generate recombinant NPVs expressing the PTTH gene under control of the polyhedrin promoter (Ppolh), each combination of recombinant transfer vector and viral DNA (pApPTTH + AnpeNPV DNA, pBMPTTH + BmNPV DNA, pHcPTTH + HycuNPV and pAcPTTH + Baculo-Gold linearized DNA) was cotransfected into respective host insect cell line (AnPe, BmN4, SpIm and Sf9) by the lipofectin method, and polyhedra-deficient recombinant NPVs (AnpePTTH, BmPTTH, HcPTTH and AcPTTH) contained in culture supernatants of the transfected cells were purified by at least three rounds of plaque assay as described previously (Huang *et al.*, 2001). The insertion of the PTTH gene at the polyhedrin locus in each recombinant virus and the production of PTTH polypeptide in each virus-infected host cells were confirmed by PCR analysis of viral DNA and Western blot analysis of culture supernatants, respectively.

The purified recombinant NPVs were amplified using the respective host cells, and the infected culture supernatants containing virions were harvested and, after measuring virus titers, stored as virus stocks at −80 ℃ until use.

Virus infection and sample preparation

To analyze N-glycosylation pattern of the PTTH produced by each virus-infected insect cell line (AnPe-AnpePTTH, BmN4-BmPTTH, Splm-HcPTTH, Sf9-AcPTTH and High5-AcPTTH), the following virus infection experiment and sample preparation were performed.

AnPe, BmN4, Splm, Sf9 and High5 cells cultured in both TC-100 (+10% FBS) and Sf-900II media were infected with the corresponding recombinant NPVs at multiplicity of infection (MOI) =1 and cultured at 27℃ for 7 to 10 days until when all the cells in culture were lysed. Then the culture supernatants containing PTTH were harvested, filtered through a 0.45μm Millex HA filter (Millipore) and, after adding 10μM (final) of trans-epoxysuccinyl-leucylamido(4-guanidino)-butane (E-64) (Sigma), desalted using Sephadex G-25-prepacked PD-10 column (Amersham Biosciences) and phosphate buffer (PB) (1 mM $Na_2HPO_4 \cdot H_20$, 10.5 mM KH_2PO_4, pH6.2). The desalted samples were applied to the High - Trap SP cation-exchange column (Amersham Bioscience) pre-equilibrated with PB. After washing the column with 0.05 M NaCl in PB, proteins in which PTTH was one of the major components were eluted with 0.08 M NaCl in PB. The eluates were again desalted using the PD-10 column and concentrated to adequate volumes by the conventional centrifugal evaporator. The concentrated eluates were designated as crude PTTH samples and used for analyzing the N-glycans as well as polypeptides as described below.

SDS-PAGE, Western and lectin blot analyses

Crude PTTH samples prepared as above were analyzed by electrophoresis in 15% SDS-polyacrylamide gels (Laemmli, 1970). Gels were stained with Coomassie brilliant blue or used for electrotransfer of proteins to PVDF membranes (BioRad) using Transblot SD Cell (BioRad) according to the manufacturer's instruction.

In Western blot analysis, the PVDF membranes were incubated in phosphate buffered saline (PBS) (20 mM NaH_2PO_4, 20 mM Na_2HPO_4, 150 mM NaCl, pH7.2) containing 0.1% gelatin at room temperature for one night and probed with a 1: 1000 dilution of rabbit anti-PTTH serum raised against purified recombinant PTTH produced by AcPTTH-infected Sf9 cells. Then the membranes were washed with PBS containing 0.05% Triton X-100 (PBST) and incubated with a 1: 5000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Tago) and visualized with ImmunoStain Kit (Konica).

In lectin blot analysis using Digoxigenin Detection Kit (Roche), the PVDF membranes were incubated in blocking solution at 4℃ for one night and probed with a 1:1000 dilution of

digoxigenin-conjugated lectins (Roche), *Galanthus nivalis* agglutinin (GNA; binds terminal α-linked mannose), *Aleuria aurantia* agglutinin (AAA; binds terminal α1,6-linked fucose), *Sambucus nigra* agglutinin (SNA; binds terminal α2,6-linked sialic acid) and *Maackia amurensis* agglutinin (MAA; binds terminal α2,3-linked sialic acid). After washing with Tris buffered saline (TBS) (0.05 M Tris, 0.15 M NaCl, pH7.5), the membranes were incubated with a 1:1000 dilution of alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragments and visualized with staining solution according to the kit manual.

In lectin blot analysis using HRP-conjugated *Ricinus communis* agglutinin (RCA120; binds terminal β 1,4-linked galactose) (Seikagaku Corporation), the PVDF membrane was incubated in blocking solution at 4℃ for one night, probed with a 1: 1000 dilution of HRP-conjugated RCA120 and, after washing with TBS, visualized with ImmunoStain Kit (Konica).

PNGaseF digestion

Flavobacterium meningosepticum peptide: N-glycosidase (PNGaseF) (Takara Shuzo) digestion for removing N-glycans from PTTH was performed essentially according to the manufacturer's instruction. The crude PTTH samples were denatured in 0.5 M Tris-HCl (pH 8.6) containing 0.5 % SDS and 1% 2-mercaptoethanol by boiling at 100 ℃ for 3 min and stabilized by mixing with equal volumes of 5% Nonidet P-40. Then 1 mU of PNGaseF was added to the samples and incubated for 15-20 hr at 37 ℃. After incubation, the digested samples were analyzed by SDS-PAGE, Western and lectin blot analysis. In the Western blot analysis, not only rabbit anti-PTTH serum but also rabbit anti-HRP serum (Biogenesis) was used as primary antibody.

Determination of the N-terminal amino acid sequence of PTTH

Bands of PTTH on the PVDF membranes were visualized by Coomassie brilliant blue stain and cut out for direct sequencing of N-terminal amino acids by a peptide sequencer (ABI, model 473A) as described in Kanaya and Kobayashi (2000).

Results

PTTH production by five lepidopteran insect cell lines

The synthetic PTTH gene inserted in each recombinant NPV encodes a fusion protein consisted of the signal peptide of sarcotoxin IA and the PTTH polypeptide (Fig. 2a) so as to facilitate virus-infected cells secreting mature PTTH of 109 amino acids into the culture medium (O'Reilly *et al.*, 1985). SDS-PACE and Western blot analyses revealed that all of the crude PTTH samples prepared from 5 insect cell lines cultured in both TC-100 (+10% FBS) and Sf-900 Ⅱ media abundantly contained PTTH polypeptides with heterogeneous molecular masses ranging between 13 and 20 kDa (Fig.3).

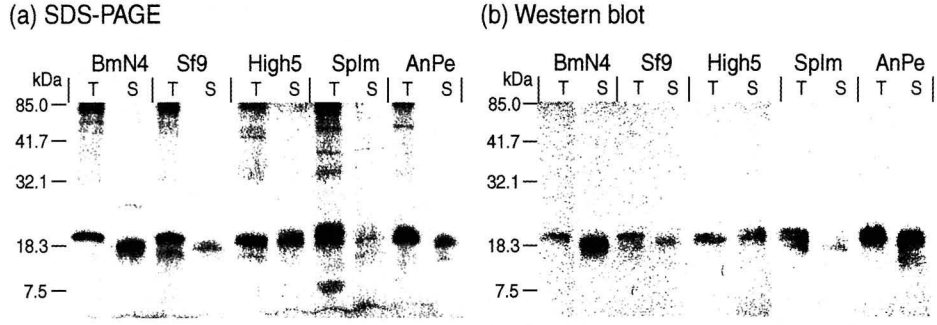


Fig. 3. SDS-PAGE (a) and Western blot (b) analyses of recombinant PTTH produced by each insect cell line (BmN4, Sf9, High5, SpIm and AnPe). T, TC-100 medium (+10% FBS). S, Sf-900 Ⅱ medium. The positions and molecular masses of prestained marker proteins are indicated on the left side of each panel.

The molecular mass distribution of PTTH was slig htly different among cell lines. In addition, for each cell line except High5, the distribution changed with culture media as follows, between 15 and 20 kDa with TC-100 (+ 10% FBS) medium and between 13 and 18 kDa with Sf-900 II medium. In spite of such variations, apparent molecular masses of most PTTHs in each sample were significantly higher than the calculated molecular mass (12,737 Da) of mature PTTH.

N-glycosylation profiles of PTTH polypeptides

Lectin blot analysis using lectins GNA, AAA, RCA120, MAA and SNA revealed that most PTTHs larger than 17 kDa in each sample were bound with GNA and AAA, whereas no PTTHs distributed between 13 and 20 kDa bound with RCA120, MAA and SNA (Fig.4; data of BmN4, High5 and Splm samples for MAA and SNA were not shown). Western blot analysis after removal of N-glycans by PNGaseF digestion showed that the size distribution of PTTH in each sample shifted to smaller sizes (Fig.5) and that PTTHs from cells cultured in TC-100 (+10% FBS) medium were converged into single bands of ca. 15 kDa, while those of Sf-900 II medium formed relatively broader bands ranging between 13 and 15 kDa. Neither lectin GNA nor MA bound with these deglycosylated PTTHs, except PNGaseF-resistant PTTHs of ca. 18 kDa which were detected only in samples from High5 and were bound with not only lectins GNA and AM but also anti-HRP serum (data not shown).

N-terminal amino acid sequence of PTTH

N-terminal amino acid sequences of deglycosylated PTTHs from AnPe and Sf9 were analyzed and compared. PTTHs produced by both AnPe and Sf9 cultured in TC-100 (+10% FBS) medium had the same G-N-I-Q-V- sequence at their N-termini, which consisted with that of mature PTTH obtained by cleavage of PTTH precursor just after the signal peptide of sarcotoxin IA (Fig. 2b). The similar but slight different N-terminal sequence, ?-N-I-Q-V- (?: non identifiable residue), was detected for PTTH from Sf9 in Sf-900 II medium. The completely

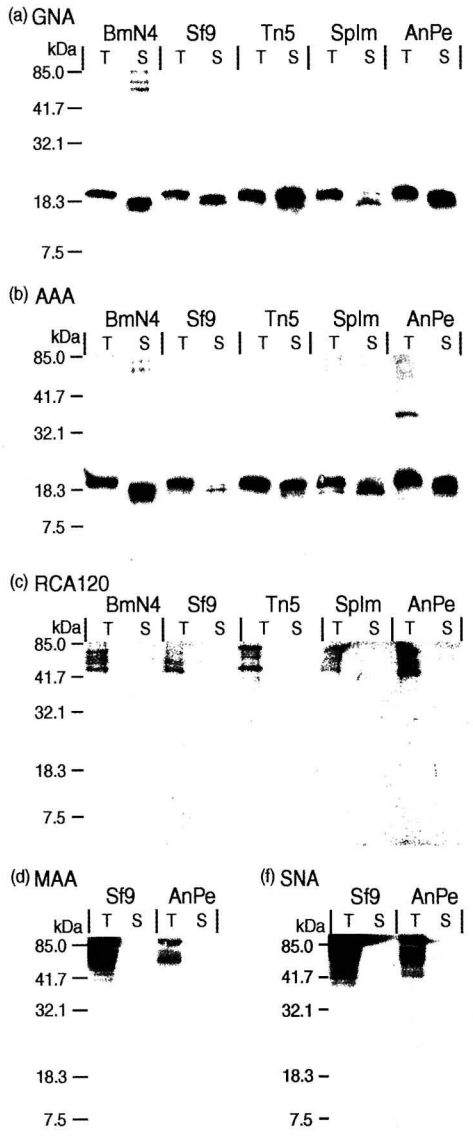


Fig. 4. Lectin blot analysis of N-glycans added on recombinant PTTH produced by each insect cell line (BmN4, Sf9, High5, Splm and AnPe) using lectins GNA (binds terminal α-linked mannose) (a), AAA (binds terminal α 1,6-linked fucose) (b), RCA120 (binds terminal β1,4-linked galactose) (c), SNA (binds terminal α2,6-linked sialic acid) (d) and MAA (binds terminal α 2,3-linked sialic acid) (e). For SNA and MAA, because of no bindings to the PTTH in each sample as shown for RCA120, only results of Sf9 and AnPe are shown. T, TC-100 medium (+10% FBS). S, Sf-900 II medium. The positions and molecular masses of prestained marker proteins are indicated on the left side of each panel.

different N terminal sequence, N-Q-A-I-P-, was detected for PTTH from AnPe in Sf900 II medium and was consistent with the sequence from the 7th to the 11th amino acids at the N-terminus of mature PTTH.

Discussion

In this study, recombinant PTTH produced and secreted by each cell line showed the significant variation in apparent molecular mass ranging between 13 and 20 kDa (Fig.3). Differences in N-glycan structure and/or proteolytic processing were considered as major reasons. In fact, lectin blot analysis revealed that lectins GNA and AAA, which are specific to terminal α-linked mannose and α1,6-linked fucose respectively, clearly bound to recombinant PTTH produced by each cell line, while lectins RCA120, MAA and SNA, which are specific to β1,4-linked galactose, α2,3-linked sialic acid and α2,6-linked sialic acid respectively, did not bind to each PTTH sample (Fig.4). The results indicated that the majority of recombinant PTTH produced by AnPe as well as other lepidopteran cell lines carried fucosylated paucimannosidic N-glycans as generally observed in recombinant glycoproteins produced by BEV systems (Fig.1).

Molecular mass of the PTTH produced in the serum containing medium (TC-100 + 10% FBS) was reduced and converged to ca. 15 kDa (Fig.5) after PNGaseF digestion, indicating the molecular mass variations were essentially caused by differences in N-glycan structure. However, this is not always applicable to the PTTH produced in the serum free medium (Sf-900 II). Although the molecular mass was also reduced after PNGaseF digestion, variations ranging between 13 and 15 kDa still remained. N-terminal amino acid sequence analysis of PNGaseF-digested PTTHs produced by Sf9 and AnPe revealed that unknown modification and removal of N-terminal amino acids, both of which seemed to be related to the degradation of PTTH polypeptide, occurred only under the serum free condition respectively (Fig. 1), indicating that not only the N-glycan structure but also the proteolytic processing varied molecular mass of the PTTH produced in Sf-900 II medium, Similar proteolytic processing could also occur in TC-100 (+10% FBS) medium but the effect on PTTH might be almost masked by some buffer action of abundant serum proteins.

In spite of perfect match of N-terminal sequence between deglycosylated PTTH produced under serum containing condition and mature PTTH. (Fig.2b), molecular mass (ca. 15 kDa) of the former was little bit higher than the latter (12,737 Da). The discrepancy is likely to be caused by slower migration speed of PTTH polypeptide in the SDS-polyacrylamide gel than the expected speed for its calculated molecular mass, or by overestimation based on inaccurate molecular masses of prestained marker proteins blotted on the PVDF membranes.

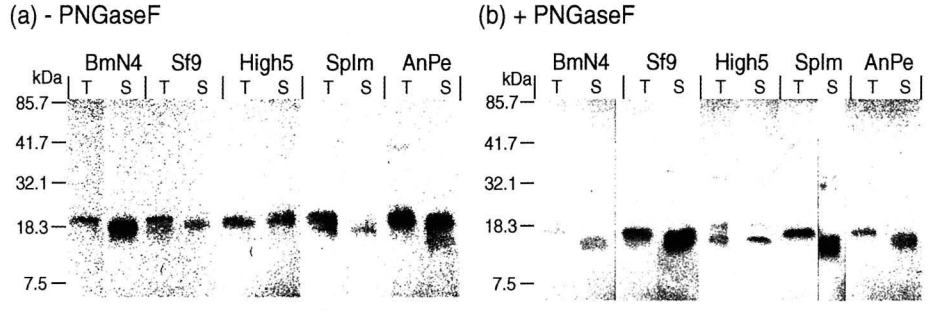


Fig. 5. Effects of PNGaseF digestion on recombinant PTTH produced by each insect cell line (BmN4, Sf9, High5, SpIm and AnPe). The intact PTTH samples (a; identical to Fig. 3b) and the PNGaseF digested samples (b) were analyzed by Western blot analysis. T, TC-100 medium (+10% FBS). S, Sf-900 II medium. The positions and molecular masses of prestained marker proteins are indicated on the left side of each panel.

Significant amounts of PTTH produced by High5 were resistant to PNGaseF digestion (Fig. 5) and bound with anti-HRP serum in Western blot analysis (data not shown), indicating that α1,3-fucosylated N-glycan was added on these PNGaseF-resistant PTTHs. The result was well agreed with other reports on a significant ability of High5 to α1,3-fucosylate N-glycans (Hsu *et al.*, 1997) in contrast to no or a low capacity of Sf9 (Staudacher *et al.*, 1992; Voss *et al.*, 1993; Kubelka *et al.*, 1994). As α1,3-fucosylate N-glycans are allergenic to mammals, such N-glycosylation property of High5 is not appropriate for the production of therapeutic glycoproteins.

In this study, all the results did not support the preliminary result indicating that AnPe added complex type N-glycans to the recombinant glycoprotein (Kobayashi, 2001). However, it may be probable that amounts of complex type N-glycan added on PTTH were below detectable limits of lectin blot analysis. In addition, there is a possibility that PTTHs with sialylated N-glycan were removed during the preparation of crude PTTH samples by ion exchange column chromatography, which was used for separation of charged N-glycans containing sialic acid from uncharged N-glycans (Hollister and Jarvis, 2001). Therefore, we cannot conclude the inability of AnPe as well as other cell lines to form complex type N-glycan. Further analysis of N-glycan structure on affinity-purified recombinant PTTH in conjunction with biochemical and molecular biological analyses of the cell lines to identify glycosyltransferases and the responsible genes required for complex type N-glycan formation will provide us reliable answers.

Acknowledgments

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