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Cloning, expression and characterization of *Bombyx mori* α1,6-fucosyltransferase

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ABSTRACT

Although core α1,6-fucosylation is commonly observed in *N*-glycans of both vertebrates and invertebrates, the responsible enzyme, α1,6-fucosyltransferase, has been much less characterized in invertebrates compared to vertebrates. To investigate the functions of α1,6-fucosyltransferase in insects, we cloned the cDNA for the α1,6-fucosyltransferase from *Bombyx mori* (Bmα1,6FucT) and characterized the recombinant enzyme prepared using insect cell lines. The coding region of Bmα1,6FucT consists of 1737 bp that code for 578 amino acids of the deduced amino acid sequence, showing significant similarity to other α1,6-fucosyltransferases. Enzyme activity assays demonstrated that Bmα1,6FucT is enzymatically active in spite of being less active compared to the human enzyme. The findings also indicate that Bmα1,6FucT, unlike human enzyme, is *N*-glycosylated and forms a disulfide-bonded homodimer. These findings contribute to a better understanding of roles of α1,6-fucosylation in invertebrates and also to the development of the more efficient engineering of *N*-glycosylation of recombinant glycoproteins in insect cells.

1. Introduction

Core α1,6-fucosylation of the asparagine-linked oligosaccharide (*N*-glycan) is distributed in eukaryotes except for plants and fungi [1,2]. Core α1,6-fucosylation has been reported to be involved in development, differentiation, growth and the development of certain types of diseases in vertebrates, including humans [3,4]. Core α1,6-fucosylation is catalyzed by the action of an α1,6-fucosyltransferase, in which fucose unit is transferred from GDP-β-L-fucose to the reducing terminal GlcNAc of an *N*-glycan [5,6]. The mammalian enzymes are referred to as FUT8, and human FUT8 has been the most extensively characterized in terms of structure, substrate specificity and reaction mechanism [7–13].

In contrast, the biological roles of core α1,6-fucosylation and the α1,6-fucosyltransferase have been much less investigated in invertebrates, albeit the enzymes from *Caenorhabditis elegans* and *Drosophila melanogaster* have been characterized using recombinant proteins [14]. The substrate specificity studies using the fly and nematode enzymes suggested that α1,6-fucosylation is predominantly inhibited by core α1,3-fucosylation in invertebrate *N*-glycan synthesis.

Core α1,6-fucosylation has also been observed in recombinant proteins produced in larvae or culture cell lines of lepidopteran [15,16]. Insect culture cells from lepidopteran species such as *Bombyx mori* and *Spodoptera frugiperda* are widely used as hosts to produce recombinant proteins although lepidopteran α1,6-fucosyltransferase has not been characterized in detail. Recently, engineering based on the manipulation of glycosyltransferase genes has been developed to artificially modify or humanize *N*-glycans of recombinant proteins that are expressed in lepidopteran cells [17–19]. For the manipulation of core α1,6-fucosylation in insect cells, it is necessary to characterize insect α1,6-fucosyltransferase in terms of structural and enzymatic properties. Such analyses would allow the roles of core α1,6-fucosylation to be investigated in more detail and to manipulate *N*-glycans by knockout, knockdown or overexpression of the responsible enzyme gene.

In this study, we report on the cloning of the cDNA for Bmα1,6-FucT from BmN cells, a culture cell line derived from *B. mori*, in order to determine the primary structure of Bmα1,6FucT and to characterize the enzyme using its recombinant protein.

Abbreviations: *N*-glycan, asparagine-linked oligosaccharide; Bmα1,6FucT, *Bombyx mori* α1,6-fucosyltransferase; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PNGase, glycopeptidase F; Enzyme, α1,6-fucosyltransferase (EC 2.4.1.68).

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2. Materials and methods

2.1. Chemicals

GDP-β-L-fucose was purchased from Wako pure chemicals (Osaka, Japan). Glycopeptidase F (Peptide: *N*-glycosidase F) was purchased from Takara Bio Inc. (Shiga, Japan). Restriction endonucleases and DNA-modifying enzymes were purchased from Takara, Toyobo (Fukui, Japan) and New England Biolabs (Hitchin, U.K.). Oligonucleotide primers were synthesized by Hokkaido system science (Sapporo, Japan). Other common chemicals were purchased from Sigma (MO, U.S.A.) and Wako.

2.2. Insect cells

Sf21 cells from *S. frugiperda* were maintained at 27 °C in Grace's insect medium supplemented (Gibco, Life technologies, CA, U.S.A.) containing 10% fetal calf serum and 100 mg/L of kanamycin. BmN cells from *B. mori* were maintained at 27 °C in TC-100 medium (AppliChem, Darmstadt, Germany) supplemented with 10% fetal calf serum, and 100 mg/L of kanamycin. BmN cell was obtained from Cell Bank, RIKEN BioResource Center (Ibaraki, Japan).

2.3. cDNA cloning of Bmα1,6FucT

Total RNA and messenger RNA (mRNA) were prepared from BmN cells by using ISOGEN II (Wako) and a FastTrack® 2.0 mRNA Isolation Kit (Invitrogen, Life technologies, CA, U.S.A.), respectively. The first-strand cDNA from total RNA of BmN cell was synthesized by using ReverTra-Plus-™ (TOYOBO) with an oligo dT primer. The cDNA fragment of the full length of Bmα1,6FucT was amplified by the polymerase chain reaction (PCR) using PrimeSTAR GXL DNA polymerase (Takara) and a primer set of Bmα1,6FucT-for and -re (Table 1). The 5'- and 3'- rapid amplification of cDNA ends (RACE) analyses were carried out by using a SMARTer® RACE cDNA Amplification Kit (Clontech, Takara Bio), mRNA from BmN cell, and the primer sets for RACE analyses (Table 1), according to the manufacturer's instructions. The products of PCR, 5'- and 3'-RACE were purified by 1% agarose gel electrophoresis, and then subcloned into the pTA2 vector for TA cloning using Target Clone™ (TOYOBO). The sequences of the TA cloning products were determined by using BigDye® Terminator v3.1 Cycle Sequencing Kit and a DNA sequencer, ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, CA, U.S.A.).

2.4. Site-directed mutagenesis

Site-directed mutagenesis experiments were performed using QuikChange II XL Site Directed Mutagenesis Kit (Agilent technologies, CA, U.S.A.), as described previously [9]. The primer sets used for mutagenesis were listed in Table 1. The resulting mutations were verified by DNA sequencing as described in Section 2.3.

2.5. DNA transfection

A 1.8 kb DNA fragment was produced by the digestion of the pTA2 vector harboring Bmα1,6FucT or their mutants with EcoRI, and was then subcloned into the pIZT vector for the insect cell expression (Invitrogen). The resulting plasmids for Bmα1,6FucT were transfected into Sf21 and BmN cells using ScreenFect™A (Wako), a transfection regent. For transient expression in Sf21 and BmN cells, the cells were harvested after 72 h post-transfection.

2.6. SDS–PAGE and immunoblot analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Laemmli's method [20]. Immunoblot analysis was performed as described previously [9]. An anti-tetra-histidine monoclonal antibody (Promega, WI, U.S.A.) and a horseradish peroxidase-conjugated anti-mouse IgG antibody (Qiagen, VI, U.S.A.) were used as the first and second antibodies, respectively. The bands of immuno-reactive proteins were detected by chemiluminescence using ECL system (GE Healthcare, Tokyo, Japan).

2.7. Glycopeptidase F digestion

Glycopeptidase F digestion was performed to examine whether Bmα1,6FucT is *N*-glycosylated, according to the manufacturer's instructions. Before digestion by glycopeptidase F, the samples were denatured by boiling for 3 min in 500 mM Tris–HCl buffer (pH 8.6) containing 0.5% SDS and 0.75% 2-mercaptoethanol. After adding 2.5% Nonidet P-40 to the sample solutions, the denatured samples were digested by treatment with glycopeptidase F (40 mU/ml) at 37 °C for 24 h, and then analyzed by SDS–PAGE and immunoblot analysis.

2.8. The assay for α1,6-fucosyltransferase activity of Bmα1,6FucT

α1,6-Fucosyltransferase activity was assayed using a fluorescence labeled asparagine-linked sugar chain as an acceptor substrate, as described [9,21]. The reaction product was separated and quantified using an HPLC system (Waters, MA, U.S.A.) equipped with TSKgel, ODS 80TM (4.6 × 150 mm) (Tosoh, Tokyo, Japan). The fluorescence of the column eluate was monitored with the fluorescence detector (2475 Multi λ Fluorescence Detector) at excitation and emission wavelengths of 315 nm and 380 nm, respectively.

2.9. Protein determination

Protein contents were determined by BCA protein assay kit (PIERCE, IL, U.S.A.) using bovine serum albumin as a standard.

3. Results

3.1. Cloning of B. mori α1,6-fucosyltransferase from BmN cell

To clone the gene coding the full length of Bmα1,6FucT, we first performed a BLAST search in the silkworm genome database, KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/) [22], using the sequence of the α1,6-fucosyltransferase from *Danaus plexippus* (AGBW01008152, genome sequence; EHJ67895, protein sequence from GenBank), which is a member of the lepidopteran family. As a result, we found the putative nucleotide sequence for Bmα1,6-FucT in the genome sequence (DF090317, the scaffold of Bm_scaf 2 from KAIKObase). The cDNA harboring Bmα1,6FucT was obtained from BmN cells by PCR with the primer sets (Table 1) that were based on the result of the BLAST search. As shown by the sequencing, the coding region of Bmα1,6FucT consists of 1737 base pairs (bp) of nucleotide sequence and encodes for 578 amino acids (Fig. 1). In addition, 5'- and 3'-RACE analyses determined 241 bp of the 5'-untranslational region (UTR), 334 bp of the 3'-UTR and poly A tail, respectively (Fig. 1). The determined sequence was registered in DNA data bank of Japan (DDBJ) with the accession number AB848715. The deduced amino acid sequence was also registered in the data bank with the accession number BAO96240.

Table 1
Sequences of the primers used in this study.

Primer	Sequence
Bmα1,6FucT-for	5'-ATGAAGAATTATTCAAATGGTCACG-3'
Bmα1,6FucT-re	5'-TTAAACGTTCTTTTGGCTCGTTTCTG-3'
5'-RACE-re	5'-GTCGCTGACGTTGGCCGCCTCTAGTTG-3'
5'-RACE-re(nest)	5'-CGAGTGGATCCTCCTCCTGAGATTCTCG-3'
3'-RACE-for	5'-AGAGTGTATCTAGCCACAGACGAC-3'
3'-RACE-for(nest)	5'-TCGTTAGATGACATTTACTACTTCGG TGGACAAAACGCGCACGAC-3'
M17-for	5'-ATGTACTTTGCGAAATGG-3'
M63-for	5'-ATGTCTTCGGAGCTGGAAG-3'
C46A-for	5'-TATCGCCTTTAAGGGCCAGCGGCAATTCAG-3'
C46A-re	5'-TCTGAATTGCCGCTGGCCCTTAAAGGCGAT-3'
R359A-for	5'-GGGTTCACATAGCGCGCACCGACAAAG-3'
R359A-re	5'-CTTTGTCGGTGCGCGCTATGTGAACCC-3'
N183Q-for	5'-CTAGAGGCGGCCCAGGTCAGCGACCTTGTC-3'
N183Q-re	5'-GACAAGGTCGCTGACCTGGGCCGCCTCTAG-3'
N341Q-for	5'-GCAGAAGGCGATCCAGGACACAATAGCC-3'
N341Q-re	5'-GGCTATTGTGTCCTGGATCGCCTTCTGC-3'
N183D-for	5'-CTAGAGGCGGCCGACGTCAGCGACCTTGTC-3'
N183D-re	5'-GACAAGGTCGCTGACGTCGGCCGCCTCTAG-3'
N341D-for	5'-GCAGAAGGCGATCGACGACACAATAGCC-3'
N341D-re	5'-GGCTATTGTGTCGTCGATCGCCTTCTGC-3'
poly His-for	5'-CAAAAGAACGTTGCTGGTGGAGGTCATCAT CATCATCATCATGGAGGTGGATAAGTATTGGG AATTCCTG-3'
poly His-re	5'-CAGGAATTCCCAATACTTATCCACCTCCATGAT GATGATGATGATGACCTCCACCAGCAACGTTCTTTTG-3'

The Bmα1,6FucT-re primer was also used as a reverse primer for the construction of M17 and M63 mutants of Bmα1,6FucT.

An alignment analysis was carried out using the CLUSTAL W program [23]. The alignment of the amino acid sequences among *B. mori*, Spodoptera frugiperda, Danaus plexippus, Manduca sexta indicated that lepidopteran α1,6-fucosyltransferases were nearly identical with one another (data not shown). The alignment of the sequences of α1,6-fucosyltransferases among various species from vertebrates and invertebrates also shows that the catalytic region is highly conserved in enzymes ranging from insects to mammals (Fig. 2).

3.2. Overexpression of Bmα1,6FucT in Sf21 and BmN cells

The plasmid DNA carrying the full length Bmα1,6FucT that was fused with the C-terminal poly histidine tag was transiently transfected into Sf21 and BmN cells in order to determine whether the cloned gene encodes an active form of the α1,6-fucosyltransferase. Based on an immunoblot analysis using anti-poly histidine tag IgG, Bmα1,6FucT was clearly expressed as about a 66 kDa protein in Sf21 cells (Fig. 3A). The enzyme was also successfully expressed in BmN cells (Fig. 3B), in spite of the lower expression level compared to the case of Sf21 cells. As evidenced by an enzyme activity assay, the Bmα1,6FucT was catalytically active in terms of catalyzing the core α1,6-fucosylation of *N*-glycans (Table 2). However, the specific activity in the cell lysate was significantly lower for Bmα1,6FucT than for human FUT8 although the protein level based on the immunoreactivity of the common tag was comparable between the silkworm and human enzymes (Fig. 3, Table 2).

The N-terminal region of Bmα1,6FucT contains four methionine residues, Met-1, Met-16, Met-17 and Met-63 (Fig. 1). To identify a potential start codon to produce functional Bmα1,6FucT, two deletion mutants were constructed and examined. The M17 and M63 mutants were constructed by the deletion of the N-terminal 16 and 62 residues, respectively, from the full length molecule, tentatively the wild type, of Bmα1,6FucT. The results indicated that the M17 mutant was comparable to the wild type in terms of protein expression and enzymatic activity (Fig. 3A and Table 2). In the case of the M63 mutant, on the other hand, no expression and no activity were detected (Fig. 3 and Table 2). As suggested by these results, it is very likely that Met-1 in the longest polypeptide is a starting residue for the translation of Bmα1,6FucT. Although Met-17 can also substitute for Met-1 to produce the catalytically active enzyme, the N-terminal region of sixteen residues could play a role in the actual and proper function of the enzyme within cells. In addition, it was found that the N-terminal 62 residues are required for the expression of active Bmα1,6FucT.

It is known that Arg-365 of human FUT8 is essential for its activity [24], and, as indicated by the alignment of amino acid sequences, the human Arg-365 residue corresponds to Arg-359 of Bmα1,6FucT (Fig. 2). In addition, the substitution of alanine for Arg-359 (R359A) led to a loss of enzymatic activity in Bmα1,6FucT while the expression level of the R359A mutant was the same as the wild type (Fig. 3A and Table 2). These results indicate that the Arg-359 of Bmα1,6FucT plays a role that is equivalent to that of Arg-365 of human FUT8.

3.3. Dimerization of Bmα1,6FucT via the disulfide bond of Cys-46

SDS–PAGE analysis followed by immunoblotting showed a band of approximately 120 kDa for Bmα1,6FucT under nonreducing conditions, while the protein displayed 66 kDa under reducing conditions (Fig. 3C). These results indicate that Bmα1,6FucT forms a homodimer via a disulfide bond. Bmα1,6FucT contains nine cysteine residues at positions 46, 201, 209, 215, 219, 227, 263, 454 and 459 (Fig. 1). Eight cysteine residues, except for Cys-46, are located in and are highly conserved in the catalytic domain of eukaryote α1,6-fucosyltransferases (Fig. 2) [9]. The additional cysteine residue, Cys-46, of Bmα1,6FucT is uniquely conserved in lepidopteran enzymes (data not shown), and appears to be located in the stem region, as indicated by the alignment. When Cys-46 of Bmα1,6FucT was replaced with alanine, covalent dimerization was not observed, as evidenced by nonreducing SDS–PAGE (Fig. 3C). Because all of the cysteine residues except Cys-46 are highly conserved in Bmα1,6FucT and human FUT8, the latter of which does not form a covalent dimer, it is likely that the homologous cysteine residues are involved in formation of intramolecular disulfide bonds. These results suggest that Bmα1,6FucT is covalently dimerized via an intermolecular disulfide bridge between two Cys-46 residues of protomer. However, this disulfide was not essential for a sufficient expression level and enzyme activity, as indicated by Fig. 3C and Table 2.

3.4. N-Glycosylation of Bmα1,6FucT

Asn-183 and Asn-341 of Bmα1,6FucT are the consensus sites for *N*-glycosylation (Fig. 1), whereas human and most mammalian enzymes contain no potential *N*-glycosylation sites. Site-directed mutagenesis and glycosidase digestion studies were carried out to ascertain whether Bmα1,6FucT is, in fact, *N*-glycosylated. The mutants were prepared by replacement of the asparagine residues with glutamine and aspartic acid, and denoted as N183Q, N341Q, N183/341Q, N183D, N341D, and N183/341D. Substitution by aspartic acid was performed to mimic acidic residues at the corresponding positions in human FUT8. All mutants showed a faster mobility in SDS–PAGE, compared to the wild type (Fig. 4A). Moreover, when the wild type and the mutants were treated with glycopeptidase F, which releases *N*-glycans from glycoproteins, the protein bands were shifted in the wild type and the single mutants (Fig. 4B). In the double mutant, on the other hand, no shift in the protein band was observed even after digestion by glycopeptidase F. These results clearly indicate that Bmα1,6FucT is actually *N*-glycosylated at Asn-183 and Asn-341 residues. This finding is in contrast to the lack of *N*-glycosylation in human FUT8. Because

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1     GTCA
      AACGTCAATCGTTCAGTTGCCTCTCGGAGTGTGTTTTGTCAGTTTTGTGCTAGGATAAAACAATCCGTTATTAACCAAT
      TTAGTCAAAGAAAACAATTACTTTACGCTATAATCATAGTGAAAATAACAACTAAATGTTTTAATGTTCATGAAAGTTC
      TAAAGTTTTCTACTTCTCTGTATACAATTTAGAAAGATTACGATCGTCCGTCGTTTGGTAACATGAAGCGTTATACATA  241

242   ATG AAG AAT TAT TCA AAT GGT CAC GTA ACG ATA CAT GAT TAT TTT ATG ATG TAC TTT GCG   60
1     Met Lys Asn Tyr Ser Asn Gly His Val Thr Ile His Asp Tyr Phe Met Met Tyr Phe Ala   20

302   AAA TGG AAG CGA GTC GCC GTC GTA TTG CTC GCC GTT TGG ATT GTC GTC ACC TAT TTA GTC   120
21    Lys Trp Lys Arg Val Ala Val Val Leu Leu Ala Val Trp Ile Val Val Thr Tyr Leu Val   40

362   ATA TCG CCG TTA AGA TGC AGC GGC AAT TCA GAT GAG ATC CCC GAC GTC CAG GAG AGG TTG   180
41    Ile Ser Pro Leu Arg Cys Ser Gly Asn Ser Asp Glu Ile Pro Asp Val Gln Glu Arg Leu   60

422   AAG CGG ATG TCT TCG GAG CTG GAA GTC CTG TGG CAG AGA AAC AGT AAG CTC ATA GCG CAG   240
61    Lys Arg Met Ser Ser Glu Leu Glu Val Leu Trp Gln Arg Asn Ser Lys Leu Ile Ala Gln   80

482   ATA AAG AAG TCT TCA GGG CCC AAT GGC AAT CTT AAA GAT TTG GAT CCG GCG TTT TGG AAC   300
81    Ile Lys Lys Ser Ser Gly Pro Asn Gly Asn Leu Lys Asp Leu Asp Pro Ala Phe Trp Asn   100

542   CTT GAA GCG GGA CTT GGT CCG TCA GAG GAA TAC GAG AAT CTC AGG AGG AGG ATC CAC TCG   360
101   Leu Glu Ala Gly Leu Gly Pro Ser Glu Glu Tyr Glu Asn Leu Arg Arg Arg Ile His Ser   120

602   AAT ACA AAG GAG TTC TGG TAC TAC GCG AAC CAT GAG CTC GGG AAG TTG ATG CAC GAG AGT   420
121   Asn Thr Lys Glu Phe Trp Tyr Tyr Ala Asn His Glu Leu Gly Lys Leu Met His Glu Ser   140

662   GAC AAG GAA GCG AAA ATA CGA GCG ATT TTG GAA CAA CTG TCT GAT AGG AAA AGC ACA TTA   480
141   Asp Lys Glu Ala Lys Ile Arg Ala Ile Leu Glu Gln Leu Ser Asp Arg Lys Ser Thr Leu   160

722   CTG TCT GAT CAA GAA AAA CTA TCG GAA ATG GAT GGT TAC CAC GAG TGG AGG CAA CTA GAG   540
161   Leu Ser Asp Gln Glu Lys Leu Ser Glu Met Asp Gly Tyr His Glu Trp Arg Gln Leu Glu   180

782   GCG GCC AAC GTC AGC GAC CTT GTC CAG AGG AGG TTG CAC TAT TTA CAG AAC CCG CCG GAT   600
181   Ala Ala Asn Val Ser Asp Leu Val Gln Arg Arg Leu His Tyr Leu Gln Asn Pro Pro Asp   200

842   TGT AGG GAG GCA AGG AAA CTT ATA TGC AAC TTG AAT AAG GGC TGC GGC TTC GGC TGT CAA   660
201   Cys Arg Glu Ala Arg Lys Leu Ile Cys Asn Leu Asn Lys Gly Cys Gly Phe Gly Cys Gln   220

902   CTC CAC CAC ATA GTG TAC TGC CTT ATA TTC GCT TAC GCC ACC GAG AGG ACT CTA ATC CTG   720
221   Leu His His Ile Val Tyr Cys Leu Ile Phe Ala Tyr Ala Thr Glu Arg Thr Leu Ile Leu   240

962   AAC TCG AAG GGG TGG CGG TAC AAC ACT AAA GGA TGG GAG TAC GTC TTC CAT CCG ATA TCG   780
241   Asn Ser Lys Gly Trp Arg Tyr Asn Thr Lys Gly Trp Glu Tyr Val Phe His Pro Ile Ser   260

1022  GAG AGC TGT TTG TCT TCG TAC GAC GAT AAA GTC GTG CAG TGG CCA GTT TCA TAC GAC GCG   840
261   Glu Ser Cys Leu Ser Ser Tyr Asp Asp Lys Val Val Gln Trp Pro Val Ser Tyr Asp Ala   280

1082  AAA GTG GTT TCG CTA CCG TTT ATA GAT TCC ATA TCG CAG AAG CCC AAG TTC TTG CCG CTG   900
281   Lys Val Val Ser Leu Pro Phe Ile Asp Ser Ile Ser Gln Lys Pro Lys Phe Leu Pro Leu   300

1142  GCT GTG CCT AAA GAC TTG GCT CAT AGG ATA GTC CGC TTC AAC GGC GAT CCG TCG TCG TGG   960
301   Ala Val Pro Lys Asp Leu Ala His Arg Ile Val Arg Phe Asn Gly Asp Pro Ser Ser Trp   320

1202  TGG GTC GGG CAA ATG CTG AAG TTC GTC CTG AAG CCG CGC CTG CCG ATG CAG AAG GCG ATC   1020
321   Trp Val Gly Gln Met Leu Lys Phe Val Leu Lys Pro Arg Leu Pro Met Gln Lys Ala Ile   340

1262  AAC GAT ACA ATA GCC AAA ATG AAT TTC AAA TCT CCC ATA GTC GGG GTT CAC ATA CGT CGC   1080
341   Asn Asp Thr Ile Ala Lys Met Asn Phe Lys Ser Pro Ile Val Gly Val His Ile Arg Arg   360

1322  ACC GAC AAA GTT GGA ACC GAA GCC GCC TTC CAC CAC ATA CAC GAG TAC ATG GCG CAC GTA   1140
361   Thr Asp Lys Val Gly Thr Glu Ala Ala Phe His His Ile His Glu Tyr Met Ala His Val   380

1382  AAG GAC TAT TAC GAC CAG CTA GAG CTG ACC CGG CCC GTC GAC GTC CGG AGA GTT TAT CTA   1200
381   Lys Asp Tyr Tyr Asp Gln Leu Glu Leu Thr Arg Pro Val Asp Val Arg Arg Val Tyr Leu   400

1442  GCC ACA GAC GAC GCT AAT GTG TTA GAC GAC GCC CGA CAA AAG TAT CCG GAG TAC ACG TTC   1260
401   Ala Thr Asp Asp Ala Asn Val Leu Asp Asp Ala Arg Gln Lys Tyr Pro Glu Tyr Thr Phe   420

1502  TTA GGG GAT CCG TCG ATA GCT AAG ACG GCG GCC ACT CAC CGT AGA TAC ACG CCG CTC TCG   1320
421   Leu Gly Asp Pro Ser Ile Ala Lys Thr Ala Ala Thr His Arg Arg Tyr Thr Pro Leu Ser   440

1562  CTT ACC GGG CTA CTG GTG GAT CTA CAC ATG CTA GCC ATG TGC GAT TAC TTA GTG TGT ACA   1380
441   Leu Thr Gly Leu Leu Val Asp Leu His Met Leu Ala Met Cys Asp Tyr Leu Val Cys Thr   460

1622  TTT AGC AGT CAA GTG GGT CGA GTG GCA TAC GAA ATG ATG CAG TCG AAC AGA GTG GAC GCG   1440
461   Phe Ser Ser Gln Val Gly Arg Val Ala Tyr Glu Met Met Gln Ser Asn Arg Val Asp Ala   480

1682  TCA AAC AGC TTC TTC TCG TTA GAT GAC ATT TAT TAC TTC GGT GGA CAA AAC GCG CAC GAC   1500
481   Ser Asn Ser Phe Phe Ser Leu Asp Asp Ile Tyr Tyr Phe Gly Gly Gln Asn Ala His Asp   500

1742  AGG GTG GCC ATC ATG CAG AAC CAC GGC GGG AAA AAC GAG GAT ATA TCT TTT GAG GTG GGC   1560
501   Arg Val Ala Ile Met Gln Asn His Gly Gly Lys Asn Glu Asp Ile Ser Phe Glu Val Gly   520

1802  GAT AAA ATA GGA GTG GCC GGC AAC CAT TGG AAC GGA TAC GGA CGC GGA ACC AAC AAG AGA   1620
521   Asp Lys Ile Gly Val Ala Gly Asn His Trp Asn Gly Tyr Gly Arg Gly Thr Asn Lys Arg   540

1862  ACT AAT ATG AAC GGT CTG ATC CCT TGG TAC AAG ACT GCC GAT CAT CTG GTG CTG TAC CCG   1680
541   Thr Asn Met Asn Gly Leu Ile Pro Trp Tyr Lys Thr Ala Asp His Leu Val Leu Tyr Pro   560

1922  TTT CCG GAA TAC AAA CAG GTG CCT ATT TAT TCA GAA ACG AGC CAA AAG AAC GTT TAA       1978
561   Phe Pro Glu Tyr Lys Gln Val Pro Ile Tyr Ser Glu Thr Ser Gln Lys Asn Val End      579

1979  TACCAAACGAATTAGGTTCAAATTACTGTAGTATTTTTTTTGGTGTTAGTTTAAATCGTTCTGTGACAGATCGAAACGT
      TGGTTATATTATGAATCATGTTGTTATACTTGTGATGTTGATTGTGAATTGTTAACGTTTGTTTCTCTTGTTACTTGTG
      ATTGTAAACAAAGTGTACCCTATTAATATTTTTTGTCTTGTGAGATTGGTATGAGCAAACAAAAATATTTTAATCGTTT
      CTCCCCACGACTGACGTTTTAAAATAATAGCAGCAAGGAAAAACTGGCAACACTAAGGCGGAAATTGCATAGTAAATTT
      CCCTTTGCCTAAAGGAGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                                  2342
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Fig. 1. The primary structure of *Bombyx mori* α1,6-fucosyltransferase. The nucleotide sequence of the cloned cDNA consists of 241 bp of 5'-UTR (1–241), 1737 bp of coding region (242–1978), and 364 bp -3'-UTR with a poly A tail (1979–2342). The deduced amino acid sequence is indicated by three-letter codes. The consensus sites for *N*-glycosylation are underlined.

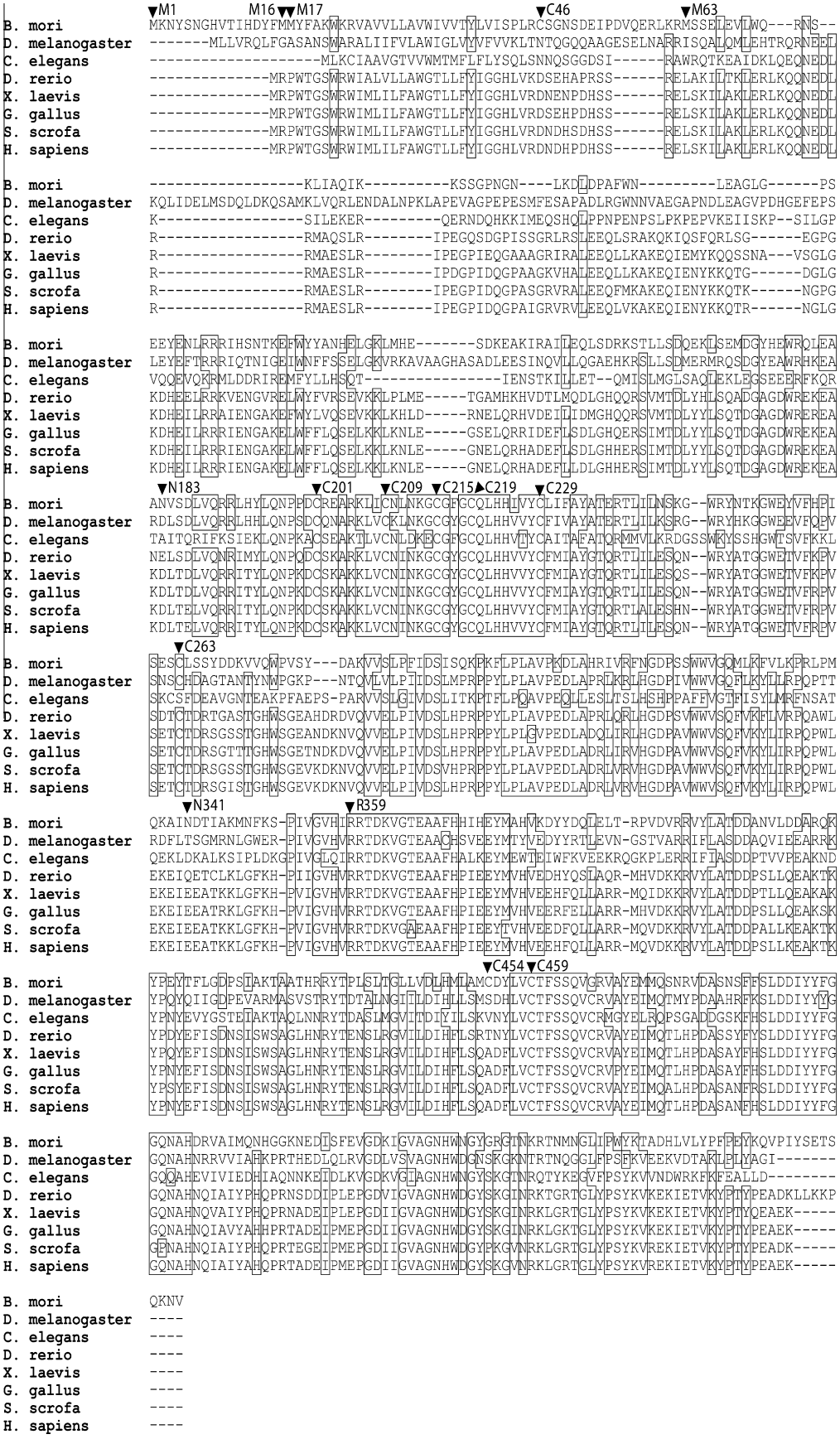

Fig. 2. Alignment analysis of amino acid sequences of eukaryotic α1,6-fucosyltransferases. The amino acid residues which are conserved with 80% similarities are enclosed in boxes. GenBank accession numbers for α1,6-fucosyltransferase are: *Bombyx mori*, BAO96240 (this study); *Drosophila melanogaster*, AAF48079; *Caenorhabditis elegans*, AAN84870; *Danio rerio*, CAH03675; *Xenopus laevis*, AAH79978; *Gallus gallus*, CAH25853; *Sus scrofa*, BAA13157; *Homo sapiens*, BAA19764.

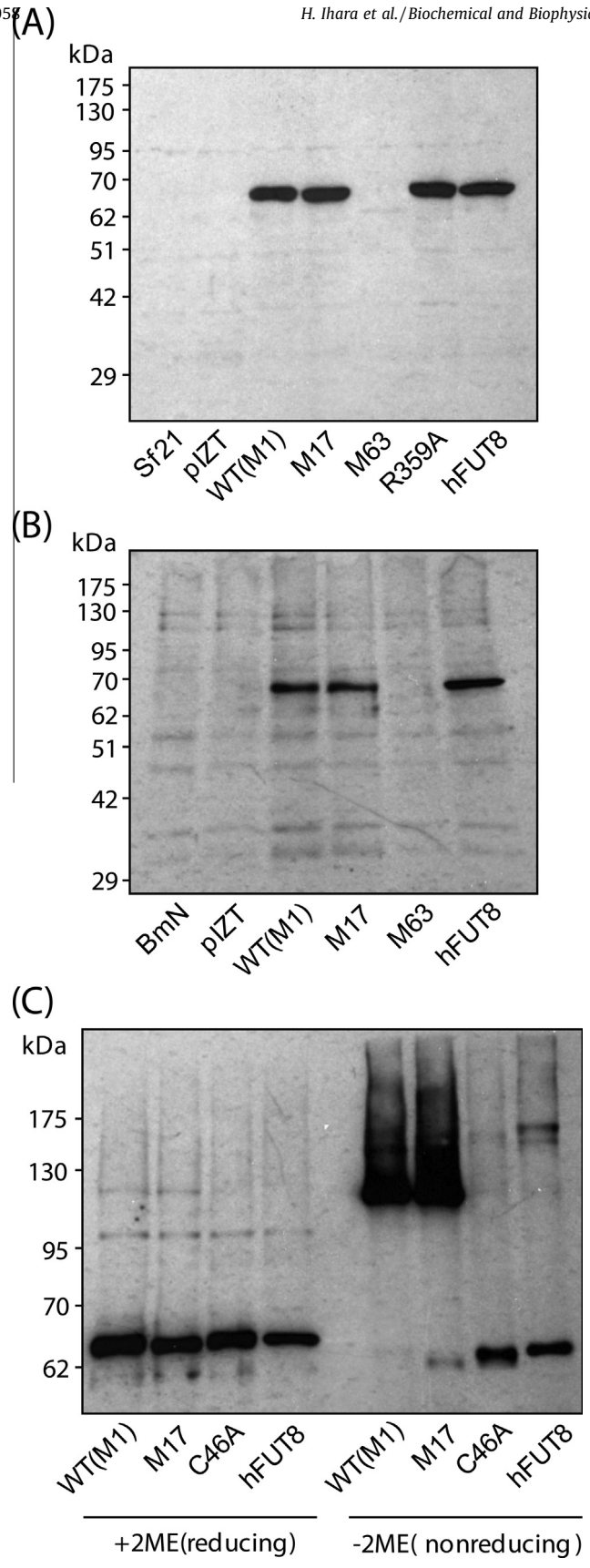

Fig. 3. Overexpression of *Bombyx mori* α1,6-fucosyltransferase in insect cells. The wild type and mutants of Bmα1,6FucT were transiently expressed in Sf21and BmN cells. Whole cell lysates from Sf21 (A) and BmN (B) were separated on 10% polyacrylamide gels under reducing conditions. Dimeric and monomeric forms of Bmα1,6FucT were separated on 6.5% polyacrylamide gel under reducing or nonreducing conditions (C). Separated proteins were analyzed by immunoblotting using anti-poly histidine antibody.

Table 2
α1,6-Fucosyltransferase activity of plasmid-transfected cells.

Cells	Plasmid	α1,6-Fucosyltransferase activity (nmol/hour/mg protein)
Sf21	none	N.D.
	pIZT	N.D.
	Bmα1,6FucT	1.6
	M17	1.6
	M63	N.D.
	R359A	N.D.
	C46A	1.6
	N183Q	1.6
	N341Q	1.2
	N183/341Q	1.0
	N183D	1.5
	N341D	1.2
	N183/341D	1.0
	human FUT8	123.0
BmN	none	N.D.
	pIZT	N.D.
	Bmα1,6FucT	0.2
	M17	0.2
	M63	N.D.
	human FUT8	18.1

N.D. means "not detectable".

enzymatic activity was sufficiently retained in all mutants (Table 2), it appears that *N*-glycosylation is not essential for enzyme activity or for the formation of the active enzyme.

4. Discussion

Based on the results of cDNA cloning and the expression of Bmα1,6FucT, the findings indicate that Bmα1,6FucT is enzymatically active despite the fact that it is less active than the human FUT8. It was also found that the silkworm enzyme is unique in terms of being *N*-glycosylated and existing in the form of a covalently linked dimer. Owing to such differences, Bmα1,6FucT may adapt to characteristic biosynthetic pathway of insect *N*-glycans. Bmα1,6FucT catalyzed the fucosylation of glycoproteins much more efficiently in vivo in insect cells than was expected from the in vitro enzyme activity, which appeared to be much less active than the mammalian enzyme.

It was recently recognized that α1,6-fucosylation is of significant importance in terms of modifying the effecter function of therapeutic immunoglobulin, since it was reported that the deletion of α1,6-fucose from IgG enhances antibody-dependent cellular cytotoxicity [25,26]. To produce α1,6-fucose-deleted IgG, the FUT8-lacking CHO cell line, which was established by gene-targeting was used as a host for expression because FUT8 expression and the α1,6-fucosylation of glycoproteins have been observed in a wide variety of cells [27,28]. On the other hand, lepidopteran insect cells and larvae such as *S. frugiperda* and *B. mori* do not serve as a host due to unavailability of α1,6-fucosyltransferase-knocked-out preparations. Alternatively, core α1,3- and α1,6-fucosylation was reduced by the expression of GDP-4-dehydro-6-deoxy-D-mannose reductase which leads to the depletion of GDP-fucose within cells [18]. In fact, lepidopteran α1,6-fucosyltransferase(s) has not been well characterized in terms of structure and function, and, because of this, this study was conducted to determine the nucleotide sequence of mRNA for Bmα1,6FucT and to partially characterize the enzyme.

The synthetic pathways for the formation of lepidopteran *N*-glycans are distinct from those of vertebrates, particularly with respect to core α1,3-fucosylation and the shortened nonreducing terminal that is produced by specific hexosaminidases [14]. Due

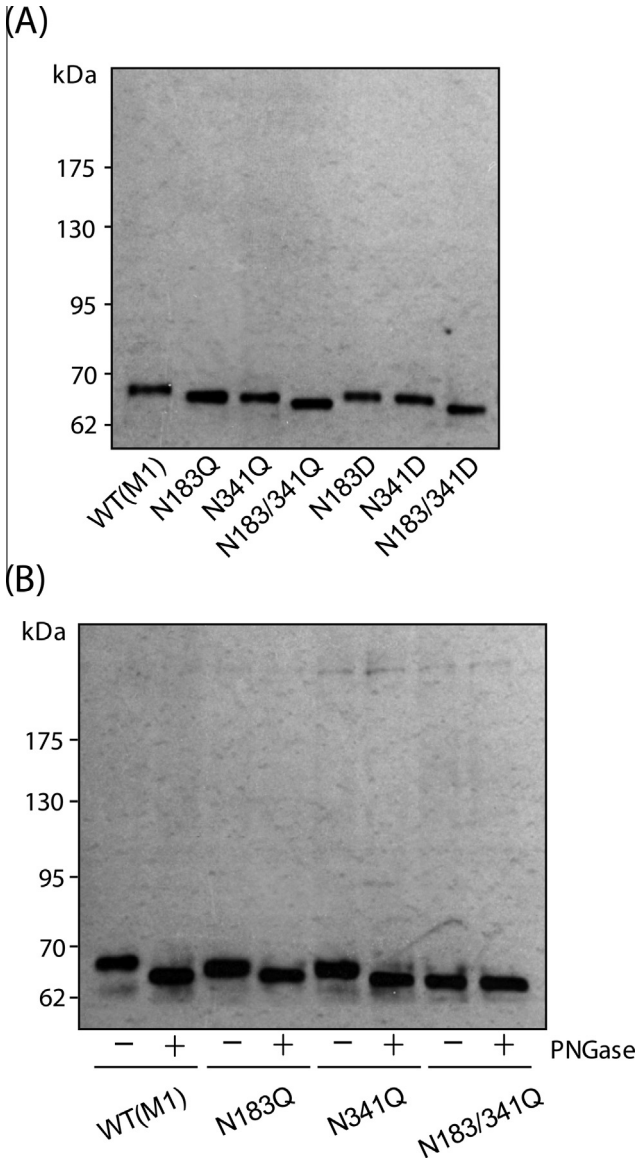

Fig. 4. Identification of *N*-glycosylation in *Bombyx mori* α1,6-fucosyltransferase. The wild type and *N*-glycosylation mutants of Bmα1,6FucT were transiently expressed in Sf21 cells. Whole cell lysates were separated were separated on 6.5% polyacrylamide gel (A). Cell lysates were digested by glycopeptidase F (displayed as PNGase in figure), and then analyzed (B). Bmα1,6FucT and mutants were detected by means of an anti-poly histidine antibody.

to these differences, recombinant glycoproteins that are produced by insect cells are likely to be immunogenic to humans, thus preventing the use of insect cells in the industrial production of therapeutic glycoproteins. We previously reported that the overexpression of human GnT-III is effective in the beneficial modification of insect *N*-glycans [19]. The core α1,6-fucose and the bisecting GlcNAc, each of which is attached to a glycan by the action of FUT8 and GnT-III, respectively, cause a steric change in the *N*-glycan [29]. Thus, the perturbation of α1,6-fucosylation by the overexpression, knockdown or knockout of Bmα1,6FucT could lead to a disruption in the ordered biosynthesis of *N*-glycans, possibly contributing to the humanization of insect *N*-glycans, as was found for the bisecting GlcNAc.

Our findings would be helpful for developing a further understanding of the α1,6-fucosylation of insect *N*-glycans, and may also contribute to the development of *N*-glycan engineering in the production of therapeutic glycoproteins using lepidopteran cells.

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