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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.