Efficient production of human $\beta$-1,3-N-acetylglucosaminyltransferase-2 fused with green fluorescence protein in insect cell

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Received 9 July 2003; accepted after revision 10 September 2003

Abstract

Human $\beta$-1,3-N-acetylglucosaminyltransferase-2 ($\beta$3GnT2) was produced in a baculovirus expression system as a secreted fusion protein with a green fluorescence protein variant, GFPuv, flanked by the (His) 6 sequence and an enterokinase cleavage site. The expression of the $\beta$3GnT2–GFPuv fusion gene was rapidly detected using a fluorescence microscope without employing complicated assay methods. When Tn-5B1–4 cells were infected with a recombinant AcMNPV–$\beta$3GnT2–GFPuv virus at MOI 10, intracellular and extracellular $\beta$3GnT activities increased to 0.26 and 0.68 mU/ml, respectively, until 3 days post-infection (d.p.i.), and decreased markedly at 3 d.p.i. In contrast to Tn-5B1–4 cell culture medium, the extracellular $\beta$3GnT activity in Sf-9 cell culture medium increased to 0.86 mU/ml at 4 d.p.i. The fusion protein obtained from Tn-5B1–4 and Sf-9 cultures was confirmed based on the GFPuv of the fusion protein. The fusion protein was purified using a Ni 2+ affinity column, and was concentrated by approximately 900-fold. The observed $\beta$3GnT activity and the specific $\beta$3GnT activity of the purified fusion protein were 77.6 mU/ml and 4.6 U/mg protein, respectively. When the purified fusion protein was treated with glycopeptidase F, its molecular weight decreased by 7–8 kDa, indicating that $\beta$3GnT2 is glycosylated.

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Keywords: Insect cell culture; Baculovirus; Recombinant DNA; $\beta$-1,3-N-Acetylglucosaminyltransferase; Green fluorescence protein; Baculovirus expression system

1. Introduction

Recently, six kinds of human $\beta$-1,3-N-acetylglucosaminyltransferase ($\beta$3GnT-6) were cloned using expression sequence tag sequences and PCR technology [1–4]. $\beta$3GnT2 exhibited 10–20 times higher activity than polylactosamine acceptors such as $\beta$3GnT3 and $\beta$3GnT4. Thus $\beta$3GnT2 is thought to be an enzyme that extends the polylactosamine acceptor chains. $\beta$3GnT5 is regarded as a lactotriosylceramide synthase that plays a key role in the synthesis of lacto- or neolacto-series carbohydrate chains of glycolipids. Their characterizations have been incomplete, however, because their expression levels in a heterogeneous expression system are too low to quantify their amounts. Still a sufficient amount of $\beta$3GnT is urgently required, not only for the investigation of biochemical and biological properties including protein structure analysis, but also for the efficient synthesis of oligosaccharides.

The baculovirus expression system (BES) is functional in the mass production of active human proteins due to the ability of insect cells to perform post-translational modification processes including the proteolytic processing, glycosylation, and phosphorylation of sugars [5–7]. The BES is valuable for the production of human glycosyltransferases such as uridine-5′-diphosphate-N-acetylglucosamine (UDP-GlcNAc)-α-6-mannoside-$\beta$-1,2-acetylglucosaminyltransferase, $\alpha$-1,3/4-fucosyltransferase III, and O-glycan core 2 $\beta$-1,6-N-acetylglucosaminyltransferase [8–10]. To secrete these glycosyltransferases extracellularly, the cytoplasmic N-terminal and transmembrane sequences are substituted for other signal sequences. The BES is suitable for the production of useful human proteins that are difficult to produce using a microorganism system from the protein functional point of view. This makes kinetic research or molecular structure analysis by crystallization of protein possible.

The green fluorescence protein (GFP) is used extensively as a reporter gene for expression and as a live marker [11,12] because GFP can be detected easily and rapidly without any substrate or cofactor. Moreover, GFP fusion proteins
are known to be particularly stable and soluble [13]. Many GFP variants have been subjected to protein purification in order to generate useful fusion partners, and GFPuv has been optimized for easy detection with UV excitation [14].

In the present study, to produce the GFPuv–β3GnT2 fusion protein and to purify it efficiently, we constructed a vector to express the β3GnT2 gene, in which the honeybee melittin signal sequence, GFPuv gene, (His)6 sequence, and an enterokinase cleavage site were inserted under the control of a polyhedrin promoter. Since GFPuv can be detected easily and rapidly without any substrates or cofactors, it enables us to easily visualize the fusion protein. The GFPuv–β3GnT2 gene was expressed in a serum-free medium as a soluble protein in the BES with a recombinant Autographa californiana multicapside nuclear polyhedrosis virus (AcMNPV). The expression levels of the GFP fusion protein and its eluent obtained by column chromatography were then investigated.

2. Materials and methods

2.1. Cell lines and media

Sf-9 cells derived from Spodoptera frugiperda and Trichoplusia ni were purchased from Invitrogen (San Diego, CA) and grown in both 25 cm² tissue culture flasks (Falcon) and 100 ml flasks. SF-900 II and Express Five (Invitrogen) media were used for the cultivation of SF-9 and Tr-SBI-4 cells, respectively, before which, 1% antibiotic–antimycotic (Invitrogen) was added. Suspension cultures were carried out in 100 ml flasks with a working volume of 20 ml in the specified medium. Agitation rate and temperature were controlled at 100 rpm and 27°C, respectively. Two million Tr-SB1–4, SF-9 cells/ml were infected at a multiplicity of infection (MOI) of 10 with a recombinant baculovirus, AcMNPV-GFPuv–β3GnT2. The cells were counted using a hemacytometer, and their viability was determined by the trypan blue exclusion method.

2.2. Construction of recombinant baculovirus

The 1264 bp DNA fragment of a truncated segment and a 3’ untranslational region of β3GnT2 was obtained by PCR using oligonucleotide primers flanked with BamHI and EcoRI restriction sites, respectively, from cDNA of Quick-Clone™ human fetal brain cDNA (Clontech, Palo Alto, CA). The designed primers were as follows: forward primer 1: 5′-CGGGATCCGGGACTCTCCTAAAGGCATTGCGCAAG-3′; reverse primer 1: 5′-CGGAAATCTGTGAGGGCCGTCCTACTAATGGG-3′. The CAT gene was removed from pBlueBacHis2-2-GFPuv/ CAT [11], and the resulting plasmid, referred to as pBlueBacHis2-2-GFPuv, was used for further experiments. The amplified PCR product of β3GnT2 was inserted between the BamHI and EcoRI sites of pBlueBacHis2-2-GFPuv to yield pBlueBacHis2-2-GFPuv-β3GnT2. To introduce a new signal peptide coding region, the GFPuv–β3GnT2 fusion fragment was amplified by PCR using reverse primer 1 and a long forward primer encoding the honeybee melittin signal peptide [15]. The long forward primer 2 used was 5′-CGGGGTTCTTCATCACATTCTTACATCACATAGCCGGCGGCG- CGGGGTTCTTCATACATCACATTCTTACATCACATAGCCGGCGGC.

2.3. Culture method for insect cells

Suspension cultures were carried out in 100 ml flasks with a working volume of 20 ml in the specified medium. Agitation rate and temperature were controlled at 100 rpm and 27°C, respectively. Two million Tr-SB1–4, SF-9 cells/ml were infected at a multiplicity of infection (MOI) of 10 with a recombinant baculovirus, AcMNPV-GFPuv–β3GnT2. The cells were counted using a hemacytometer, and their viability was determined by the trypan blue exclusion method.

2.4. Purification of GFPuv–β3GnT2 fusion proteins

After a post-infection time of 2 days, the cultures were sampled and centrifuged at 8000 rpm for 5 min to remove the cells. Fifty milliliters of the cell culture supernatant was added to 10 ml of 50 mM Tris–HCl (pH 7.5) containing 150 mM NaCl and 40 mM imidazole. The histidine-tagged GFPuv–β3GnT2 fusion protein was eluted with 3 V of 50 mM Tris–HCl (pH 7.5) containing 150 mM NaCl and 200 mM imidazole.

2.5. Assay of β3GnT and protease activities

The β3GnT activity assay was carried out using 50 mM Tris–HCl (pH 8.0), 15 mM MnCl₂, 19 mM UDP-GlcNAc, 4 mM UDP-GalNAc, 1 mM UDP-Glc, 1 mM UDP-Gal, and 2.5 mM UDP-

22 mM Gal β1–4 GlcNAc β-pNP, and 5 μl of enzyme solution (total volume 25 μl). The reaction was started by the addition of the β3GnT sample. After every sampling, 5 μl of the reaction mixture was added to 195 μl of distilled water, followed by boiling for 5 min. The resulting solution was filtered with a 0.45 μm nitrocellulose filter (Millipore, Bedford, MA), and the filtrates were analyzed by HPLC. MightySil RP-18 (H) GP 150-4.6 (Kanto Chem., Tokyo, Japan) was used as the column. Reaction products were eluted with 10% methanol and detected at the absorbance of 300 nm. HPLC was performed at 40 °C with a flow rate of 1.0 ml/min. One unit of enzyme activity is defined as the amount of an enzyme capable of catalyzing the transfer of 1 μmol of GlcNAc per min.

The protease assay was performed as described by Slack et al. [17]. Seventy microliters of sample (culture medium and cell lysate) was gently mixed with 430 μl of AUE buffer (0.2% azocasein, 3 M urea, 5 mM cysteine, 5 mM EDTA, and 50 mM citrate, pH 5.4). The mixture was incubated at 37 °C for 1 h, after which 500 μl of 20% trichloroacetic acid was added to stop the protease reaction. The mixture was centrifuged at 15,000 rpm for 5 min, and the absorbance of the supernatant was determined at 405 nm using a spectrophotometer (UVmini 1240, Shimadzu, Tokyo, Japan). One unit of protease activity was defined as the amount of an enzyme capable of increasing the absorbance by 1 at 405 nm within 1 h.

2.6. Detection, visualization, SDS-PAGE, and fluorescence image analysis

Extracellular fluorescence levels of GFPuv were detected with a Fluoromark Microplate Fluorometer (Bio-Rad, Hercules, CA) by excitation (390 nm) and emission (538 nm). Intracellular green fluorescence in cells was observed using a fluorescence microscope (IX-70, Olympus, Tokyo, Japan) equipped with a CCD camera (U-CMT, Olympus) at 400 x magnification.

To detect the expression of the recombinant protein and to assess its purity, cell lysates, supernatants, and partially purified preparations were subjected to SDS-PAGE [18] on 10 or 12% polyacrylamide gel with the Mini-protein II system (Bio-Rad). Cell lysates were prepared with lysis buffer (50 mM Tris–HCl, pH 7.5, 1% Triton X-100). To detect specific GFPuv–β3GnT2 protein on SDS-PAGE gel, the samples were only mixed with sample buffer without boiling [19], and the molecular bands were then directly observed using Molecular Imager FX (Bio-Rad). The non-specific detection of proteins on the SDS-PAGE gel was performed with Coomassie Brilliant blue (CBB) R-250.

2.7. Characterization of GFPuv–β3GnT2 fusion protein

The purified fusion protein was cleaved with an enterokinase (Stratagene, La Jolla, CA) and deglycosylated with...
Glycopeptidase F (Takara Bio., Otsu, Shiga, Japan) according to the procedure or as described in the handbook supplied. For the denaturation of the purified fusion protein 2.5 μl of the sample was heated at 100 °C for 3 min using a denaturing buffer, and subjected to deglycosylation with Glycopeptidase F. On the other hand, to investigate β3GnT activity, the sample was used for the deglycosylation with Glycopeptidase F. Fusion proteins cleaved with each enzyme were confirmed by SDS-PAGE under the same conditions. Protein concentration was determined using protein assay kit II (Bio-Rad) according to the Bradford method[20].

3. Results

3.1. Construction of recombinant baculovirus

When the GFPuv–β3GnT2 fusion protein was expressed without a signal sequence as an intracellular protein in Sf-9 cells, the molecular weight of the GFPuv–β3GnT2 fusion protein was approximately 77 kDa, but β3GnT did not show biological activity (data not shown). This lack of activity may have been caused by the absence of a signal sequence in the GFPuv–β3GnT2 fusion protein. Because human β3GnT is a type II transmembrane protein usually found in the Golgi apparatus, it has N-glycosylation sites. Therefore, in this construct, we introduced a signal sequence as shown in Fig. 1. The insertion of a 1264 bp PCR fragment containing a truncated human β3GnT2 coding region (amino acids 26–397) and 3′-untranslational sequence into pBlueBacHis2–GFPuv derived from pBlueBacHis2–GFPuv/CAT yielded the plasmid pBlueBacHis2–GFPuv/β3GnT2. Here, we added the melittin signal sequence to the GFPuv–β3GnT2 fusion gene to post-translationally modify β3GnT. PCR was performed with long oligonucleotides encoding the honeybee melittin signal peptide. Finally, the resulting recombinant baculovirus, which had signal peptide sequences from honeybee prepromelittin[15], was designated AcMNPV–GFPuv–β3GnT2. The final constructed expression vector contained a (His)6 tag and GFPuv at the N-terminus of the truncated β3GnT2 to facilitate detection and purification, respectively.

3.2. Expression of fusion protein in BES

Because β3GnT2 was fused to GFPuv, the expression of β3GnT2 was confirmed rapidly by observing GFPuv green fluorescence using fluorescence microscopy, as shown in Fig. 2. Intracellular green fluorescence was observed 18–20h after infection in Sf-9 cells (A) and 16–18h after infection time in Tn-5B1–4 cells (B). A high fluorescence intensity was detected on the edge of both cells due to the accumulation of β3GnT2 in the secretory pathway. Next, at 2–4 days post-infection (d.p.i.), green fluorescence was observed all around the cells and also in the culture broth.
Fig. 3. Time courses of activities of βGnT and protease, cell viability and GFPuv fluorescence intensity in Sf-9 cells (A) and (B) and Tn-5B1–4 cells (C) and (D). The cultivation and virus infection were performed as described in Section 2. Aliquots of suspension were collected every 24 h and cells were separated from culture broth, and then cells were disrupted with lysis buffer. The culture supernatants and cell lysates were used as samples. Symbols in (A) and (C): extracellular (▲) and intracellular (△) βGnT activity, extracellular (■) and intracellular (●) protease activity. Symbols in (B) and (D): extracellular (●) and intracellular (▲) GFPuv fluorescence intensity, (▲) cell viability.

Fig. 4. Fluorescence image analysis of GFPuv–βGnT2 fusion protein in culture supernatants from Sf-9 (lanes 1–4) and Tn-5B1–4 (lanes 5–8) suspension cultures. The methods of cultivation and virus infection were described in Section 2. Proteins were separated by SDS-PAGE (12% gel) and detected by fluorescence image analyzer. Lanes 1–4 denote supernatant of 1–4 d.p.i. of Sf-9, and lanes 5–8 are supernatant of 1–4 d.p.i. of Tn-5B1–4. Arrow indicates molecular weight of purified fusion protein.
proteins were investigated by observing green fluorescence on the SDS-PAGE gel (Fig. 4). In Sf-9 cells, degraded products appeared clearly at 3 d.p.i., but high-molecular weight products still existed at 4 d.p.i. In the case of Tr-5B1–4 cells, degraded fusion proteins appeared at 2 d.p.i. Most of the band products were approximately 40 kDa, but the full-length fusion protein disappeared at 3 d.p.i. This result corresponds to the time course of β3Gnt activity (Fig. 3).

3.3. Purification and characterization of GFPuv–β3Gnt2 fusion protein

To obtain a full-length fusion protein (non-degraded protein), Sf-9 cells that showed a protease activity less than that of Tr-5B1–4 cells were used for protein production. Sf-9 cells were infected with AcMNPV–GFPuv–β3Gnt2 at MOI 10. After 2 days of infection, the fusion protein was purified with a Ni2
d+ NTA agarose resin column. When the resins that absorbed the fusion protein were washed with 40 mM imidazole, 80% of the fusion protein was eluted, with the remaining 20% being eluted with 200 mM imidazole. Using this Ni2
d+ affinity column, the specific activity of β3Gnt2 increased to 4.6 U/mg protein, which was approximately 900-fold in comparison with the activity of the crude enzyme (Table 1). The recovery yield was 20%.

The purified fusion protein was applied to a SDS-PAGE gel, and a major band was observed at 77 kDa, as shown in Fig. 5. Simultaneously degraded proteins at around 31 and 42 kDa were detected. The molecular weights of the purified fusion proteins obtained from Sf-9 cells and Tr-5B1–4 cells were the same (data not shown). The soluble β3Gnt2 was separated from the Histidine tag and GFPuv using an enterokinase, as shown in lane 3 of Fig. 5. Two bands were observed: 45 kDa of β3Gnt2 and 31 kDa of (His)6–GFPuv.

To confirm the glycosylation of β3Gnt2, the fusion protein was treated with Glycopeptidase F, and the results are shown in Fig. 6A. The molecular weight shifted from 77 kDa (lane 2 in Fig. 6A) to 69–70 kDa (lane 3 in Fig. 6A) in response to that of a treatment with Glycopeptidase F. The fusion protein treated with Glycopeptidase F exhibited 63% β3Gnt activity, and contained two low-molecular weight bands than that in the case without the Glycopeptidase F treatment (Fig. 6B). Deglycosylation under the native condition was likely performed incompletely, thus the nature of saccharides is being further investigated.

4. Discussion

Shiraishi et al. [1] have reported the cloning of β3Gnt2 and identification of β3Gnt2, and expressed the β3Gnt2 gene in the BES. However, they did not obtain a sufficient amount of the protein for purification, because of the very low expression level. The expression level of heterologous proteins in the BES greatly depends on the genetic construction of the recombinant baculovirus and culture conditions for insect cells including the type of cell line and medium used. To solve the low expression level of β3Gnt2, we used the honeybee prepromelittin signal sequence for protein secretion. Furthermore, the GFPuv gene was fused into the β3Gnt2 gene for a rapid protein analysis. As for the culture, we used serum-free medium that is suitable
for high protein production, and cultured the cells in shaking flasks with agitation at 100 rpm for sufficient oxygen supply. These modifications contributed to a marked increase in extracellular β3GnT2 production from 0.017 [1] to 0.86 mU/ml. Moreover, β3GnT2 fused to its N-terminal with GFPuv exhibited β3GnT activity, with its specific activity being approximately 50-fold higher than that of a recombinant N-acetylglucosaminyltransferase (LgtA) from Neisseria meningitidis [21]. Although its activity and specific activity cannot be directly compared because of the use of different substrates, GFP fusion techniques and the BES have been found to be suitable for the production of human β3GnT2.

Moreover, by expressing the GFP fusion protein, complicated and time-consuming assays including Western blotting and ELISA can be bypassed. Fluorescence microscopy analysis showed the expression level of β3GnT2 for 16–20 h after infection in Tn-5B1–4 and Sf-9 cells. Higher green fluorescence intensity was detected extracellularly in Tn-5B1–4 cells than in Sf-9 cells (Fig. 2), but the β3GnT activity was less in Tn-5B1–4 (Fig. 3). In Tn-5B1–4 cells, extracellular protease activity reached a maximum after 3 d.p.i., which may explain the decrease in β3GnT activity and β3GnT degradation. The difference between β3GnT activity and green fluorescence intensity proves that protease activity is present, judging from the several low-molecular weight bands on the SDS-PAGE gel. These results indicate that fusion proteins are degraded randomly. In contrast to the extracellular activity, the intracellular protease activity of Tn-5B1–4 cells was as high as that of Sf-9 cells. Sf-9 cells, however, exhibited higher intracellular β3GnT activity than Tn-5B1–4. β3GnT2 activity gradually decreased during cultivation in Tn-5B1–4 cells. Three kinds of protease associated with the BES have previously been found [17,22]. The 40 kDa protease was found at both pre- and post-infections in Sf-9 cell extracts, and the 40 and 36 kDa proteases appeared only at 48 h post-infection with AcM-NPV. A similar viral protease of 36 kDa has been identified.
in *Bombyx mori* nuclear polyhedrosis virus BmNPV [23]. A protease in TsB1–4 cells has not yet been identified. Different types of protease from those in Sf-9 cells may exist in TsB1–4 cells. The addition of protease (carboxyl and cystein protease) inhibitors to a culture and the use of a protease-deficient recombinant baculovirus should contribute to the more efficient production of the GFOUV-β3GT2 fusion protein [24–27].

The molecular weight difference between the intact GFOUV-β3GT2 fusion protein and its deglycosylated form was estimated to be 7–8 kDa (see Fig. 6A). This result suggests that the fusion protein is glycosylated at several sites in the β3GT2 region. In Fig. 5, however, the apparent molecular weight of truncated β3GT2 is 43 kDa and the calculated mass is 43 kDa, indicating that there is one estimated glycosylation site. The fusion protein was incompletely deglycosylated with Glycopeptidase F, resulting in a decrease in β3GT activity only by 23% as compared to that without the Glycopeptidase F treatment (Fig. 6B).

To confirm this, 10 μg/ml tunicamycin was added to the culture, the fusion protein was obtained, but the β3GT activity was not detected (data not shown). These findings suggest that glycosylation is required for active β3GT2.

In the present study, the GFOUV-β3GT2 fusion protein was produced in the BES and was purified. Fusion of GFOUV to β3GT2 can facilitate the rapid observation of its expression level using a fluorescence microscope and is expected to elucidate the protein secretory pathway. Moreover, the investigation of culture conditions, either utilization of a protease-deficient virus or the addition of a protease inhibitor to the culture, should enable us to produce β3GT2 more efficiently.

Acknowledgements

We are very grateful to Professor H. Cha for kindly providing plasmid pBlueBacHis2-GFPuv/CAT.

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