Improvement of GFPuv-β3GnT2 Fusion Protein Production by Suppressing Protease in Baculovirus Expression System

Tatsuya Kato,1 Takeomi Murata,2 Taichi Usui,2 and Enoch Y Park1,†

1Laboratory of Biotechnology, 2Laboratory of Biochemistry, Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan

Received June 2, 2003; Accepted August 22, 2003

The effects of protease inhibitors on the production of recombinant protein were investigated using a recombinant baculovirus containing GFPuv-human β1,3-N-acetylgalactosaminytranslerase 2 (β3GnT2) connected to the prepromelittin signal sequence. The addition of leupeptin as a cysteine protease inhibitor at 2.5 μg/ml improved intra- and extracellular β3GnT activities 5- and 3-fold, respectively, compared to those without addition, which was due to a suppression of protease activity. With the leupeptin addition only four degraded molecular bands lower than 32 kDa appeared, but 9 degraded molecular bands between 29 kDa and 41 kDa existed without addition. In contrast, pepstatin A as a carboxyl protease inhibitor had no influence on the improvement of β3GnT production, judging from SDS-PAGE. Moreover, when 50 μM carboxenzyoxy-L-leucyl-L-leucyl-L-leucinal (MG-132), known as a proteasome inhibitor, was used in combination with the leupeptin, a ladder of low molecular mass bands of fusion protein was diminished. The intracellular β3GnT activity increased 9-fold, to as high as that without addition of two kinds of protease, but the extracellular activity was not different from that with the addition of only leupeptin. These findings indicate that the decrease in cell viability causes the decrease in the secretion rate of intracellular fusion protein, resulting in the accumulation of the full-length of fusion protein.

Key words: baculovirus-insect cell expression system; human β1,3-N-acetylgalactosaminytransferase 2 (β3GnT2); leupeptin; carboxenzyoxy-L-leucyl-L-leucyl-L-leucinal (MG-132); protease inhibitor

Human β1,3-N-acetylgalactosaminytransferase (β3GnT) is indispensable to the synthesis of lacto-N-tetraose II converted into lacto-N-tetraose and lacto-N-neotetraose,10 so that several kinds of human β1,3-N-acetylgalactosaminytransferases (β3GnT2-7) have recently been cloned using EST sequences and PCR technology.2-9 Their characterizations have been incomplete, however, because the expression levels in a heterogeneous expression system have been too low to measure the amount of purified protein.10 Still, a sufficient amount of β3GnT is needed as soon as possible, for not only investigation about biochemical and biological properties including protein structure analysis, but also for efficient syntheses of oligosaccharides.

The baculovirus expression system (BES) is a very adequate tool for efficient production of eukaryotic proteins that require correct folding and posttranslational modifications such as processing of signal peptides,6 phosphorylation,7 and glycosylation.8 However, Autographa californica nuclear polyhedrosis virus (AcNPV) has a cysteine protease, V-CATH, which plays an important role in the liquefaction of virus-infected hosts. The Sf-9 cells commonly used in the BES also have a protease different from V-CATH, a carboxyl protease. These proteases cause the degradation of expressed recombinant proteins, which hinders their efficient production in the BES.9,11 The V-CATH has a cathepsin L-like sequence, and its substrate specificity is similar to that of cathepsin B.12,13 When insect cells are infected with baculoviruses, the V-CATH accumulates as a propeptide, proV-CATH in infected cells, and the death of infected cells provokes activation of the V-CATH.14 Bombyx mori nuclear polyhedrosis virus (BmNPV) also has a V-CATH-like cysteine protease.15

To prevent proteolytic degradations of expressed proteins, cysteine protease-deficient viruses were constructed.16 In fact, efficient productions of firefly luciferase and human growth factor in silkworm with cysteine protease-deficient BmNPV were accomplished.17 Alternatively, the addition of protease inhibitors to the culture medium was also effective. Cysteine protease and carboxyl protease inhibitors were selected, commonly. In the case of expression of human proapolipoprotein A-I in SF-21 cells, the addition of a cysteine protease inhibitor, leupeptin, and a carboxyl protease inhibitor, pepstatin A, allowed its production to increase.10 However, some

† To whom correspondence should be addressed. Tel/Fax: +81-54-238-4887; E-mail: yspark@agr.shizuoka.ac.jp
protease inhibitors have been reported to be toxic to host cells.\textsuperscript{18,19} Moreover, protease inhibitors are expensive and have relatively short half-lives. Therefore, appropriate timing of the addition of protease inhibitors as well as their concentrations and combinations are important for improving recombinant protein production in BES.

In this study, we expressed human $\beta$3GnT2 fused with honeybee prepromelittin signal peptide and GFPuv in suspension culture of insect cells, and investigated the effects of several protease inhibitors on the production of $\beta$3GnT2-GFPuv fusion protein. Since GFPuv can be detected easily and rapidly without any substrate or cofactor, fusion with GFPuv enables us to visualize the degradation of fusion protein and to analyze the effects of proteases and protease inhibitors on SDS-PAGE gel. This technique is useful for improving protein production in the BES, and studies of proteases and protease inhibitors.

Materials and Methods

Cell lines and media. SF9 cells derived from Spodoptera frugiperda and Tn-5Bl-4 cells from Tricopila ni were purchased from Invitrogen (San Diego, CA, USA) and grown in 25 cm\textsuperscript{2} tissue culture flasks (Falcon) and 100 ml flasks. SF-900 II and Express Five media (Invitrogen) with the addition of 1\% antibiotic-antimycotic (Invitrogen) were used in the insect cell culture.

Construction of recombinant baculoviruses. The 1,264 bp DNA fragment of a truncated segment and 3'-untranslational region of $\beta$3GnT2 were obtained by PCR with oligonucleotide primers with BamH I and EcoR I restriction sites, respectively, from cDNA of Quick-Clone human fetal brain cDNA (Clontech, Palo Alto, CA, USA). The designed primers are as follows: the forward primer 1, 5'-CGGGATCCGGAACTTCTCCAAAGCATGACCAAG-3'; the reverse primer 1, 5'-CGGAATTCGAGGTTTGAGGGCCTCATAATGG-3'. The CAT gene was excised from pBlueBacHis2-GFPuv/CAT,\textsuperscript{20} and the resulting plasmid was named pBlueBacHis2-GFPuv. The amplified fragment of $\beta$3GnT2 was inserted between the BamH I and EcoR I sites of pBlueBacHis2-GFPuv to yield pBlueBacHis2-GFPuv/$\beta$3GnT2. To enable introduction of a new signal peptide coding region, the GFPuv-$\beta$3GnT2 fusion fragment was amplified by PCR using reverse primer 1 and long forward primer, which encode the honeybee prepromelittin signal peptide.\textsuperscript{21} The forward primer 2 was used as follows: 5'-CACCATGAAATTTTCTTATGAACTTCTGGCCTGGCTTTGAGTTGCTGATACATTTCTTATGCTTGGCCCTCCGCGGTTTCTCATC-3'. This PCR product including the signal sequence and the GFPuv-$\beta$3GnT2 fusion gene was inserted into the entry vector, pENTR/D-TOPO (Invitrogen). From the resulting plasmid, named pENTR/D/GFPuv-$\beta$3GnT2, the GFPuv-$\beta$3GnT2 fusion gene connected to the signal sequence was removed and inserted into the pDEST8 donor vector according to the protocol provided by Gateway Cloning Technology (Invitrogen). The production of recombinant baculovirus was done according to the protocol of Bac-to-Bac Expression System (Invitrogen). In brief, the recombinant donor vector, pDEST/GFPuv-$\beta$3GnT2, was used to transform Escherichia coli DB10 containing a bacmid. Next, transposition between the bacmid and the recombinant donor vector occurred, and the recombinant bacmid was produced, which was then extracted from E. coli and transfected into Sf-9 cells. Finally, the recombinant baculovirus obtained was recombinant AcNPV-GFPuv-$\beta$3GnT2 containing the honeybee melittin signal sequence and the GFPuv-$\beta$3GnT2 fusion gene under the control of the viral polyhedrin promoter. All PCR fragments were confirmed by dideoxynucleotide chain terminating sequence\textsuperscript{22} using Thermo Sequenase Cycle Sequencing Kit (USB Co., Cleveland, Ohio, USA).

Infection with recombinant viruses. Two or three million cells/ml of SF9 and Tn-5Bl-4 cells were infected at a multiplicity of infection (M.O.I) of 10 with a recombinant baculovirus. The suspension culture was done in a 100-ml flask with a working volume of 20 ml in the specified medium. Agitation and temperature were controlled at 100 rpm and 27°C, respectively. The protease inhibitors were added to the culture 1 d post infection time. Aliquots of the suspension culture were taken every 24 h and centrifuged to separate the culture supernatant and cells.

$\beta$3GnT and protease assay. The $\beta$3GnT activity was measured in 50 mM Tris-HCl (pH 8.0), 15 mM MnCl\textsubscript{2}, 19 mM UDP-GlcNAc, 22 mM Gal\textsubscript{1}-4 GlcNAC\textsubscript{β}-pNP, and 5 $\mu$l of enzyme solution (total volume 25 $\mu$l). The reaction started as the $\beta$3GnT sample was added. At each sample time, 5 $\mu$l of the reaction mixture was added to 195 $\mu$l of distilled water, followed by boiling for 5 min. After filtration through a 0.45-μm nitrocellulose filter (Millipore, Bedford, Massachusetts, USA), the filtrates were analyzed by HPLC. Mightsyil RP-18 (H) GP 150-4.6 (KANTO CHEMICAL Co., Inc.) was used as the column. Reaction products were eluted with 10\% methanol and detected at the absorbance of 300 nm. HPLC was done at 40°C with a flow rate of 1.0 ml per min. One unit of enzyme activity is defined as the amount of enzyme capable of the transfer of 1 μmol of GlcNAc per minute.

A protease assay under neutral pH was done as described in the method of Hom et al.\textsuperscript{14} First, 83 $\mu$l
of sample was added to 583 μl of 0.2% azocasein in 50 mM sodium phosphate buffer (pH 7.2). The mixture was incubated at 37°C for 4 h, followed by the addition of 27 μl of 100% TCA to stop the reaction. Samples were centrifuged at 15,000 rpm for 5 min, and 333 μl of 9 M urea was added to the supernatant before measuring the absorbance at 410 nm.

**SDS-PAGE and fluorescent image analysis.** To detect the expression of recombinant fusion protein, cell lysates and supernatants were separated by SDS-PAGE on 10 or 12% polyacrylamide gel with the Mini-protean II system (Bio-Rad Co., Ltd., Hercules, CA, USA). Cell lysates were prepared with lysis buffer (50 mM Tris-HCl pH 7.5, 1% TritonX-100). The detection of GFPuv-β3GnT2 fusion protein on SDS-PAGE gel was done with Coomassie Brilliant blue (CBB) R-250. In the case of detection of fluorescent GFPuv-β3GnT2 fusion protein on SDS-PAGE gel, samples were suspended with sample buffer, and their fluorescent bands for GFPuv-β3GnT2 fusion protein were detected with a Molecular Imager FX (Bio-Rad). The quantitative analysis of GFPuv intensity of fusion protein on SDS PAGE gel was done using Quantity One software (ver. 4, Bio-Rad).

**Results**

**Difference in expression of GFPuv-β3GnT2 fusion protein between Sf-9 and Tn-5B1-4 cells**

Human β3GnT2 is a transmembrane protein in the Golgi apparatus and has five putative N-glycosylation sites. The β3GnT2 requires a signal sequence in its N-terminus to modify posttranslationally. In this study, the cytosolic and transmembrane region of human β3GnT2 was replaced with a signal sequence from honeybee prepromelittin, a histidine-tag, and GFPuv. Sf-9 and Tn-5B1-4 cells were infected with AcNPV-GFPuv-β3GnT2 at M.O.I. 10 and sampled at each post-infection time. Culture supernatants and cell lysates were analyzed by SDS-PAGE (Fig. 1). Fusion proteins were detected by its GFPuv fluorescence. In the case of Sf-9 cells, 4 d post infection time the full-length fusion protein (77 kDa) remained in the culture supernatant and cell lysate, but in the case of Tn-5B1-4 cells without addition of protease inhibitor, only low molecular weight proteins appeared at 2 d post infection time, while the fusion protein disappeared at the 3 d post infection time (Fig. 1). These results indicate that the Tn-5B1-4 cell culture was affected more severely by protease than the Sf 9 cell culture.
Effects of protease inhibitors on degradation of GFPuv-β3GnT2 fusion protein

The production of recombinant protein in the BES frequently decreased after 3 days of post infection time. When GFPuv genes are expressed in BES with SF-9 cells, Gotoh et al. have reported that carboxyl and cysteine proteases are released into the culture medium. To avoid the proteolytic degradation of β3GnT2, the effects of protease inhibitors on β3GnT2 production were investigated visually and biologically in the culture of Tn-5B1-4 cells because the resulting proteolytic degradation of β3GnT activity is more than that in Sf-9 cell culture. After Tn-5B1-4 cells were infected with AcNPV-GFPuv-β3GnT2, protease inhibitors were added to the culture medium 1 d post infection time at the concentration of 0-2.5 of μg/ml. From these preliminary experiments, 2.5 of μg/ml of protease inhibitor was found to be enough to investigate the effects of protease on production of the fusion protein (data not shown). When 2.5 of μg/ml of leupeptin as a cysteine protease inhibitor and 2.5 of μg/ml pepstatin A as a carboxyl protease inhibitor were added separately, the changes in the molecular bands of GFPuv-β3GnT2 fusion protein on SDS-PAGE gel are also shown in Fig. 1. When leupeptin was added, the production of GFPuv-β3GnT fusion protein was improved intracellularly and extracellularly because of the suppressed protease activity. The 4 kinds of molecular mass protein lower than 32 kDa appeared (Fig. 2) and their total amount was considerably lower than that without addition of protease inhibitor. In contrast, in the case of the addition of pepstatin A 9 kinds of molecular mass protein were shown in the supernatant, but the full-length fusion protein band had disappeared at 4 d post infection time. Eighty percent of the total amount was existed between 32 kDa and 39 kDa (Fig. 2). The molecular weight distribution of fluorescent bands was almost the same as that of the control. Although pepstatin A and leupeptin were added to the culture, synergistic effects on β3GnT activity were not observed, only activity similar to that occurring with the addition of leupeptin (data not shown).

Effects of protease inhibitors on production of GFPuv-β3GnT2 fusion protein

The β3GnT activity of the culture supernatant in SF-9 cells increased to 0.81 mU/ml (Fig. 3A). However, that of Tn-5B1-4 cells increased to 0.68 mU/ml, but decreased drastically at 3 d post infection time (Fig. 3C). Although the intracellular protease activities of Tn-5B1-4 cells were similar to that of SF9 cells, the extracellular protease activity at 3 d post infection time increased three-fold in comparison with that of SF9 cells (B and D in Fig. 3), which might have caused the decrease in β3GnT activity by proteolytic degradation.

The β3GnT activity of the culture supernatant with the addition of leupeptin was 2.1 mU/ml (Fig. 3C), which was approximately three-fold higher than that without its addition. Moreover, the β3GnT activity of the cell lysate was 1.78 mU/ml, which was approximately five-fold higher. Extracellular protease activity was almost completely suppressed by leupeptin, but intracellular protease activity was still detected even with the addition of 2.5 μg/ml of leupeptin (Fig. 3D). When 0.25 μg/ml of leupepeptin
was added, the extracellular $\beta 3$GnT activity was also 2.1 mU/ml, the same as that with 2.5 $\mu$g/ml of leupeptin (data not shown), indicating that leupeptin is an effective protein inhibitor in the BES for the production of gene products.

Effects of 26S proteasome inhibitor on production of GFPuv-$\beta 3$GnT fusion protein

If cysteine and carboxyl proteases are active in the BES, cysteine and carboxyl protease inhibitors should suppress intracellular protease activity; nevertheless, protease activity was detected, as shown in Fig. 3. These results indicate that a protease different from cysteine and carboxyl proteases may exist actively in the Tn-5Bl-4 cell. If these intracellular proteases were suppressed completely, the production of GFPuv-$\beta 3$GnT fusion protein would be improved.

Degraded molecular bands of fusion protein on SDS-PAGE gel in Fig. 1 were similar to those in the ubiquitin-proteasome degradation pathway. To investigate this, Tn-5Bl-4 cells infected with recombinant virus were cultivated with the addition of both 2.5 $\mu$g/ml leupeptin and 0-200 $\mu$M of proteasome inhibitor, carboxybenzoy-L-leucyl-L-leucyl-L-leucinal (MG-132) at 1 d post-infection time. The results are shown in Fig. 4. A prominent decrease in the levels of fluorescent low-molecular-mass proteins was observed in the culture supernatant and cell lysate 2-4 d post infection time. With the addition of both 50 $\mu$M MG-132 and leupeptin, amounts of low-molecular-mass proteins were less than those of leupeptin. Moreover, when 100 mM MG-132 was added, only a small amount of fusion protein was degraded. However, with the addition of 200 $\mu$M MG-132 there were few fluorescent low-molecular-weight-mass proteins, and the full-length fusion protein still remained, though in small quantities. With the increase in the amount of MG-132 in the presence of leupeptin the degradation of the full-length fusion protein was lessened (Fig. 5). When 0, 50, and 100 mM MG-132 were added, 15, 54, and 97% of the full-length fusion protein, respectively, remained at the 3 d post-infection time; 10, 21, and 27% of the full-length fusion protein, respectively, remained at 4 d post-infection time.

The intracellular $\beta 3$GnT activity in the presence of 50 or 100 $\mu$M MG-132 increased approximately two-fold as compared to that without addition of MG-132, but decreased at 4 d post-infection time (Fig. 6A). The intracellular protease activity was suppressed to 0.06 U/ml by MG-132, which corresponded to 30% and 15% in comparison with the activity occurring with the addition of only leupeptin and without the addition of any protease inhibitor, respectively (Fig. 6B). In contrast, the extracellular $\beta 3$GnT activity did not change drastically during the culture, except with the addition of 200 $\mu$M MG-132 (Fig. 6C). The addition of 200 $\mu$M MG-132 forced large decreases in $\beta 3$GnT activity (A and C in Fig. 6), despite the disappearance of proteolytic degradation.

Fig. 4. GFPuv Fluorescent Analysis of GFPuv-$\beta 3$GnT2 Fusion Protein in Culture Supernatant from Virus-infected Tn-5Bl-4 Cell Suspension Culture Supplemented with 0-200 $\mu$M MG-132 on SDS-PAGE Gel.

Numbers and arrow are the same as those of Fig. 1.

Fig. 5. GFPuv Fluorescent Intensity of the Full-length Fusion Protein at 1 d (■), 2 d (□), 3 d (△), and 4 d (□) Post-infection Time of Each Experiment.

GFPuv fluorescent intensity of the full-length fusion protein as shown in Fig. 4 was measured using Quantity One software and is presented as a relative value to that at 1 d post-infection time.
on SDS-PAGE gel (see Figs. 4 and 5). Therefore, 50 μM MG-132 may be sufficient to avoid the intracellular proteolytic degradation of recombinant protein.

Cell viability was 85%, 55%, 25%, and 2% at 1 d, 2 d, 3 d, and 4 d post-infection time, respectively, which was irrespective of MG-132 addition. These results indicate that MG-132 dose not have an effect on cell viability.

**Discussion**

The BES has been widely used for production of foreign gene products, but the expression of heterologous proteins is greatly dependent on the genetic construction of recombinant baculovirus and culture conditions of the insect cells including cell line and media. Shiraishi *et al.* reported the identification of the β3GnT2 and expressed the β3GnT2 gene in the BES. However, they could not obtain enough of the protein for purification, because of very low expression (Table 1). To solve this low expression of β3GnT2, we used a honeybee prepromelittin signal sequence for protein secretion. Furthermore, the GFP<sub>pr</sub> gene was fused into the β3GnT2 gene for a rapid protein analysis. As for the culture, we used a Tn-5B1-4 cell line combined with serum-free Express Five medium, which was suitable for high protein production, and cultured the cells in shaking flasks with agitation of 100 rpm for a sufficient oxygen supply. These modifications contributed to a drastic increase of extracellular β3GnT2 production from 0.017 mU/ml to 0.68 mU/ml (Table 1).

However, problems in using the BES for the production of heterologous proteins are the markedly decreased productivities of proteins occurring due to protein degradation during the later stages of infection. Baculoviruses have cysteine proteases by which host degradation after death occurs.<sup>14,15</sup> Moreover, a carboxyl protease from insect cells has been observed in the culture supernatant of infected

---

**Table 1.** Comparison of Genetic Construction and Culture Method on the Production of β3GnT2 Fusion Protein with Previous Study

<table>
<thead>
<tr>
<th>Genetic construction of recombinant baculovirus</th>
<th>Previous work&lt;sup&gt;30&lt;/sup&gt;</th>
<th>This work</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino acids of β3GnT2</strong></td>
<td>human gastric cDNA library</td>
<td>human fetal brain cDNA</td>
</tr>
<tr>
<td><strong>Origin of cDNA</strong></td>
<td>—</td>
<td>GFP&lt;sub&gt;pr&lt;/sub&gt;</td>
</tr>
<tr>
<td><strong>Fused gene</strong></td>
<td>—</td>
<td>honeybee prepromelittin signal sequence</td>
</tr>
<tr>
<td><strong>Signal sequence</strong></td>
<td>human immunoglobulin κ</td>
<td>his tag</td>
</tr>
<tr>
<td><strong>Tag for purification</strong></td>
<td>FLAG peptide</td>
<td></td>
</tr>
</tbody>
</table>

| **Culture**                                   | Sf9, Sf21                 | Sf9, Tn-5B1-4 |
| **Media**                                     | TNM-FH                    | Express Five |
| **Serum in media**                            | unknown                   | free        |
| **Culture method**                            | monolayer                 | suspension in shaker |

| **Production**                                |                          |             |
| **β3GnT2 production**                         | 0.017<sup>a</sup>        | 0.81<sup>b</sup> |
| **(mU/ml of medium)**                         | extracellular activity    | 0.68<sup>c</sup> |
| **with the addition of leupeptin**            | —                        | 2.09<sup>c</sup> |

<sup>a</sup> Cited from reference 2. Unit was converted from pmol/ml of medium/h to mU/ml of medium.
<sup>b</sup> Data from the culture of Sf9 cells (Fig. 3).
<sup>c</sup> Data from the culture of Tn-5B1-4 cells (Fig. 3).
Those proteases degrade recombinant proteins in virus-infected cell cultures. Several researchers have increased protein production by adding cysteine and carboxyl protease inhibitors to the culture medium. In these experiments, with the addition of 2.5 µg/ml of leupeptin the extracellular \( \beta \)-3GnT activity was 3-fold higher than that without addition (Table 1). The extracellular protease activity in Tn-5B1-4 cells with the addition of leupeptin after viral infection was completely suppressed (Fig. 3). However, a carboxyl protease inhibitor, pepstatin A, had no effect on the productivity of GFPuv-\( \beta \)-3GnT2 fusion protein. These findings suggest that Tn-5B1-4 cells may lack carboxyl protease or may have little activity.

From GFPuv fluorescent analyses on SDS-PAGE gels a ladder of GFPuv-fused proteins was also observed in culture supernatant of recombinant virus-infected Tn-5B1-4 cells with the addition of 2.5 µg/ml leupeptin to the culture medium. This ladder was supposed to form as the result of proteolytic degradation by ubiquitin-proteasome pathway. In the case of hWRS TYMV-RDRPs expression in Tn-5B1-4 and Sf-9 cells, a ladder of proteins with low molecular weight was also observed in the cell lysate. However, its ladder of the GFPuv-\( \beta \)-3GnT fusion protein appeared mainly in the culture supernatant. It is speculated that the extracellular ladder of the fusion protein may result from the secretion of intracellular proteins degraded through the ubiquitin-proteasome pathway or extracellular degradation by proteasome leaked from cells. The former appears more likely because with the addition of MG-132, the intracellular protease activity was suppressed to one-fourth that without the addition (Fig. 6). Ju and Cummings have reported previously that unfolded human core I \( \beta \)3-galactosyltransferase in the endoplasmic reticulum (ER), known as type II transmembrane protein, is the same type of \( \beta \)3GnT2 that is degraded by proteasome in Jurkat cells. Therefore, some amounts of GFPuv-\( \beta \)-3GnT2 fusion protein expressed in Tn-5B1-4 cells appear to be unfolded in the ER and might be degraded by a proteasome. Alternatively, 20S proteasome, a part of the 26S proteasome, may also be involved in ubiquitin-dependent and ubiquitin-independent pathways in intracellular proteolysis.

When both MG-132 and leupeptin were added, only the intracellular \( \beta \)-3GnT activity improved, while the extracellular activity did not increase. However, with the increase in the MG-132 amount the full-length of the fusion protein remained without degradation (Fig. 5), suggesting that increased intracellular fusion protein due to the presence of MG-132 as a protease inhibitor remained undegraded. This indicates that, though the MG-132 suppressed the degradation of fusion protein, the ability of protein secretion decreased due to decrease in cell viability, the intracellular fusion protein might have accumulated in the cell. From these results, therefore, if the cell viability was maintained longer, the fusion protein production would be improved with addition of MG-132 in the presence of leupeptin.

In this study, the use of the honeybee prepromelitin signal sequence and the improved culture with the addition of a cysteine protease inhibitor, leupeptin, allowed intracellular and extracellular \( \beta \)-3GnT activities to be 5- and 3-fold higher than those without addition. Moreover, in the presence of a proteasome inhibitor, MG-132, in Tn-5B1-4 cell culture, the results obtained strongly suggest that the GFPuv-\( \beta \)-3GnT2 fusion protein expressed in the BES was degraded through the ubiquitin-proteasome pathway. The MG-132 suppressed the ladder of fusion protein observed on SDS-PAGE (Fig. 4). Though the extracellular activity did not differ significantly based on the addition of MG-132, the intracellular \( \beta \)-3GnT activity increased 9-fold higher than that without addition (Fig. 6) and the full-length of fusion protein remained undegraded (Fig. 5). The combination of leupeptin as a cysteine protease inhibitor and MG-132 as a proteasome inhibitor was useful to improve the production of GFPuv-\( \beta \)-3GnT2 fusion protein in BES.

Acknowledgments

The authors thanks professor H. J. Cha for kindly providing the plasmid pBlueBacHi52-GFPuv/CAT.

References


4) Iwai, T., Inaba, N., Naundorf, A., Zhang, Y., Gotoh, M., Iwasaki, H., Kudo, T., Togayachi, A., Ishizuka, Y., Nakanishi, H., and Narimatsu, H., Molecular cloning and characterization of a novel UDP-GlcNAc: GalNAc-peptide \( \beta \),1,3-N-acetyl-