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Effect of 1-deoxynojirimycin on the Replication of Baculoviruses, *Bombyx Mori* Nucleopolyhedrovirus and *Autographa Californica* Multiple Nucleopolyhedrovirus

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1-Deoxynojirimycin (DNJ) is an alkaloid that is found at relatively high concentrations in mulberry leaf and tissues of the silkworm, *Bombyx mori*. DNJ is a well known inhibitor of α-glucosidase, an enzyme that is involved in the early stages of the *N*-linked glycoprotein synthesis pathway. α-Glucosidase activity in the cell extract from *B. mori*-derived Bm5 cells showed approximately 40-fold less sensitivity to DNJ than α-glucosidase activity in the cell extract from *Spodoptera frugiperda*-derived Sf9 cells. The replication of *B. mori* nucleopolyhedrovirus (BmNPV) was not inhibited when it was propagated in BmN cells that were grown in medium containing up to 10 mM DNJ. In contrast, the replication of *Autographa californica* multiple NPV (AcMNPV) was reduced by 67% when it was propagated in Sf9 cells that were grown in medium containing 10 mM DNJ. The viability of Bm5 and Sf9 cells that were grown in medium containing up to 10 mM DNJ was not affected. Our results suggested that the reduced replication of AcMNPV was the result of the higher sensitivity of α-glucosidase activity in Sf9 cells to DNJ.

Key words: 1-Deoxynojirimycin, *Bombyx mori* nucleopolyhedrovirus, BmNPV, Baculovirus, α-Glucosidase inhibitor

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Introduction

Glycosylation is an essential process in eukaryotic cells that attaches glycans to proteins and other biomolecules. In many proteins, appropriate glycosylation is essential for proper folding, authentic biological activity, and/or for normal protein stability. The most common types of glycoproteins are *N*-linked (glycans attached to a nitrogen of asparagine or arginine) and *O*-linked (glycans attached to the hydroxyl oxygen of serine, threonine, tyrosine, hydroxylysine or hydroxyproline). α-Glucosidase and mannosidase are the key enzymes involved in the *N*-linked glycosylation pathway (Francis *et al*., 2002). The first step of *N*-linked glycosylation generally involves the transfer of $Glc_3Man_9GlcNAc_2$ (a 14-sugar precursor formed by 3 glucose, 9 mannose, and 2 *N*-acetylglucosamine molecules) to a nascent protein in the endoplasmic reticulum (ER). Subsequently, the attached $Glc_3Man_9GlcNAc_2$ is processed by glycosidases such as α-glucosidase I, α-glucosidase II, α-mannosidase I, α-mannosidase II, and glycosyltransferases such as acetylglucosaminyltransferase I (Ren *et al*., 1997). The initial steps in the processing of complex oligosaccharides occur in the ER lumen and involve the removal of the terminal glucose of $Glc_2Man_9GlcNAc_2$ by α-glucosidase I. Subsequently, α-glucosidase II removes the next two glucose residues and α-mannosidase I removes one mannose residue. The resulting glycoprotein precursor ($Man_8GlcNAc_2$–protein) moves to the Golgi complex where three more mannose residues are removed by α-mannosidase I found within the Golgi. *N*-acetylglucosamine is then attached by *N*-acetylglucosaminyltransferase I and subsequently the

terminal mannose residues are removed by α-mannosidase II. The product, $GlcNAc_2Man_3GlcNAc_2$–protein can be further processed by various enzymes to generate more complex oligosaccharide structures.

1-Deoxynojirimycin (DNJ) is a naturally occurring alkaloid that inhibits glycosidases such as α-glucosidase I and α-glucosidase II that are involved in the early processing steps of $Glc_3Man_9GlcNAc_2$ (Moremen *et al.*, 1994). Relatively high concentrations of DNJ are found in the leaves of mulberry trees and in silkworm, *Bombyx mori* (Asano *et al.*, 2001), a caterpillar that feeds exclusively on mulberry. DNJ can also be purified from the culture medium of microorganisms including some species of *Bacillus* and *Streptomyces* (Kim *et al.*, 2011). DNJ can inhibit α-glucosidase activity in the small intestine of man and other mammals. By inhibiting this α-glucosidase activity the amount of free glucose that is generated after a meal is reduced. This reduction in free glucose results in a reduction in the rate of glucose uptake and corresponding reduction in blood glucose levels after a meal. DNJ also functions as an antiviral of enveloped viruses, such as Hepatitis B virus (HBV), Hepatitis C virus (HCV), and bovine virus diarrhea virus (BVDV) (Dwek *et al.*, 2002). Since glycoproteins play important roles in the attachment of enveloped viruses to host cells DNJ may also be involved in altering viral surface glycoproteins so that recognition by receptor(s) on the host cell surface is altered.

Nucleopolyhedroviruses (NPVs) belong to the family Baculoviridae. NPVs are characterized by enveloped nucleocapsids and large, circular, double-stranded DNA genomes (Rohrmann, 2011). Baculoviruses have a unique biphasic replication cycle that involves the formation of two types of progeny virions: budded virus (BV) and occlusion-derived virus (ODV). BV is produced during an early stage of the replication cycle and obtains its envelope as it buds through the cell plasma membrane. ODV on the other hand is formed during a late stage of the replication cycle and obtains its envelope "de novo" while in the cell nucleus. The ODV is subsequently occluded in a protein matrix resulting in the formation of a granule (containing a single ODV in the case of granulovirus) or a polyhedron (containing multiple ODVs in the case of NPV). ODVs are primarily involved in insect-to-insect transmission, whereas BVs are involved in cell-to-cell transmission within a single host. A virus-encoded glycoprotein called GP64 is the major glycoprotein found in the envelope of BVs (Jarvis and Garcia 1994). GP64 is required for binding of the BV to a host cell and subsequent fusion of the BV envelope and endosomal membrane during the penetration phase of the virus infection cycle (Volkman *et al.*, 1984). During a later phase of baculovirus infection, the amount of GP64 that is found in the host cell membrane is related to the efficiency of BV production and rate of systemic infection in insects (Nagai *et al.*, 2011).

In this study, we investigated the effect of DNJ on the replication of *B. mori* NPV (BmNPV) and *Autographa californica* multiple NPV (AcMNPV). The host of BmNPV is the silkworm a specialist that feeds exclusively on mulberry, a plant with relatively high levels of DNJ. AcMNPV on the other hand replicates on generalist insects that commonly feed on plants with relatively low DNJ levels (Hirayama *et al.*, 2007). In addition, we characterized α-glucosidase activity in *B. mori*-derived Bm5 and *Spodoptera frugiperda*-derived Sf9 cell extracts. Bm5 and Sf9 are specific hosts of BmNPV and AcMNPV, respectively. We found that the replication of BmNPV on Bm5 cells was unaffected by the addition of DNJ into the culture medium, whereas AcMNPV replication on Sf9 cells was sensitive to DNJ in the culture medium.

Materials and Methods

Chemicals

DNJ was purified from *Bacillus subtilis* MORI as described previously (Kim *et al.*, 2011). The α-glucosidase inhibitors acarbose and voglibose were purchased from BayerKorea (Korea) and CJ (Korea), respectively. The α-glucosidase substrate *p*-nitrophenyl α-D-glucopyranoside (PNPG) was purchased from Sigma (USA).

Cells and viruses

Bm5 and Sf9 cells were maintained in TC-100 medium (Welgene, Korea) supplemented with 5% and 10% fetal bovine serum, respectively, at 27°C. *B. subtilis* MORI was propagated in TSB medium at 37°C as described previously (Kim *et al.*, 2011). BmNPV and AcMNPV were propagated on Bm5 and Sf9 cells, respectively. Viral titer was determined by the end-point dilution method as described previously (O'Reilly *et al.*, 1992).

Preparation of cell extract

Extracts of Bm5 and Sf9 cells were prepared from 1×10^7 cells that were pelleted by centrifugation at 3,000 x g for 5 min at 4°C. Following the centrifugation the cells were resuspended in 50 mM sodium phosphate buffer (pH 7) and the cells were sonicated on ice for 1 min using a VCX130 ultrasonic processor (Sonics & Materials, USA). The extracts were centrifuged at 3,000 x g for 5 min at 4°C and then stored at –20°C prior to use. The protein concentration of the cell extract was measured by the method of Bradford (1976).

Virus replication assay

Bm5 or Sf9 cells were seeded into the wells of a 24 well plate (1×10^5 cells per well) and allowed to attach for 2 h. Subsequently, the cells were inoculated with virus at a multiplicity of infection (MOI) of 1. Following a 1 h-long viral attachment period, the inoculum was removed by aspiration and the cells were cultured in 0.5 mL of fresh medium containing 0, 1, 2.5, 5 or 10 mM DNJ. After a 4 day-long incubation period at 27°C, the virus titer in the cell culture supernatant was determined by the end-point dilution method.

Cell viability assay

Cell viability was determined using a colorimetric XTT assay (Cell Proliferation Kit; Biological industries, Israel) following the manufacturer's protocol. Bm5 or Sf9 cells were seeded into the wells of a 96 well plate (1×10^4 cells per well) and allowed to attach for 2 h. Following cell attachment, the old cell culture supernatant was replaced with 100 µL of fresh medium containing 0, 1, 2.5, 5 or 10 mM DNJ and incubated at 27°C for 48 h. After the 48 h-long incubation, 50 µL of activated XTT reagent was added to each well of the 96-well plate and incubated at 27°C for 4 h. Subsequently, the formation of reduced XTT was measured at 450 nm using a microplate reader (Molecular devices, USA) as described previously (Wu *et al*., 2002).

α-Glucosidase activity assay

α-Glucosidase activity in the cell extract was measured using PNPG as a substrate in the wells of a 96 well plate. The ability of the cell extract to hydrolyze PNPG was determined in a 150 µL reaction containing 80 µg of cell extract and 4 mM PNPG in 100 mM sodium phosphate buffer, pH 7. The reaction mixture was incubated for 30 min at 37°C and then 50 µL of 234 mM Na_2CO_3 was added to terminate the reaction. Absorbance was measured at 405 nm using a microplate reader as described previously (Kang *et al*., 2010).

The effect of pH on α-glucosidase activity was determined in 100 mM citrate buffer (pH 3, 4, 5, and 6), 100 mM sodium phosphate buffer (pH 7 and 8) or 100 mM glycine-NaOH buffer (pH 9, 10, 11). The effect of temperature on α-glucosidase activity was determined in 100 mM sodium phosphate buffer, pH 7, at 5°C intervals from 25°C to 70°C.

Inhibition of α-glucosidase activity by DNJ, acarbose, and voglibose

The ability of DNJ (0.03 – 1000 µM), acarbose (200 – 1,000 mM), and voglibose (3 – 200 mM) to inhibit α-glucosidase in Bm5 and Sf9 cell extracts was determined using PNPG as a substrate in 100 mM sodium phosphate buffer, pH 7, as described above. Each inhibitor, dissolved in water, was preincubated with the Bm5 or Sf9 cell extract for 5 min at 37°C prior to the addition of PNPG. Following the addition of the PNPG, the reaction was incubated for 30 min at 37°C and then read at 405 nm as described above. The median inhibitory concentration (IC_{50}) of each compound was determined by regression analysis (SigmaPlot; SPSS Scientific, USA).

Results

Effect of DNJ on the replication of BmNPV and AcMNPV

DNJ is a known inhibitor of α-glucosidase activity as well as the replication of various viruses. The ability of DNJ to inhibit the replication of BmNPV and AcMNPV was determined in Bm5 and Sf9 cells that were grown in medium containing 1 to 10 mM DNJ. After 4 days, DNJ showed no significant effect on BmNPV replication in Bm5 cells at a concentration as high as 10 mM (Fig. 1). In contrast, AcMNPV replication was significantly reduced in the presence of 2.5 mM or higher concentrations of

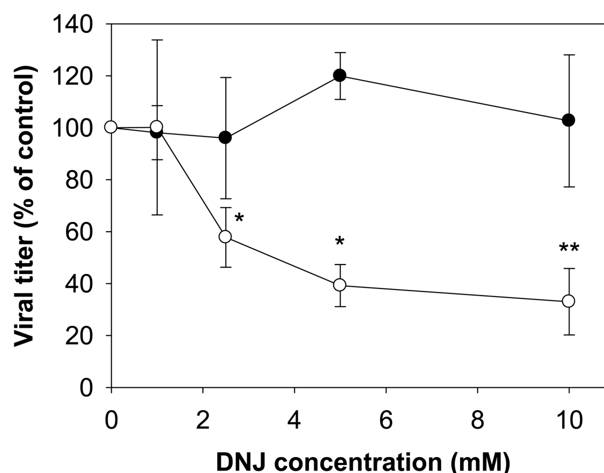

Fig. 1. The effect of DNJ on the replication of BmNPV (●) and AcMNPV (○). Bm5 or Sf9 cells were inoculated with BmNPV or AcMNPV, respectively, at an MOI of 1. Following inoculation, the cells were maintained in medium containing 0, 1, 2.5, 5 or 10 mM DNJ. At 4 days postinfection, the viral titer in each of the DNJ containing treatments was determined and compared to the viral titers in non-DNJ containing control infections. Each point represents the mean value of three independent experiments. The error bars indicate standard deviation of the mean. The asterisk indicates a significant difference between the mean viral titer in non-treated and DNJ-treated groups (*$P < 0.05$, **$P < 0.01$).

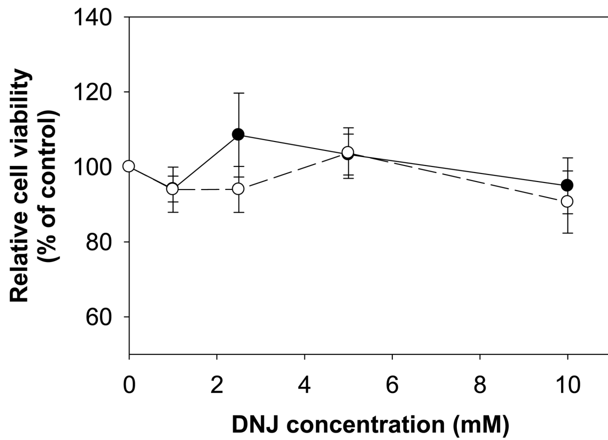

Fig. 2. Viability of Bm5 (●) and Sf9 (○) cells following treatment with DNJ. The viability of cells that were grown for 48 h in medium containing 0, 1, 2.5, 5 or 10 mM DNJ was compared to the viability of control cells that were grown in medium lacking DNJ. Cell viability was measured using XTT reagent. Each point represents the mean value of three independent experiments. The error bars indicate standard deviation of the mean. No statistically significant differences were found between cells grown in the absence or presence of DNJ up to 10 mM.

DNJ (in comparison to control Sf9 cells that were not treated with DNJ). AcMNPV showed a dose-specific inhibitory response up to 5 mM DNJ (i.e., no apparent inhibition at 1 mM DNJ, 40% inhibition at 2.5 mM DNJ, and 60% inhibition at 5 mM DNJ). The replication of AcMNPV, at 4 days post inoculation, was inhibited by approximately 67% when the host Sf9 cells were grown in medium containing 10 mM DNJ.

Effect of DNJ on the viability of Bm5 and Sf9 cells

The viability of Bm5 and Sf9 cells was measured by the colorimetric XTT assay. DNJ did not show any statistically significant affects on the viability of Bm5 and Sf9 cells that were grown for 48 h in medium containing up to 10 mM DNJ (Fig. 2). The results indicated that the viability of Bm5 and Sf9 cells is not affected by DNJ.

Effect of pH and temperature on α-glucosidase activity from Bm5 and Sf9 cell extracts

α-Glucosidase activity in Bm5 and Sf9 cell extracts was measured using PNPG as a substrate in 100 mM sodium phosphate buffer, pH 7. Under this condition α-glucosidase specific activity in the Sf9 cell extract (3.39 μmol/min/mg) was roughly 2-fold higher than that in the Bm5 cell extract (1.73 μmol/min/mg) (Table 1). When the effect of pH on α-glucosidase activity was analyzed, the highest activity in both Bm5 and Sf9 cell extracts was found at pH 10 (Fig. 3A). In comparison to the Bm5 cell extract, α-glucosidase activity in the Sf9 cell extract

Table 1. α-Glucosidase activity in extracts from Bm5 and Sf9 cells

Extract source	Specific activity (μmol/min/mg protein)[a]
Bm5	1.73 ± 0.42[b]
Sf9	3.39 ± 0.58

[a] *p*-Nitrophenyl α-D-glucopyranoside was used as a substrate to measure α-glucosidase activity in 100 mM sodium phosphate buffer, pH 7.

[b] Values represent the mean ± standard deviation separate experiments.

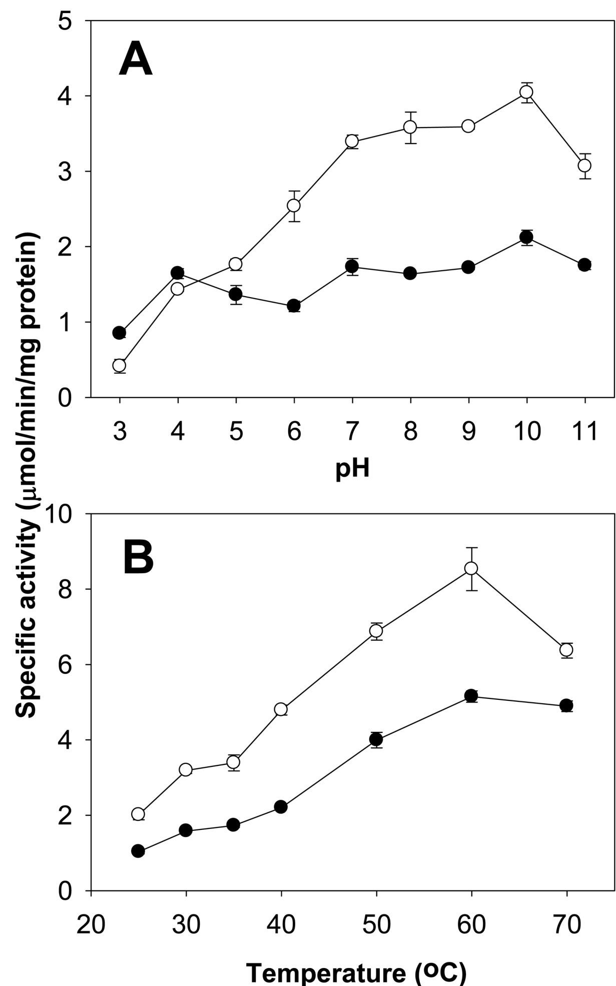

Fig. 3. The effect of pH (A) and temperature (B) on α-glucosidase activity in extracts of Bm5 (●) and Sf9 (○) cells. The α-glucosidase activity was measured with *p*-nitrophenyl α-D-glucopyranoside substrate. Each point represents the mean value of three experiments. The error bars indicate standard deviation of the mean.

Table 2. Inhibition of α-glucosidase activity in Bm5 and Sf9 cell extracts by DNJ, acarbose, and voglibose

Extract source	IC_{50} (μM)[a]		
	DNJ	acarbose	voglibose
Bm5	26.1 ± 3.0[b]	NI[c]	27.9 ± 2.1
Sf9	0.64 ± 0.06	NI	13.3 ± 4.0

[a]*p*-Nitrophenyl α-D- glucopyranoside was used as a substrate to measure α-glucosidase activity in 100 mM sodium phosphate buffer, pH 7.
[b]Values represent the mean a standard error of three separate experiments.
[c]NI: No Inhibition (less than 50% inhibition at 1000 μM).

showed greater sensitivity to increases in pH. α-Glucosidase activity in the Bm5 cell extract showed relatively little sensitivity to changes in pH. Surprisingly, α-glucosidase activity in the Bm5 and Sf9 cell extracts showed strong thermo stability with increased activity up to 60°C (Fig. 3B).

Inhibition of α-glucosidase activity in Bm5 and Sf9 cell extracts by DNJ, acarbose, and voglibose

The ability of DNJ, acarbose, and voglibose to inhibit α-glucosidase activity in Bm5 and Sf9 cell extracts was measured using PNPG as a substrate in 100 mM sodium phosphate buffer, pH 7. α-Glucosidase activity in the Sf9 cell extract was 40-fold more sensitive to DNJ than α-glucosidase activity in the Bm5 cell extract (Table 2). Similarly, α-glucosidase activity in the Sf9 cell extract was more sensitive to inhibition by voglibose but by only 2-fold in comparison to α-glucosidase activity in the Bm5 cell extract (Table 2). Acarbose did not show any detectable inhibitory activity with Bm5 and Sf9 cell extracts.

Discussion

α-Glucosidase I removes the terminal glucose residue of $Glc_3Man_9GlcNAc_2$ that is attached to a nascent glycoproteins, a critical initial step of the *N*-linked glycosylation process. The inhibition of α-glucosidase interrupts the maturation of glycoproteins that are found on the envelope of enveloped viruses resulting in reduced viral particle assembly, suppression of virion secretion, and interference of the attachment of virions to host cells (Pelletier *et al*., 2000; Jacob *et al*., 2007). DNJ is an iminosugar which can competitively inhibit α-glucosidase I and II in the ER (Moremen *et al*., 1994; Wojczyk *et al*., 1995). Inhibition of the formation of mature virions and reduced infectivity have been found in enveloped viruses such as HBV, human immunodeficiency virus (HIV), herpes simplex virus type 1, influenza virus, bovine viral diarrhea virus (BVDV), dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus, and HCV following exposure to DNJ and its derivatives (Durantel *et al*., 2001; Dwek *et al*., 2002; Mehta *et al*., 2002; Woodhouse *et al*., 2008). In this study, we found that the presence of DNJ reduces the budded virus titer of AcMNPV but not BmNPV. This suggested that α-glucosidase activity that processes the glycoproteins of AcMNPV is dramatically more sensitive to DNJ.

In our previous work, we showed that α-glucosidase activity in the midgut of *B. mori*, a specialist insect that feeds exclusively on mulberry leaf, is less sensitive to DNJ in comparison to α-glucosidase activity in the midgut of *Antheraea yamamai*, a generalist insect (Kang *et al*., 2010). Hirayama *et al.* (2007) have also shown that sucrase and trehalase in the midgut of *B. mori* are less sensitive to DNJ in comparison to the corresponding enzymes of *Samia ricini*, a generalist herbivore. We found similar results in this study. The sensitivity of α-glucosidase activity in the Bm5 cell extract was significantly lower (i.e., 40-fold higher IC_{50}) in comparison to that found in the Sf9 cell extract when PNPG, a known substrate of α-glucosidase II (Moremen *et al*., 1994), was used as a substrate. Since it appeared that DNJ did not induce any effects on cell viability, we hypothesized that the reduction in AcMNPV titer that was found in Sf9 cells grown in DNJ containing medium resulted from the inhibition of α-glucosidase I and/or α-glucosidase II that are important for the formation and/or release of mature BVs. In contrast, in Bm5 cells these enzymes appeared to be less sensitive to the effects of DNJ, so that the formation and/or release of BmNPV BVs from Bm5 cells that were grown in DNJ containing medium was unaffected. Our findings are the first to show a differential response in terms of the inhibitory effects of DNJ on α-glycosidase activity in insects and on the ability of baculoviruses to efficiently replicate. Elucidation of the molecular mechanism behind this differential sensitivity to DNJ will provide a probe for the study of the comparative evolution of insects and nucleopolyhedroviruses.

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