Paucimannosidic glycans are often predominant in N-glycans produced by insect cells. However, a β-N-acetylhexosaminidase responsible for the generation of paucimannosidic glycans in lepidopteran insect cells has not been identified. We report the purification of a β-N-acetylhexosaminidase (Sfhex) from the culture medium of *Spodoptera frugiperda* SF9 cells. The purified Sfhex showed ten times higher activity toward a terminal N-acetylglucosamine on the N-glycan core than tri-N-acetylchitotriose. Sfhex was found to be a homodimer of 110 kDa in solution, with a pH optimum of 5.5. With a biantennary N-glycan substrate, it exhibited a five fold preference for removal of the β(1,2)-linked N-acetylglucosamine from the Manα(1,3)-branch than the Manα(1,6)-branch. We isolated two corresponding cDNA clones for Sfhex that encode proteins with greater than 99% amino acid identity. A phylogenetic analysis suggests that Sfhex is an ortholog of mammalian lysosomal β-N-acetylhexosaminidases. A recombinant Sfhex expressed in SF9 cells exhibited the same substrate specificity and pH optimum as those of the purified enzyme. While a larger amount of newly synthesized Sfhex was secreted into the culture medium from SF9 cells, a significant amount of Sfhex was also found to be intracellular. Under a confocal microscope, cellular Sfhex exhibited punctate staining throughout the cytoplasm, but did not co-localize with a Golgi marker. Since secretory glycoproteins and Sfhex are co-transported through the same secretory pathway and Sfhex is active at the pH of the secretory compartments, our study suggests that Sfhex may play a role as a processing β-N-acetylhexosaminidase acting on N-glycans from Sf-9 cells.

β-N-acetylhexosaminidase (hexosaminidase) (EC 3.2.1.52) catalyzes hydrolysis of non-reducing terminal N-acetyl-D-hexosamine residues in N-acetyl-β-D-hexosaminides. Hexosaminidases belong to either the glycosyl hydrolase family 3, family 20, or family 84 (1-4). Of these, family 20 hexosaminidases include mammalian lysosomal hexosaminidases, fungal exo-chitinases, bacterial chitobiases, and insect chitinolytic hexosaminidases.

The hexosaminidase activity of insects and insect cells is of particular interest because of the role that the enzyme may play in altering the structures of N-glycans generated by these cells. The N-glycan synthesis pathway in insects differs from that in mammals in that insects and insect cells produce appreciable amounts of paucimannosidic glycans [reviewed in (5)]. The intracellular N-glycan processing pathway in the ER of insects has been observed to include the addition of a Glc3Man9GlcNAc2 group onto the acceptor Asn followed by the subsequent trimming of the initial oligosaccharide to generate the Man3GlcNAc2. Insect cells also contain significant levels of N-acetylgalcosaminyl transferase I which adds a GlcNAc residue to the Manα(1,3)-branch, followed by the removal of two Man residues to
produce the GlcNAcβ(1,2)Manα(1,3)[Man(1,6)]Manβ(1,4)Glc NAcβ(1,4)GlcNAc structure. Unlike mammalian cells, which subsequently modify this intermediate to yield complex, often sialylated oligosaccharides, insect cells typically hydrolyze the non-reducing terminal GlcNAc attached to the Manα(1,3)-branch of the N-glycan core by the action of a hexosaminidase, leading to generation of paucimannosidic structures with one or two Man attachments. Indeed, N-glycans which do not contain this GlcNAc residue are often prevalent on glycoproteins expressed in insect lines such as Mb-0503 cells from *Mamestra brassicae* (Cabbage moth), Sf21 cells from *Spodoptera frugiperda* (Fall armyworm), and Bm-N cells from *Bombyx mori* (Silk moth) (6), cells from *Trichoplusia ni* (Cabbage looper) (7,8), and Ld652Y cells from *Lymnantria dispar* (Gypsy moth) (9), and in adult bodies of *Drosophila melanogaster* (10).

Hexosaminidases are widely distributed in insects including species of Lepidoptera, Coleoptera, Hemiptera and Orthoptera (11), and Diptera (12). This activity was found not only in tissues, but also in the blood and the molting fluid of *B. mori* L (13) and the secretion fluid of female accessory glands of *Ceratitis capitata* (mosquito) (14). Several studies reported hexosaminidase activity in culture media as well as in cell extracts of lepidopteran insect cell lines such as Sf9, *T. ni* TN-368, *Malacosoma disstria* MD108 and *B. mori* cells (15), *T. ni* TN-5B1-4 cells (16), and a dipteran insect cell line, *D. melanogaster* Kc cells (17,18). The culture media of *Culex quinquefaciatus* (Southern house mosquito) cells was also found to contain hexosaminidase activity (19). A few studies reported purification of a hexosaminidase from the haemolymph (20) and larval integument tissue of *B. mori* (21), the larval or pupal molting fluid, hemolymph, and integument tissue of *Manduca sexta* (Tobacco hornworm) (11,22), the secretion fluid of female accessory glands of *C. capitata* (14), the culture media and the cell extract of *D. melanogaster* Kc cells (17), and the culture media of *C. quinquefaciatus* (19). Two closely related genes which encode insect hexosaminidases were cloned from *B. mori* (21) and *M. sexta* (23). The former is known to be an exo-chitinase. However, since these studies used synthetic substrates and not N-glycan substrates in the enzyme assay, it is not known whether previously purified or cloned insect hexosaminidases can hydrolyze the terminal GlcNAc linked to the N-glycan core or not.

In addition to the above mentioned studies, a microsomal membrane-associated hexosaminidase activity in lepidopteran insect cells that could catalyze such a reaction was reported (24), and the importance of a cellular hexosaminidase activity in the N-glycan processing in insect cells was supported by the finding of an inverse relationship between the level of cellular hexosaminidase activity and the level of GlcNAc-containing N-glycans in glycoproteins expressed in *S. frugiperda* Sf9 cells and *Estigmene acrea* cells (25).

In this paper, we describe the purification of a hexosaminidase (SfHex) from the culture broth of Sf9 cells. This enzyme, a homodimer of 110 kDa with maximal activity at pH 5.5, was found to preferentially remove terminal GlcNAc residues on the Manα(1,3)-branch of the N-glycan core. The N-terminal sequence of the purified enzyme was subsequently used to isolate two corresponding cDNA clones from *Spodoptera frugiperda* mRNA. The Sfhex cDNA when introduced into insect cells resulted in enhanced hexosaminidase activity in the media. Based upon our characterization of SfHex, we believe this hexosaminidase from lepidopteran insect cells is capable of removing the terminal GlcNAc linked to the N-glycan core, and therefore of generating paucimannosidic N-glycans. The identification of this type of hexosaminidase not only contributes to a better understanding of N-glycan processing in insect cells, but will be important in engineering insect cells capable of generating complex N-glycans, that can be used for baculovirus expression of heterologous mammalian proteins, (26,27).

**MATERIALS AND METHODS**

The following materials were obtained from the sources indicated: β-galactosidase (Jack bean), β-N-acetylgalcosaminidase (Jack bean), α-L-fucosidase (bovine kidney), 2-aminopyridine, borane-dimethylamine complex, apo-human transferrin, human IgG, and bovine pancreatic ribonuclease B, 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside (MU-
GlcNAc, 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-galactopyranoside (MU-GalNAc), 2-acetamido-2-deoxy-β-D-glucopyranoside-6-sulfate (MU-GlcNAc-6SO4), Nonidet P-40 (NP-40) (Sigma-Aldrich, St. Louis, MO); tri-N-acetylchitotriose (Seikagaku America, Falmouth, MA); 2-acetamido-1,2-dideoxynojirimycin (2-ADN) (Toronto Research Chemicals, North York, ON, Canada); Sephacryl S-200HR (Amersham Biosciences, Piscataway, NJ); Shim-pack CLC-ODS column (6×150 mm; Shimadzu USA, Columbia, MD); Amide-80 column (2×250 mm; Tosoh Biosep LLC, Montgomeryville, PA); SF-900 II SFM medium (Invitrogen, Carlsbad, CA). Peptide-N-glycosidase F (Prozyme, San Leandro, CA).

**Oligosaccharides** – Structures of oligosaccharides used in this work are shown in Table I. The oligosaccharides were derivatized with 2-aminopyridine by the method of Kondo et al. (28). GnGn-PA was prepared from a PA-derivative of a de-sialylated biantennary oligosaccharide prepared from human transferrin (Sigma-Aldrich, St. Louis, MO). Briefly, sialic acids were removed by treating the PA-derivatized sialylated biantennary oligosaccharide in 20 mM HCl for 1 h at 80°C, and then terminal Gal residues were removed with Jack bean β-galactosidase. MGn-PA and GnM-PA were prepared from PA-derivatives of two positionally isomeric mono-galactosylated biantennary oligosaccharides from human IgG (29) by a sequential digestion with Jack bean β-N-acetylglucosaminidase, β-galactosidase, and bovine kidney α-L-fucosidase. M2-PA and M3-PA were prepared from quail ovomucoid (30) and bovine pancreatic ribonuclease B, respectively. M2Gn-PA was synthesized from M2-PA by the action of GlcNAc-transferase I (a kind gift from Dr. H. Schachter). (GlcNAc)3-PA was prepared from tri-N-acetylchitotriose. All PA-oligosaccharides were successively purified with normal-phase (Amide-80) and reversed-phase (Shim-pack CLC-ODS) HPLC columns before use.

**Enzyme assay** – When 4-methylumbelliferyl (MU) glycosides were used as substrates, the substrates (10 nmol) were incubated in a 96-well plate with an appropriately diluted enzyme in 100 μL of 50 mM sodium citrate – phosphate buffer (pH 5.5) at 37°C for 30 min. The reaction was quenched by adding 200 μL of 0.4 M glycine-NaOH buffer (pH 10.5), and released 4-methylumbelliferone was measured by fluorescence (Eₐ=355 nm, Eₘ=460 nm) using a Victor Multilabel Counter, model 1420 (Wallac, Gaithersburg, MD) in a fluorometric mode. When pNP-GlcNAc (p-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside) was used as a substrate, the substrate was incubated in a 96-well plate with an appropriately diluted enzyme in 100 μL of 30 mM sodium citrate – phosphate buffer (pH 5.5) at 37°C for 30 min. The reaction was terminated as describe above, and released p-nitrophenol was measured at 415 nm by a Benchmark microplate reader (Bio-Rad, Hercules, CA). When PA-oligosaccharides were used as substrates, the reaction mixture contained substrates (500 pmol in 5 μL), an appropriately diluted enzyme solution (10 μL), and 0.1 M sodium citrate – phosphate, pH 5.5 (10 μL). The mixture was incubated at 37°C for predetermined periods of time. The reaction was terminated by heating the sample for 5 min in boiling water, centrifuged to obtain clear supernatant. Substrates and products were analyzed by a normal-phase HPLC column, Amide-80. Enzyme activity was determined by decrease of a substrate or production of a product upon reaction. When GnGn-PA was used to determine branch specificity of hexosaminidase, the enzyme reaction mixture was first subjected to normal-phase HPLC to separate the substrate, mono-N-acetylglucosaminylated M3-PA products, and M3-PA product. A fraction containing mono-N-acetylglucosaminylated M3-PA products was isolated, and then subjected to reversed-phase HPLC to separate MGN-PA and GnM-PA. When asialo, aglacto-human transferrin was used as a substrate, the protein (24 μg) was incubated with an appropriately diluted enzyme solution (1 μL) in 20 mM sodium citrate – phosphate, pH 5.5, at 37°C for 5.5 h. After the reaction, 6-O-methyl galactose was added to the sample as an internal standard, and the sample was heated for 5 min in boiling water, centrifuged to obtain clear supernatant. The released GlcNAc was measured by high-performance anion-exchange chromatography using a CarboPac PA20 column (3×150 mm, Dionex, Sunnyvale, CA) and 10 mM sodium hydroxide as an eluent. Sugars were detected by pulsed amperometry. One unit of hexosaminidase is defined as the amount of
Enzyme required to catalyze the release of 1 µmol of terminal GlcNAc residue from substrates in 1 min at 37°C.

**High-performance liquid chromatography** – All HPLC separations were performed on a LC-10Ai HPLC system (Shimadzu USA, Columbia, MD). Substrate PA-oligosaccharides and their degradation products were routinely analyzed by a normal-phase HPLC using an Amide-80 column (2 mm×250 mm). A sample (4 µL) was injected to an Amide-80 column pre-equilibrated with Solvent A (acetonitrile:10 mM ammonium formate, pH 7, 80:20, v/v), and PA-oligosaccharides were eluted at a flow rate of 0.2 mL/min using a gradient consisting of Solvent A and Solvent B (acetonitrile:10 mM ammonium formate, pH 7, 50:50, v/v) by increasing a proportion of Solvent B [0% (t=0 min), 50% (t=20 min), and 100% (t=50 min)]. For reversed-phase HPLC, a sample (10 µL) was injected to a Shim-pack CLC-ODS (6 mm×150 mm) column pre-equilibrated with Solvent C (10 mM ammonium formate, pH 4.3, containing 0.1% 1-butanol). PA-oligosaccharides were eluted by increasing a concentration of 1-butanol from 0.1 to 0.175% in 30 min at a flow rate of 1.0 mL/min. PA-oligosaccharides were monitored by fluorescence using E_s = 300 nm and E_m = 360 nm (for normal-phase HPLC) or E_s =315 nm and E_m = 380 nm (for reversed-phase HPLC).

**Preparation of GlcNAc-amidine affinity adsorbent** – GlcNAc-amidine immobilized Toyopearl 650M was prepared by the reported procedures (31,32) with a minor modification. TSK Gel Carbobox Toyopearl 650M (wet volume 50 mL) modified with maleimido groups via a spacer (4,7,10-trioxatridecane-1,13-diamine) was treated with 2-acetamido-2-deoxy-β-D-glucopyranosylamine (5.5 g, 25 mmol) and 2-iminothiolan-HCl (3.4 g, 25 mmol) in dry pyridine (100 mL) (33). The mixture was shaken at room temperature for 24 h and was filtered. The resin was washed with ethanol and water successively and was suspended in 0.1 M sodium citrate buffer (pH 6) until use.

**Cell culture** – Serum-free adapted SF9 cells (Invitrogen, Carlsbad, CA) were routinely grown in serum free SF9-900 II SFM medium in shaker flasks at 140 rpm, 27°C. The cells were passed every 4 days at a seeding density of 0.8×10^6 cells/mL.

**Cell extract and supernatant preparation** – The suspension culture was typically harvested at 96 h post seeding, and a clarified cell culture supernatant and a cell pellet were obtained by centrifugation at 350g for 10 min. The cell pellet was washed with chilled PBS (phosphate-buffered saline) (Invitrogen, Carlsbad, CA). Cells were lysed by re-suspending the cell pellet in chilled PBS containing 0.5% NP-40 followed by two cycles of sonication with a Tekmar sonic disruptor (Cincinnati, OH) for 30 s at 50% duty cycle and a power setting of 5. The cell debris was removed by centrifugation and the clear extract was used for analysis.

**Purification of hexosaminidase** – At 96 h post seeding, the suspension culture (1.8 L) was centrifuged at 350g for 10 min, and the cell free supernatant was collected. The purification was carried out at 4°C. To the clarified cell culture supernatant, sodium chloride and sodium citrate were added to a final concentration of 1 M and 10 mM, respectively, and the pH was adjusted to 6.0 with a dilute sodium hydroxide solution. The sample was loaded at a flow rate of 5 mL/min to the GlcNAc-amidine affinity column (2.5×6 cm, 30 mL), pre-equilibrated with 10 mM sodium citrate (pH 6.0) containing 1 M sodium chloride. After washing the column with 3 bed volumes of the same buffer, bound protein was eluted with 10 mM sodium citrate – phosphate buffer (pH 7.0) containing 1 M D-GlcNAc at a flow rate of 5 mL/min by pumping the elution buffer in a reverse direction, and 5 mL fractions were collected. Hexosaminidase activity in each fraction was measured with MU-GlcNAc as a substrate, and protein concentration was determined by the method of Bradford (34) using bovine serum albumin as a standard.

**SDS-polyacrylamide gel electrophoresis** – The purified Sfhex protein (0.5 µg) was analyzed by SDS-PAGE (10% acrylamide gel) under reducing conditions, and proteins were visualized by staining with Coomassie brilliant blue R-250.

**Gel filtration chromatography** – The purified Sfhex (30 µg in 0.5 mL) was applied to a Sephacryl S-200HR column (1.6×60 cm) pre-equilibrated with 10 mM sodium citrate – phosphate (pH 6.5) containing 0.15 M sodium chloride. After loading a sample, protein was eluted with the same buffer at a flow rate of 25 mL/h, and 1 mL fractions were collected.
Hexosaminidase activity in each fraction was measured with MU-GlcNAc substrate as described above. The column was calibrated with bovine thyroglobulin, human IgG, bovine serum albumin, chicken ovalbumin, bovine β-lactoglobulin, bovine ribonuclease A, and uridine.

**De-glycosylation** – N-glycans attached to Sfhex was released by digesting the purified Sfhex protein with PNGase F. The reaction was performed according to the manufacture’s protocol.

**Sequencing of N-terminal amino acids** – The N-terminal sequence analysis of the protein sample was carried out by Edman degradation using an Applied Biosystems model 494A Procise protein sequencer.

**Isolation of a cDNA clone encoding Sfhex, and DNA sequencing** – Aligning the amino acid sequences of hexosaminidases from human, mouse, *D. melanogaster*, *B. mori*, *M. sexta*, and *T. ni* showed a highly conserved internal peptide, HL/MGGDEV (aa 318-324 of human hexosaminases α-chain) that forms a part of the catalytic domain. This segment is common to all of these hexosaminidases and many other hexosaminidases in glycosyl hydrolase (GH) family 20. Degenerate primers derived from both the N-terminal (LSIVNPGPQYPPTKGSWPRP) and the internal sequences were used for RT-PCR of Sf9 RNA to amplify a cDNA corresponding to a portion of the Sfhex gene. It was reasoned that although the downstream primer was common to all known hexosaminidase proteins, the upstream primer was specific for Sfhex, and therefore, only the cDNA corresponding to Sfhex would be amplified. The forward primer (SF1’), 5’-CACTAGCTTAAAYCCNGNCCNCARTAYCC, contained a HindIII site (italics) and sequence corresponding to aa 23-29 (Supplemental Figure S3). The reverse strand primer, (SF5’), 5’-AGTGAAGCTTACYTCTCNCCNCCADRTG, contained a HindIII site (italics) and sequence corresponding to aa 333-339. Total RNA prepared by the TRIzol method (Life Technologies, Rockville, MD) from Sf9 cells, treated with amplification grade DNaseI (Life Technologies) was used as the template. RT-PCR was performed using the 3’-SMART RACE Kit (BD Biosciences Clontech, Palo Alto, CA) to perform first strand cDNA synthesis using 0.6 µg template RNA. Subsequently, 2.5 µL (out of the 110 reverse transcription reaction) was introduced into a 100 µL PCR reaction using the following cycle settings: 94°C for 5 min; 40 cycles at 94°C for 1 min, 55°C for 1.5 min, 72°C for 2 min; 72°C for 10 min; hold at 4°C. PCR reagents were purchased from Applied Biosystems (Foster City, CA) and PCR was performed using Taq Gold in 3.5 mM MgCl2 using an Applied Biosystems GeneAmp 2400 thermal cycler. The 948-bp product was subcloned into pUC18 and sequenced on both strands using BigDye terminators (Perkin-Elmer, CA) by the Nucleic Acid/Protein Core Research Facility of the Children’s Hospital of Philadelphia. The DNA sequence of the partial cDNA fragment matched the seven N-terminal amino acids used for designing the upstream degenerate primer, as well as the next ten amino acids of the determined N-terminal sequence of Sfhex, confirming that the desired cDNA had been isolated. The full length cDNA for Sfhex was obtained by performing both 5’ and 3’ RACE using the SMART RACE cDNA Amplification and BD Advantage 2 PCR Kits (BD Biosciences Clontech). For 3’ RACE, first strand cDNA synthesis was performed as described, and 2.5 µL (out of 110 µL) was introduced into a 50 µL PCR reaction using the gene specific upstream primer, SF6 (5’-GCTCTGGGGCGTTGCGTATCCAA, from aa 280-288) and the universal primer, UPM, as the downstream primer using the following cycle settings: 94°C for 5 min; 5 cycles at 94°C for 5 sec, 72°C for 3 min; 5 cycles at 94°C for 5 sec, 70°C for 10 sec, 72°C for 3 min; 30 cycles at 94°C for 5 sec, 68°C for 10 sec, 72°C for 3 min, 72°C for 10 min and hold at 4°C. A 900-bp fragment was obtained with high background. Nested PCR was performed using 1 µL of the initial PCR reaction in a 50 µL reaction using the upstream primer NSF6 (5’-CGCGAATTGGGATTGGGACCAATGGA, from aa 289-306) and the universal primer, UPM, as the downstream primer using the same cycle settings as before except that only 25 cycles were performed. The resultant 840-bp fragment was purified using GeneClean (Bio 101, Sorrento, CA) and subcloned into pTOPO (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer’s instructions except that the ligation reaction was for 1 hr at room temperature. For 5’ RACE, first strand synthesis was performed according to the manufacturer’s instructions in the
presence of the SMART II oligo, and 2.5 µl (out of 110 µl) was used in a 50 µl PCR reaction using the universal primer, UPM, as the upstream primer and SF7 (5'-AGCCCTCTTAGCACGCCCATATGGAGT, from aa 147-156) as the downstream primer using the following cycle settings: 94°C for 5 min; 5 cycles at 94°C for 5 sec, 72°C for 3 min; 5 cycles at 94°C for 5 sec, 70°C for 10 sec, 72°C for 3 min; 35 cycles at 94°C for 5 sec, 68°C for 10 sec, 72°C for 3 min; 72°C for 10 min and hold at 4°C. The resultant 519-bp fragment was subcloned into pTOPO. DNA sequencing of the fragments generated by both 3' and 5' RACE yielded the sequence of a full length 1784-bp cDNA that terminated in a poly A tail. A full length coding region of Sfhex was obtained by RT-PCR using the forward primer SF9 (5'-CACTGGATCCATGTTACGGACGT AATATTGTTATTT) that contained a BamHI site (italics), a Kozak sequence (bold) and sequence corresponding to the first nine codons of Sfhex and the reverse strand primer SF10 (5'-AGTGGAATTCTCACTAAAGTAATTCCTGG TTAGCAAAA) that contained an EcoRI site (italics), two in frame stop codons (bold) and sequence representing the last eight codons of Sfhex without the stop codon, using pBlueBac-Sfhex-3 (version A) as the template. PCR was performed as above except that the reaction contained 2.5 mM MgCl₂ and only 12 cycles were used. The resultant Sfhex coding region was cloned into the BamHI/EcoRI site of vector pIB/V5-His (Invitrogen Corporation), creating pIB/V5-His-Sfhex.

Confocal Microscopy – Sf9 cells in mid-log phase were seeded into Lab-Tek #1 Borosilicate coverglass 2 chamber slides (Nalge Nunc International Corp., Naperville, IL) at a density of 0.4×10⁶ cells/chamber and allowed to adhere for 40 min at room temperature. A transfection mixture was made by adding and gently mixing 1.2 µL of Cellfectin (Invitrogen, Carlsbad) to 200 µL of Sf900 II SFM containing 2 µL of 1 µg/µL pIB-V5/His-ShHex plasmid or the control pIB-V5/His plasmid. The medium was aspirated, and the transfection mixture (pre-incubated for 15 min at room temperature) was added, and the plates were rocked at 6 side to side motions per min for 4 h at room temperature. After 4 h, fresh Sf900 II SFM (0.4 mL) was added, and the slides were incubated at 27°C for an additional 44 h. The medium was then removed, and the cells were gently washed with PBS (Invitrogen, Carlsbad). The cells were fixed with 10% (v/v) neutral buffered formalin (Richard-Allan Scientific) for 20 min, washed with PBS, and any remaining aldehyde was quenched by incubating for 4 min in 50 mM NH₄Cl in PBS (1 mL). The cells were then washed twice with PBS, and permeabilized with 0.05% Triton X-100 in PBS for 3 min. The fixed cells were washed again with PBS, and blocked overnight with PBS – 8% BSA, followed by incubation with a 1/1000 dilution of a rabbit
polyclonal anti-V5 antibody (Abcam, Cat# ab9116) in PBS – 2% BSA for 2 h. The cells were then washed four times with PBS, and incubated in a 1/1000 dilution of a goat anti-rabbit IgG antibody conjugated with Alexa 546 in PBS – 2% BSA for 1 h. The Golgi apparatus was stained by incubating permeabilized cells with 5 μM of NBD C₆-ceramide-BSA complex (Molecular Probes, Eugene, OR) for 30 min at 4°C, followed by a PBS wash and incubation in PBS – 2% BSA for 1 h. After staining, the cells were examined under a Carl Zeiss LSM 510 META confocal laser-scanning microscope (Thornwood, NY) equipped with an imaging system.

**Immunoblotting** – Sf9 cells in mid-log phase were seeded into 60 mm tissue culture grade Petri-plates at a density of 2.25×10⁶ cells/plate and allowed to adhere for 40 min at room temperature. The medium was removed by aspiration, and 1 mL of the transfection mixture containing pIB-V5/His-ShHex plasmid or the control pIB-V5/His plasmid (prepared as described above) was added, and the plates were rocked at 6 side to side motions per min at room temperature. After 4 h, fresh Sf900 II SFM (2 mL) was added, and the plates were incubated at 27°C. Cells and medium were harvested at 1 day, 2 days or 3 days post transfection. A cell extract was prepared by resuspending the cells in 0.2 mL 2× SDS-denaturation buffer [0.1 M Tris - 4% SDS (w/v) - HCl, pH 6.8 containing 5% (v/v) 2-mercaptoethanol] using a cell scraper, diluted with an equal volume of water, and sonicated for 30 s at 50% duty cycle using a Tekmar sonic disrupter (Tekmar, Cincinnati, OH). The cell extract was then immediately boiled for 5 min. Medium (3 mL) was incubated with phenylmethylsulfonyl fluoride and N-ethylmaleimide (each 1 mM) at room temperature for 15 min, and dialyzed overnight against 5 mM Tris-HCl, pH 6.8 containing the same protease inhibitors at 4°C to remove excess salts. Then the sample was lyophilized, reconstituted in 0.2 mL of ice-cold water, mixed with an equal volume of 2× SDS-denaturation buffer, and boiled for 5 min. Proteins were separated by SDS-PAGE (12% acrylamide gel) and transferred onto a nitrocellulose membrane. The V5-His tagged SfHex was detected with Penta-His HRP conjugate (Qiagen, Valencia, CA) and SuperSignal® West Pico chemiluminescence kit (Pierce, Rockford) according to the manufacturers' instructions.

**Homology search and phylogenetic analysis** – A BLAST search was performed against the complete database “EXPASY/UniProtKB” at the SIB (Swiss Institute of Bioinformatics) using the BLAST network service. A multiple sequence alignment was performed with the ClustalX program (35) using the Gonnet series protein weight matrix provided with the program. An unrooted phylogenetic tree was generated using the Neighbor-Joining method (36), and 1000 bootstrapping trials were performed.

**RESULTS**

**Hexosaminidase activity in the culture media of Sf9 cells** – Insects or insect cells are known to contain exo-type chitinolytic hexosaminidases (exo-chitinases) that release GlcNAc residues from the non-reducing terminus of chitooligosaccharides (11,17,20,21). It is also known that several insect cell lines contain hexosaminidases which can hydrolyze the terminal GlcNAc linked to the N-glycan core (24). Since both chitinolytic enzymes and non-chitinolytic enzymes can hydrolyze synthetic substrates such as pNP-GlcNAc and MU-GlcNAc, it was essential to measure activities against an N-glycan substrate, GnGn-PA, and a chitooligosaccharide substrate, (GlcNAc)₃-PA, (see Table I for structures) to differentiate these two types of enzymatic activities. The PA-oligosaccharide substrates were incubated with either the culture media or the extract of Sf9 cells harvested at 96 h post seeding, and the reaction products were analyzed on an Amide-80 HPLC column. Shown in Figures 1A and 1B are HPLC chromatograms of the hydrolysis products of GnGn-PA and (GlcNAc)₃-PA produced by the action of hexosaminidase(s) in the culture media of Sf9 cells. Considerable amounts of GnGn-PA and (GlcNAc)₃-PA were hydrolyzed to mono-N-acetylgalactosaminylated M₃-PA (Figure 1A) and (GlcNAc)₂-PA (Figure 1B) by incubating these substrates in the media for 24 h or 30 min, respectively. The different incubation periods were required because of the relative activities of the two hexosaminidases against the specific substrates. Shown in Figures 1C and 1D are time-courses of hydrolysis monitored by loss of the
substrates upon incubation. GnGn-PA and (GlcNAc)3-PA were hydrolyzed in the culture media of Sf9 cells in a time-dependent manner with the initial velocity of 0.14 pmol/min (t₁/₂=40 h) and 14 pmol/min (t₁/₂=0.4 h), respectively. The data shown in Figures 1C and 1D fit well (R² > 0.99 for both cases) to simulated curves assuming pseudo first order reactions for the hydrolysis. Consequently, we found the hexosaminidase activity in the culture media putatively responsible for the hydrolysis of the GlcNAc residue(s) in GnGn-PA is stable for up to 50 hrs at 37°C. While the hexosaminidase(s) in the culture media showed 1.5-times higher activity at pH 5.5 than at pH 6.5 (an actual pH of the culture media) against GnGn-PA, it showed 4.3-fold higher activity at pH 6.5 than at pH 5.5 against (GlcNAc)3-PA.

In order to see if the hexosaminidase in question is normally secreted from viable Sf9 cells, the level of this enzyme activity in the media was monitored at various time points of the culture in relation to cell viability and total cell numbers (Figure 2). The number of viable cells continually increased up to Day 6. However, while cell viability was nearly 100% until Day 4, it started to decline on Day 5. In agreement with an earlier study with T. ni Tn-5B1-4 cells using pNP-GlcNAc as a substrate (16), hexosaminidase activity against GnGn-PA increased steadily until Day 6 in nearly direct proportion to the increase in number of viable cells. This direct proportion between the hexosaminidase activity and number of viable cells is reflected in the relative constant level of the specific activity (on per cell basis) up to Day 6. After that time point, enzyme activity remained at a constant level until Day 8, whereas, the number of viable cells dropped quickly after Day 6. These data suggest that the hexosaminidase(s) that hydrolyzes GnGn-PA is secreted into the media from viable Sf9 cells and not released from the cells upon lysis.

The hexosaminidase activity measured at pH 6.5 with GnGn-PA was 0.8±0.06 µU/1×10⁶ cells and 1.4±0.2 µU/1×10⁶ cells in the culture medium and the cell extract, respectively, while exo-chitinase activity measured at the same pH with (GlcNAc)3-PA was found almost exclusively in the cell culture broth (190 μU/1×10⁶ cells in the media vs 8 μU/1×10⁶ cells in the cell extract). This suggests that Sf9 cells express at least two different hexosaminidases; one that prefers N-glycans and one that prefers chitooligosaccharides, with the latter being preferentially secreted.

Purification of a secreted hexosaminidase from Sf9 cells – Although, the extract of Sf9 cells contained more hexosaminidase activity capable of hydrolyzing the terminal GlcNAc on GnGn-PA than the Sf9 culture broth, we were unable to purify the responsible enzyme from either a detergent extract of cells or microsomes. This may have been due to competitive N-glycans present in the extracts that may have interfered with the affinity purification, or instability of the enzyme activity caused by the lysis methods used. On the other hand, a secreted hexosaminidase showing similar activity against GnGn-PA could be purified to homogeneity.

Glycosylamidine derivatives have been developed as affinity adsorbents for several kinds of β-glycosidases (31,32). We synthesized several types of GlcNAc-containing affinity adsorbents for purification of a hexosaminidase from Sf9 cells. After we examined their efficacy, we found that a GlcNAc-amidine affinity adsorbent could capture a secreted hexosaminidase from Sf9 cells efficiently. Shown in Figure 3 is an elution profile of hexosaminidase activity from the GlcNAc-amidine column, monitored with a fluorogenic substrate, MU-GlcNAc. Bound hexosaminidase activity was eluted as a sharp peak with 1 M GlcNAc in the elution buffer. A low level of activity was detected in flow-through fractions. This activity was again recovered in a flow-through fraction and no activity was detected in a 1 M GlcNAc eluate, when it was applied to a regenerated affinity column. Thus, the activity which did not bind to the affinity column would represent a different enzyme. The activities of hexosaminidase in the unbound fraction and the eluate with 1 M GlcNAc were measured with GnGn-PA and (GlcNAc)3-PA as substrates. Since GlcNAc acts as an inhibitor, it was removed, prior to the assay, from the latter fraction by repeating ultrafiltration and dilution with 10 mM sodium citrate – phosphate buffer (pH 6). The hexosaminidase activity in each purification step was measured at pH 5.5 where the enzyme showed its maximum activity against the N-glycan substrate. A relatively lower activity against (GlcNAc)3-PA in the initial culture medium in Table II, compared to the 100-times difference in the initial velocities described above, is due a
difference in the assay pH and a difference in pH-dependency of the activity against GnGn-PA and (GlcNAc)_3-PA. As shown in Table II, approximately 40% of the hexosaminidase activity in the media, measured with GnGn-PA, was found in the eluate with 1 M GlcNAc. The hexosaminidase in this fraction showed 11-fold higher activity toward GnGn-PA than (GlcNAc)_3-PA. The bound fraction was concentrated and subjected to SDS-PAGE followed by Coomassie blue staining (Figure 4). The purified enzyme (lane 2) appeared as a single band with an apparent molecular weight of 67 kDa under reducing conditions. We named this N-glycan specific hexosaminidase, Sfhex. In order to determine whether Sfhex is glycosylated, a portion of the purified Sfhex protein was digested with PNGase F, an enzyme that removes N-linked glycans, and the digest was analyzed by SDS-PAGE. As shown in lane 3 (Figure 4), PNGase F-digested Sfhex migrated faster (Mr = 61 kDa) than untreated Sfhex, indicating that native Sfhex is N-glycosylated.

In contrast to Sfhex, hexosaminidase activity in the culture media detected with (GlcNAc)_3-PA did not bind to the affinity resin and was recovered in an unbound fraction exclusively. The enzyme(s) in this fraction showed 42-fold higher activity toward (GlcNAc)_3-PA than GnGn-PA (Table II). Furthermore, it could release GlcNAc residues sequentially from the non-reducing terminus of (GlcNAc)_6-PA (data not shown).

**Substrate specificity** — Activities of the purified Sfhex towards substrates with N-acetylglucosaminide, N-acetylgalactosaminide, and a sulfated N-acetylgalactosaminide were compared using fluorogenic substrates, MU-GlcNAc, MU-GalNAc, and MU-6-sulfo-GlcNAc (Supplemental Table SI). The purified Sfhex (0.2 µg) hydrolyzed MU-GlcNAc and MU-GalNAc at a rate of 5.9 and 3.0 pmol/min, respectively. However, no detectable hydrolysis was observed with MU-6-sulfo-GlcNAc.

The substrate specificity of the purified Sfhex was further investigated with several N-glycan substrates with terminal β(1,2)-linked GlcNAc residues (Table III, see Table I for structures). Activities shown in Table III were measured with 20 µM of the substrates. Since the reaction was proportional to the substrate concentration from 4 to 20 µM, relative activity will be constant within this range of the substrate concentration. When we compared two positionally isomeric mono-N-acetylglucosaminylated substrates (MGn-PA and GnM-PA), the terminal GlcNAc on the Manα(1,3)-branch was released two-times faster (1.55 pmol/min/µg protein) than the GlcNAc on the Manα(1,6)-branch (0.71 pmol/min/µg protein). Sfhex also released the GlcNAc in M_6Gn-PA at a slower rate (1.14 pmol/min/µg protein) than the corresponding GlcNAc residue in MGn-PA. When a biantennary N-glycan substrate with two terminal GlcNAc residues (GnGn-PA) was digested with Sfhex, the GlcNAc on the Manα(1,3)-branch was released five times faster (2.39 pmol/min/µg protein) than the GlcNAc on the Manα(1,6)-branch (0.46 pmol/min/µg protein), and it was faster than that of the GlcNAc at the same position on the mono-antennary substrate, MGn-PA. On the contrary, the GlcNAc on the Manα(1,6)-branch in GnGn-PA was released at a slower rate, compared to the GlcNAc at the same position in GnM-PA (0.46 vs 0.71 pmol/min/µg).

We compared activities of Sfhex, the hexosaminidase activity in the media and the extract of Sf9 cells, and hexosaminidases from bovine kidney and jack bean toward different N-glycan substrates (Supplemental Table SII). In all cases, MGn-PA was hydrolyzed at a faster rate than GnM-PA. The relative activity of the hexosaminidase in the culture media of Sf9 cells showed essentially the same pattern as that obtained with the purified enzyme. In agreement
with an earlier study (24), the enzyme activity in the extract of SF9 cells was highly specific for the GlcNAc on the Manα(1,3)-branch with very low activity toward the GlcNAc in GnM-PA and M5Gn-PA.

Activity of the purified Sfhex was also examined with a glycoprotein substrate by measuring the released GlcNAc after incubation of asialo, agalacto-human transferrin (12 µM) with the purified Sfhex (3 µU) at 37°C for 5.5h at pH 5.5. Human transferrin has two N-glycosylation sites. The asialo, agalacto-human transferrin used in this experiment contained biantennary glycans which accounted for 90% of the total N-glycans (data not shown). Therefore, the assay contained approximately 20 µM of biantennary glycans terminated GlcNAc residues. Sfhex released 25% of the terminal GlcNAc residues from asialo, agalacto-human transferrin. The same amount of the enzyme released 60% of the terminal GlcNAc residues from GnGn-PA under the same reaction conditions.

Inhibitors  
Effect of D-GlcNAc and 2-acetoamido-1,2-dideoxynojirimycin (2-ADN) on the activity of Sfhex was measured using pNP-GlcNAc as a substrate. D-GlcNAc and 2-ADN showed competitive inhibition with Kᵢ values of 2.0 mM and 0.22 µM, respectively. D-GlcNAc and 2-ADN also inhibited the activity of Sfhex with IC₅₀ values of 1.8 mM and 0.23 µM, respectively, when GnGn-PA was used as a substrate (Supplemental Figure S2).

N-terminal sequence  
Protein sequencing of the purified Sfhex identified the N-terminal 21 amino acids to be LSIVNPGPQYPPTKGSIWPRP. A BLAST search using this sequence as a query yielded a match with a similar sequence near the N-terminus of a B. mori putative hexosaminidase (SWISS PROT acc. no., Q3L6N4) [Identities = 15/21 (71%), Score = 50.3 bits, and Expect = 7e⁻⁰⁶]. We also noticed that one of the previously purified hexosaminidases from the integument tissue of B. mori larva, called β₂-enzyme (21), has a similar N-terminal sequence, LXIVEPPEYPAXKGAIWP (X: unidentified).

Sfhex subunit characterization  
The apparent molecular weight of Sfhex in solution was determined to be ~110 kDa by gel filtration using a Sephacryl S-200HR column (data not shown). Since amino acid sequencing of the N-terminus of the purified Sfhex showed only one species of N-terminal sequence, the results suggest that Sfhex is most likely to be present as a homodimer in solution.

Isolation of a cDNA encoding Sfhex  
Information on the N-terminal amino acid sequence of the purified Sfhex and a predicted internal peptide derived from a highly conserved region in the catalytic domain (Motif 3, see below and Figure 5) of mammalian and insect hexosaminidases was used to design degenerate primers, which were used for RT-PCR of SF9 RNA. A 948-bp cDNA fragment was obtained whose encoded amino acid sequence perfectly matched the N-terminal sequence of the purified Sfhex. A full length cDNA clone encoding Sfhex was obtained by performing both 5’ and 3’ RACE (rapid amplification of cDNA ends). The full length cDNA was 1,784-bp and was predicted to encode a protein of 555 amino acids shown in Supplemental Figure S3 (SWISS PROT acc. no., Q3LS76), with a molecular weight of 63.5 kDa. We obtained the same sized fragments from independent 5’ and 3’ RACE reactions and six separate full length cDNA clones were sequenced. All full length cDNA clones initiated the same number of bp upstream of the empirically determined N-terminus of the purified Sfhex and terminated in a polyA tail, confirming they were full length. As shown in Supplemental Figure S3, the Sfhex protein has a characteristic hydrophobic signal sequence for entry into the secretory pathway. The N-terminal sequence analysis of the mature protein, indicated that an 18 aa (or 17 aa in Version B, see below) signal sequence was cleaved, yielding a 61.4 kDa mature protein. This predicted molecular weight is in close agreement with the molecular size of the de-N-glycosylated Sfhex (Figure 4). The Sfhex protein has four potential N-glycosylation sites (Asn-X-Ser/Thr) at Asn116-Leu-Ser, Asn174-Ala-Thr, Asn309-Ile-Thr, and Asn357-Met-Thr. The protein also has an Asn51-Pro-Ser sequence but the Asn51 is probably not occupied since X is a Pro residue. Since PNGase F treatment of the purified Sfhex reduced its apparent molecular weight on SDS-PAGE gels (Figure 4, lane 3), it is likely that the Sfhex protein is indeed glycosylated at some or all of these sites. Although we selected the conserved internal peptide sequence, HL/MGGDEV, to design the degenerate reverse strand primer, the sequence of Sfhex in this region is actually...
HVGGDEV (aa 333-339). We found two closely related genes in cDNA clones. They encode nearly identical proteins, which we will call versions A and B in this paper (Supplemental Figure S3). Versions A and B differ at 24 nucleotide positions within the 1,664-bp coding region, but these resulted in amino acid substitutions at only three positions and one deletion in the cleaved leader sequence. Therefore the mature Sfhex proteins share 99.5% amino acid identity. We recovered cDNA clones for versions A and B at equal frequencies and therefore their RNA levels were comparable.

**Expression of Sfhex from its cDNA in Sf9 cells**

In order to confirm that the cloned cDNA, Sfhex, actually encodes the protein which was isolated from the culture media of Sf9 cells, we expressed Sfhex by infecting Sf9 cells with a recombinant baculovirus containing the full length Sfhex gene (AcSfhex). Expression of Sfhex resulted in a 60-fold increase in the hexosaminidase activity measured with the GnGn-PA substrate, in the culture media (65 μU/1×10^6 cells), compared to the activity in the media from Sf9 cells with no virus infection (1.1 μU/1×10^6 cells) and the media from Sf9 cells infected with a blank baculovirus lacking a foreign gene (1.8 μU/1×10^6 cells). The recombinant Sfhex (rSfhex) displayed the same substrate specificity with GnGn-PA, GnM-PA, MGn-PA, and M5Gn-PA substrates, and the same pH optimum at 5.5 as Sfhex isolated from the culture media. These data confirm that the Sfhex gene encodes a functional hexosaminidase that is capable of hydrolyzing the terminal β-(1,2)-linked GlcNAc on the N-glycan core.

**Distribution of Sfhex**

In the above experiment, 20% and 80% of the total (intracellular and secreted) hexosaminidase activity was found in the cell extract and the medium, respectively. Since baculovirus infection may disturb normal intracellular sorting, that could potentially result in an atypical enzyme distribution, we further examined the distribution of Sfhex by expressing the V5-His<sub>6</sub> tagged Sfhex in Sf9 cells. Sf9 cells were transfected with the pIB/Shex-V5-His DNA, and immuno-stained with a rabbit anti-V5 antibody followed by a goat anti-rabbit IgG antibody conjugated with Alexaflour 546, and a Golgi-specific dye, NBD ceramide (Molecular Probes). Stained cells were examined with a confocal microscope. Sf9 cells expressing the V5-His<sub>6</sub> tagged Sfhex exhibited punctate red or green fluorescence representing the tagged Sfhex (Figure 7, A and E) or the Golgi apparatus (Figure 7, B and F), respectively, throughout the cytoplasm. However, there was no significant overlap between the two patterns (Figure 7, C and G). These results suggest that intracellular Sfhex localizes within vesicles throughout the cytoplasm but the majority of these vesicles are not part of the Golgi apparatus. No red fluorescent signal from Sfhex was observed at the cell surface.

**Sequence homology**

Hexosaminidases (EC 3.2.1.52) are found in three GH families: family 3, family 20, and family 84 (1-4). The amino acid sequence of Sfhex was searched against the Pfam protein families database (37) to find regions of the sequence that belong to known domain families. Sfhex was found to have a catalytic domain that is conserved in GH family 20 hexosaminidases, such as mammalian lysosomal hexosaminidases. A pairwise sequence alignment using the Blast 2 sequences program (38) at NCBI (National Center for Biotechnology Information) detected identity of 52% (228 aa out of 552 aa, Score = 595 bits, Expect = e^{-108}) between Sfhex and significant Sfhex band was observed at about 70 kDa in both the cell extract and the medium. Its estimated molecular weight is in close agreement with the expected size of the purified Sfhex plus a C-terminal tag. While Sfhex is present in both the cells and medium, a significantly higher fraction accumulates in the medium with days post-transfection, consistent with our results for the baculovirus infection. In addition, a number of proteolytically processed fragments appeared upon prolonged culture (see Day 2 and Day 3) for both the Sfhex from the medium and the cells. Interestingly, fragments at approximately 60 kDa were observed in only the medium to suggest differences in processing of the intracellular and secreted Sfhex.
and a putative *B. mori* hexosaminidase (Q3L6N4). Furthermore, it detected identities of 39% (213 aa out of 535 aa, Score = 379 bits, Expect = e\(^{-100}\)) and 42% (239 aa out of 562 aa, Score = 418 bits, Expect = e\(^{-115}\)) between Sfhex and human hexosaminidase α-chain (39,40) and β-chain (41), respectively. A multiple sequence alignment was performed with the precursor forms of Sfhex (ver A) and hexosaminidases from human and mouse (both α- and β-chains) (Figure 5). Amino acids in the eight motifs known as a signature for GH family 20 enzymes (SPRINTS, http://umber.sbs.man.ac.uk/dbbrowser/sprint/) are highly conserved in Sfhex. Furthermore, Arg211, Asp354, Tyr450, Asp452, and Glu491 which are known to be involved in the substrate binding, and Asp-240, His-294, and Glu-355 which form the catalytic triad in human hexosaminidase B (HexB) (42,43) are conserved. Six cysteine residues are known to form three disulfide bonds (Cys91 – Cys137 , Cys309 – Cys360, Cys534 – Cys551) in human HexB (44). Of these, the third disulfide bond is known to be essential for enzymatic activity of human HexB (45). All of these cysteine residues are completely conserved in Sfhex. Tyr456 plays an important role for dimerization of two subunits in human HexB (42), where Tyr456 of one subunit forms hydrophobic interactions with Ile454 and Tyr492 of the other subunit. The importance of Tyr456 was indicated by the absence of active HexB from cultured fibroblasts of a Sandhoff disease patient having a Tyr456Ser mutation and a defect in homodimerization of the mutant β-chains (46). As shown in Figure 5, this Tyr492 is replaced by Gly436 in Sfhex which is a homodimer of two identical polypeptides and is active.

**Phylogenetic relationship to other hexosaminidases** – We performed an initial phylogenetic analysis using the ClustalX program (35) with about 200 proteins that cover almost of all known proteins in GH family 20, and found that hexosaminidases in this family can be categorized into several groups (data not shown). Then, we performed the analysis on selected proteins which are closely related to Sfhex. Shown in Figure 8 is an unrooted phylogenetic tree created by the Neighbor-Joining method (36), based on the result of a multiple sequence alignment. Due to the lack of an accepted common ancestor, the relationship between groups as shown by the branching order at the center of the tree is unreliable, but we found that there are two groups of hexosaminidases in the 32 proteins. Each of Group 1 (branches with thick solid lines) and Group 2 (branches with dotted lines) is defined by a highly significant node, found in >90% of bootstrap trials, near the center of the tree. While Group 1 includes hexosaminidases from a wide range of organisms such as mammals, insects, nematodes, slime molds, and ascidia, Group 2 includes hexosaminidases mostly from insects and fungi. As shown in Figure 8, Sfhex was found to be more closely related to mammalian lysosomal hexosaminidases than several other insect hexosaminidases previously reported from *B. mori* (21), and *M. sexta* (23), and in sequence databases for *T. ni*, *B. mandarina* (Wild silk moth), *D. melanogaster*, and *A. gambiae*.

In addition to the above mentioned proteins, a putative hexosaminidases from *B. mori* (Q3L6N4) and an *A. gambiae* (Q7A0Z2) were identified by performing BLAST searches. These two Sfhex orthologues were also found to belong to Group I, similar to Sfhex. The genome of *C. elegans* contains a single gene that encodes a hexosaminidase (Q22492), and it also belongs to Group I. However, all three *Drosophila* hexosaminidases were found to belong to Group 2.

**DISCUSSION**

Insect cells are known to produce substantial amounts of paucimannosidic N-glycans that lack the GlcNAc residue on the Manα(1,3)-branch of the N-glycan core in contrast to mammalian cells which often produce complex type N-glycans with sialic acid groups present on both antennae (5). A previous study (24) reported that lepidopteran insect cell lines contain a membrane-bound “N-glycan processing β-N-acetylgalactosaminidase”, but that study did not use purified enzyme nor did it examine whether a similar activity was also secreted.

In this study, we report the purification and characterization of an insect hexosaminidase, Sfhex, that is active against N-glycans, from the culture medium of Sf9 cells, and have cloned two corresponding cDNAs for the enzyme. By using an epitope-tagged Sfhex, we have investigated its distribution between intracellular and secreted
forms and its intracellular localization. We detected possible differences in intracellular processing between the secreted and intracellular forms. Because our study used purified enzyme and substrates, the characterization and properties of the enzyme may differ from previous studies that used crude extracts of cells or enriched microsomal fractions for enzyme assays.

We show that an N-glycan active hexosaminidase was secreted from viable Sf9 cells, and accumulated in the culture medium in a time-dependent manner (Figure 2). We found that Sf9 cells secrete at least two different hexosaminidases. One enzyme that bound to the GlcNAc-amidine affinity column showed a preference for GnGn-PA, while the activity in the unbound fraction showed a preference for (GlcNAc)3-PA. Since the unbound fraction contained a high level of exo-chitinase activity, this enzyme may also be responsible for the relatively low activity observed with GnGn-PA, or the unbound fraction may include another enzyme that is capable of hydrolyzing the terminal GlcNAc on N-glycans.

A previously cloned B. mori hexosaminidase (P49010) is known to be an exo-chitinase (21). D. melanogaster Hexo1 and Hexo2 are also exo-chitinases (47). In contrast, purified Sfhex showed higher activity towards the terminal GlcNAc on the N-glycan core than the GlcNAc on chitotriose. Sfhex could hydrolyze both MU-GlcNAc and MU-GalNAc at a comparable rate. Therefore, Sfhex should be called a hexosaminidase rather than \(\beta\)-N-acetylglucosaminidase. Humans possess three forms of hexosaminidase: HexA is a heterodimer of \(\alpha\)- and \(\beta\)-polypeptide chains; HexB is a homodimer of two \(\beta\)-polypeptide chains; and HexS is a homodimer of two \(\alpha\)-polypeptide chains. Of these, HexA and HexS show activity with negatively charged substrates such as MU-6-sulfo-GlcNAc, but HexB does not (48,49). Sfhex showed no detectable activity with the sulfated substrate. This result, together with its sequence similarity to human lysosomal hexosaminidases, suggests that Sfhex may be a \(S.\ frugiperda\) ortholog of human lysosomal HexB.

Similar to a previously reported hexosaminidase activity present in Sf21 cells (24), Sfhex showed a preference for the terminal GlcNAc linked to the Man\(\alpha(1,3)\)-branch on the N-glycan core, rather than the GlcNAc linked to the Man\(\alpha(1,6)\)-branch (Table III). Interestingly, Sfhex showed higher activity toward the GlcNAc on the Man\(\alpha(1,3)\)-branch of GnGn-PA than the GlcNAc at the same position on M\(\alpha\)n-PA (Table III). This suggests that a GlcNAc on the Man\(\alpha(1,6)\)-branch either increased affinity for the substrate or enhanced the catalytic rate. Jack bean hexosaminidase, but not bovine kidney hexosaminidase, also exhibited higher activity with GnGn-PA than M\(\alpha\)n-PA. On the other hand, Sfhex showed lower activity with M\(\alpha\)n-PA compared with M\(\alpha\)n-PA. Since, the former substrate has two additional Man residues on the Man\(\alpha\) (1,6)-branch of M\(\alpha\)n-PA, the result suggests that one or both of the two extra Man residues sterically hinders the action of Sfhex.

We have cloned a gene that encodes Sfhex, using sequence information from the N-terminus of the purified Sfhex and a predicted internal peptide derived from a highly conserved region in the catalytic domain of mammalian and insect hexosaminidases. We identified two closely related genes encoding proteins which share greater than 99% amino acid identity (Supplemental Figure S3). This high degree of similarity suggests that they represent polymorphic alleles of the locus, and not two different genes, as is the case for the \(\alpha\)- and \(\beta\)-polypeptide chains of human hexosaminidase, which share only 56% identity. Indeed, insect genomes, such as the genome of \(D.\ melanogaster\), are noted for having a high degree of polymorphism. The amino acids of Sfhex versions A and B are different at three positions in their mature forms. We inspected their locations in a three dimensional structure of Sfhex modeled by a homology modeling method, based on the crystal structure of human hexosaminidase B (43) as a template. These three amino acids were found to be far away from the substrate binding site (data not shown). Thus, it is unlikely that the substitution at these three positions would affect the activity and specificity of Sfhex.

C. elegans, an organism whose N-glycans are also primarily of the oligomannosidic and paucimannosidic-type, are reported to possesses a membrane-associated, Man\(\alpha(1,3)\)-branch specific hexosaminidase activity (50). Our database search of sequences sharing homology with known GH family 20 hexosaminidases revealed that C. elegans has only a single gene encoding a hexosaminidase, and therefore it likely encodes
the enzyme responsible for this activity. Our phylogenetic analysis suggests the presence of two distinct groups of hexosaminidase in the GH 20 family. Group 1 contains Sfhex, its orthologues in B. mori and A. gambiae, the above C. elegans enzyme, and several mammalian lysosomal hexosaminidases. Several enzymes in this group, including Sfhex, are known to be capable of hydrolyzing the terminal GlcNAc on the N-glycan core. On the other hand, Group 2 contains a B. mori exo-chitinase and its orthologues in M. sexta, B. mandarina, T. ni, and D. melanogaster, and several fungal enzymes, such as a T. harzianum exo-chitinase and a T. virens chitobiase. Our phylogenetic analysis suggests that lepidopteran insects contain both exo-chitinases (in Group 2) and lysosomal enzyme-type hexosaminidases (in Group 1).

Following our initial manuscript submission and subsequent to our depositing the Sfhex sequences with GenBank, two other studies reported the characterization and cloning of hexosaminidases active against N-glycans from insect cells (47,51). One of these studies has reported that D. melanogaster FDL protein (DmFDL) (in Group 2 in our classification) is active on an N-glycan substrate, but not a chitooligosaccharide, and is responsible for producing paucimannosidic N-glycans in Drosophila (47). We note that the FDL protein is atypical of other Group 2 hexosaminidases in having His and Tyr at positions 212 and 550 (numbers refer to the positions in the human hexosaminidase β-chain), respectively, similar to Group 1 enzymes, but in contrast to the conserved Asn and Trp at these positions, typical of Group 2 enzymes. Substitution of these amino acids may affect its substrate specificity. It is possible that the FDL protein evolved a separate specificity from other group 2 proteins or the algorithm has mis-categorized this protein.

We observed intracellular Sfhex as well as more abundant secreted Sfhex using the V5-His6 tagged recombinant Sfhex expressed in Sf9 cells (Figure 6). This distribution can not be directly compared to the observation of 64% intracellular activity and 34% secreted activity for the endogenous hexosaminidase activity due to differences in cell culture condition in the two experiments. However, it is clear that some of the newly synthesized V5-His6 tagged Sfhex resides in Sf9 cells. Furthermore, the overexpression of heterologous Sfhex results in a significantly higher fraction of secreted protein compared to endogenous ratios. If the intracellular sorting machinery is saturated, then the fraction of a protein that is secreted will increase; a situation that is amplified when proteins such as those targeted to the lysosome are overexpressed in insect cells using cDNA constructs such as human HexB (52), human GM2-activator protein (53), and human sphingomyelinase (54).

Interestingly, hexosaminidase B, normally a lysosomal protein, is used as a marker for regulated secretion in some mammalian cell lines and its extracellular release can parallel total protein secretion in stimulated cell lines (55). Thus, lysosomal proteins may be secreted into the medium, present in the secretory pathway compartment, or active in the lysosomes depending on cell type, culture conditions, and expression levels (55). As a result, it is not surprising to observe Sfhex, a homolog of mammalian hexosaminidase, in both the intracellular and secreted fraction in the current study.

The transport mechanism and proteolytic processing of mammalian hexosaminidases have been well characterized [reviewed in (56-59)]. As is typical of secretory and plasma membrane glycoproteins, a pro-form of hexosaminidases are transported through cis-Golgi → medial-Golgi → trans-Golgi to the trans-Golgi network (TGN) where hexosaminidases are sorted from secretory glycoproteins by mannose-6-phosphate receptors (MPRs) that recognize a mannose-6-phosphate (Man-6-P) on hexosaminidases. Thereafter, hexosaminidases are routed to endosomes, where hexosaminidases are released from the receptors and subsequently transported to lysosomes. A series of proteolytic and glycosidic processing events occur to the pro-from of hexosaminidases in endosomes or lysosomes to form their mature structure (60-67). Lysosomal processing of the α and β polypeptides by proteases and glycosidases results in smaller fragments ranging in size from 6 to 56 kDa held together by disulfide bonds (60).

Proteolytic fragmentation of Sfhex was observed in both the medium and extract of Sf-9 cells upon prolonged culture periods. While some of the fragment sizes were similar in the cell extract and medium, the medium also contained an
additional fragment (~60 kDa) not observed in the cell extract. This difference in Sfhex protease digestion is due either to different processing pathway for proteins in medium and Sf9 cells or the presence of additional non-specific proteases in the medium. The presence of multiple fragments in the cell extracts is also consistent with possible intracellular processing observed for other intracellular hexosaminidase proteins (63), although the principal intracellular band remained the full length protein. The observation of some similar protein fragments in the cell extracts and medium may be due to leakage of intracellular fragments or similar protease digestion in the two environments.

Examination of the intracellular location of the tagged Sfhex using confocal microscopy and differential interference contrast (DIC) microscopy revealed the presence of punctate cytoplasmic staining. However, the Sfhex did not co-localize significantly with the Golgi marker to suggest another possible principal intracellular location. The *Drosophila* hexosaminidase DmFDL was only observed minimally in the Golgi but was found to predominate in the plasma membrane, late endosomes, and extracellular medium. The localization and secretion of both Sfhex and DmFDL is also different from the original report by Altmann *et al.*, (24), who hypothesized that the Sf21 hexosaminidase would be a membrane-bound Golgi protein. Our findings and those of Leonard *et al.* (47) suggest that intracellular Sfhex may localize to endosomes/multivesicular bodies (MVBs) and/or possibly lysosomes. Unlike DmFDL (47), Sfhex was not observed at the cell surface. Aumiller *et al.* (51) also expressed Sf9 hexosaminidase in insect cells using a baculovirus vector and noted that the protein did not co-localize with a commercial Lysotracker probe (Molecular Probes). However, this probe labels a number of acidic organelles and lysosomal fluorescence may represent only a small fraction of the total fluorescence obtained with the dye. These authors suggest that the protein is a degradation enzyme but not an "N-glycan processing enzyme" (51). We believe that Sfhex, like *Drosophila* FDL (47), is likely to be responsible for generating paucimannosidic N-glycans in insect cells for the following reasons: 1) a homologous lysosomal hexosaminidase B is responsible for hydrolysis of the terminal GlcNAc on the Man0(1,3)-branch of the N-glycan core for the membrane protein BA-1 expressed in mouse brain (68), 2) Sfhex is active at the pH of secretory compartments, such as trans-Golgi [pH 6.17 – 6.36 (69)], TGN [pH 5.91 – 5.95 (70,71)], and secretory granules [pH 5.2 (72,73)], 3) the majority of N-glycan containing proteins, i.e., secretory glycoproteins, are co-transported with Sfhex, 4) *Drosophila* flies carrying a deficiency in the chromosomal region including the FDL gene showed a sevenfold reduction in paucimannosidic N-glycans (47), 5) the optimum pH of DmFDL with GnGn-PA (pH 5.5) (47) is same as that of Sfhex, and 6) a single *C. elegans* hexosaminidase likely to be responsible for producing paucimannosidic glycans (50) is also homologous to Sfhex. Indeed, given the absence of other likely hexosaminidase candidates against N-glycans in the genomes of either *D. melanogaster* or *C. elegans*, we hypothesize that the Sfhex and DmFDL proteins serve the multifunctional roles of both N-glycan processing and glycan degradation. The N-glycan processing capability of Sfhex in insect and insect cells may result from the requirement that functional Sfhex be transported along the same pathway as that of secreted glycoproteins. Similar to what has been observed for many lysosomal proteins (52-54), we propose that a fraction of Sfhex likely fails to be marked for intracellular sorting and/or be captured by the lysosomal receptor, resulting in its secretion into the extracellular medium from which it was purified. However, as the *Spodoptera* genome is not yet fully sequenced, another hexosaminidase is possible within the Sf genome that may function separately or in concert to modify secreted N-glycans.

If the same hexosaminidase is used both for N-glycan processing and degradation in insects and perhaps *C. elegans*, the question arises as to why mammalian N-glycans retain complex structures, since they are also exposed to lysosomal hexosaminidases in the secretory pathway. The difference may be due to the observation that the mammalian lysosomal hexosaminidases have a lower pH optimum, pH 4.5 (see Supplemental Figure S1), so that they may not be as active in the Golgi compartments prior to arrival in the lysosome. This results in insect cells having a 14-fold higher hexosaminidase activity toward a Gn-Gn-PA substrate measured at pH 6.0 than
mammalian tissues (see Table V in ref. (24). Secondly, the level of β1,4-galactosyltransferase in the Golgi is much higher in mammalian cells (74), serving to cap the N-glycans and protect them from the action of hexosaminidases. Indeed, we have observed that overexpression of this galactosyltransferase protects the exposed GlcNAc from cellular hexosaminidases (7). Finally, the mammalian intracellular transport machinery may be more efficient than insects in delivering hexosaminidase and other degradation enzymes to the proper degradation compartments. Interestingly, Drosophila has a homolog of mammalian mannose 6-phosphate receptors, the lysosomal enzyme receptor protein (LERP), but the ligand recognition by LERP does not depend on mannose 6-phosphate (75). Therefore, a different mechanism may be used for lysosomal targeting in insects. Clearly, evolution has favored the ability of mammalian N-glycans to retain the GlcNAc residues on both biantennary branches that allows for the generation of more complex N-glycan structures while insects trim these structures to the paucimannosidic form.

With our identification of a hexosaminidase, Sfhex, that is active against N-glycans from Sf9 cells and a recent report of a second Sf9 hexosaminidase identified by Aumiller et al. (51) with properties very similar to Sfhex, it should be possible to eliminate their activity in cells using targeted RNA interference to engineer insect cells with improved ability to reproduce mammalian type N-glycans. Such experiments are in progress in our laboratories.

Note: After we submitted the original manuscript of this paper to J. Biol. Chem. (Aug 2, 2005) and deposited the nucleotide sequence for Sfhex in GenBank, two similar studies were recently published (47,51).

ACKNOWLEDGMENT

\(^1\)We thank Dr. Hao-Chia Chen at Endocrinology and Reproduction Research Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892 for protein sequencing. \(^2\)The nucleotide sequences reported in this paper has been submitted to the GenBank Data Bank with accession numbers DQ183186 and DQ183187 for Sfhex version A and B, respectively.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Structure</th>
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<tr>
<td>GnGn-PA:</td>
<td>GlcNAcβ2(\text{Man})α6</td>
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<td>Manα6</td>
</tr>
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<td>M Gn-PA:</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>GlcNAcβ2(\text{Man})α3</td>
</tr>
<tr>
<td>(GlcNAc)3-PA:</td>
<td>GlcNAcβ4GlcNAcβ4GlcNAc-PA</td>
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Table II. Purification of hexosaminidase from the culture media of Sf9 cells.
Enzyme activity was measured at pH 5.5 with the GnGn-PA and (GlcNAc)$_3$-PA substrates. Recovery of
the enzyme activity is shown and the recovery percentages are in parentheses.

<table>
<thead>
<tr>
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<td>Initial culture sup.</td>
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<td>23.0 (100)</td>
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<td>350 (100)</td>
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<td>300 (85)</td>
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<td>GlcNAc-amidine bound Fr./desalted</td>
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<td>8.8 (38)</td>
<td>35</td>
<td>0.8 (0.2)</td>
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Table III. Substrate specificity of Sfhex for N-glycan substrates with terminal GlcNAc residues. N-Glycan substrate (500 pmol) was incubated with the purified Sfhex (0.1 µg) in 40 mM sodium citrate – phosphate buffer, pH 5.5 (25 µL) at 37°C, and reaction velocity was determined by analyzing the substrates and products by HPLC. See Table I for structures. Standard error in the assay was <5%.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>$v$ (pmol/min/µg)</th>
<th>$k$ (s$^{-1}$)</th>
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<tr>
<td>MGn-PA</td>
<td>M$_3$-PA</td>
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<td>GnGn-PA</td>
<td>M Gn-PA</td>
<td>0.46</td>
<td>0.0005</td>
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FIGURE LEGENDS

Figure 1: Hydrolysis of GnGn-PA and (GlcNAc)3-PA by a hexosaminidase in the culture media of Sf9 cells. The culture media of Sf9 cells was harvested when the cell density reached late-log phase. The substrates were incubated in the cell culture media (pH 6.5) at 37°C for different periods of time. After terminating the reaction by boiling, the samples were analyzed on an Amide-80 column. A and B, HPLC chromatograms of the reaction mixtures (solid line) with GnGn-PA and (GlcNAc)3-PA, respectively, and those of a negative control sample using boiled cell culture media (dotted line). The elution positions of the substrates and the expected products are marked as follows. 1, M3-PA; 2, MGn-PA and GnM-PA; 3, GnGn-PA; 4, (GlcNAc)-PA; 5, (GlcNAc)2-PA; 6, (GlcNAc)3-PA. MGn-PA and GnM-PA eluted at the same position on the Amide-80 column under the elution conditions used in this experiment. C and D, time-courses of hydrolysis of GnGn-PA and (GlcNAc)3-PA, respectively, in the cell culture media of Sf9 cells. The data were fitted by a non-linear regression curve assuming a pseudo first order reaction.

Figure 2: Elevation of hexosaminidase activity in the culture media of Sf9 cells with increasing culture time. A portion of a suspension of Sf9 cells was taken at various times of culture, cells were removed by centrifugation and hexosaminidase activity in the culture media was measured using GnGn-PA as a substrate, as described in the legend of Figure 1. Open and closed circle, hexosaminidase activity expressed in μU/1×10^6 cells and μU/mL, respectively; closed square, total cell number in the culture; open square, cell viability.

Figure 3: Elution profile of hexosaminidase from a GlcNAc-amidine affinity column. The culture media of Sf9 cells (1.8 L) was adjusted to 1 M sodium chloride and 10 mM sodium citrate and the pH of the sample was adjusted to 6.0. The mixture was applied to a GlcNAc-amidine immobilized Toyopearl 650M column (2.5×6 cm) equilibrated with 1 M NaCl – 10 mM sodium citrate, pH 6.0. After loading the sample, the column was washed with the same buffer, and bound proteins were eluted with the same buffer containing 1 M GlcNAc. The point of GlcNAc addition is indicated with an arrow. hexosaminidase activity was measured at pH 5.5 with MU-GlcNAc as a substrate, and protein concentration was determined by the Bradford method (34). Solid line, hexosaminidase activity; dotted line, protein concentration.

Figure 4: SDS-PAGE of the purified Sfhex and de-N-glycosylated Sfhex. Purified Sfhex (lane 2) or Sfhex treated with PNGase F (lane 3) were analyzed by 10% SDS-PAGE under reducing conditions, followed by Coomassie blue staining. Lane 1, molecular weight markers.

Figure 5: Multiple sequence alignment of Sfhex and mammalian hexosaminidases. A multiple sequence alignment was performed with the ClustalX program (35). Eight conserved motifs in GH 20 family hexosaminidase are indicated with underlines. Amino acids involved in the substrate binding and the catalytic reaction (42,43), and formation of disulfide bonds (44) are indicated with residue numbers.

Figure 6. Expression of V5-His<sub>6</sub> tagged Sfhex. Sf9 cells were transfected with pIB/V5-His-Sfhex or control pIB/V5-His DNA and cultured for 1, 2, or 3 days. Samples were prepared from both cell extracts (L) and cell culture medium (M). The V5-His<sub>6</sub> tagged Sfhex was detected with a mouse anti-His tag antibody conjugated with horse radish peroxidase using a SuperSignal West Pico chemiluminescence kit. Positions of marker proteins are indicated at the left with their molecular weight. An arrow at the right indicates V5-His<sub>6</sub> tagged Sfhex. Asterisks indicate non-specific staining of bands also present in the control samples, unrelated to the tagged Sfhex.

Figure 7. Intracellular localization of the V5-His<sub>6</sub> tagged Sfhex. Sf9 cells were transfected with the pIB/V5-His-Sfhex DNA, and stained with: A, a rabbit anti-V5 antibody followed by a goat anti-rabbit IgG antibody conjugated with Alexaflour 546, and B, NBD ceramide. C, a merged image of A and B; D,
a differential interference contrast (DIC) microscopy image; E, F, and G, magnified images of a cell expressing a high level of Sfhex indicated with an arrow in A, B, and C, respectively; H, superposition of image G onto a DIC microscopy image of the same cell. Bar, 10 μm

Figure 8: An unrooted phylogenetic tree of selected hexosaminidases in GH family 20. A multiple sequence alignment was performed using the ClustalX program (35), and phylogenetic tree was generated using the Neighbor-Joining method (36). Nodes found with >90% confidence values by 1000 bootstrapping trials are indicated with closed circles. Two groups of proteins referred in the text are shown with thick solid (Group 1) or dotted (Group 2) lines. The scale indicates branch length in number of substitution per site. SWISS PROT accession numbers of the proteins are shown in parenthesis. Ag, *Anopheles gambiae* str. PEST; At, *Arabidopsis thaliana*; Bma, *Bombyx mandarina*; Bmo, *Bombyx mori*; Ca, *Candida albicans*; Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum* (Slime mold); Dm, *Drosophila melanogaster*; Eh, *Entamoeba histolytica*; Fs, *Felis silvestris catus* (Cat); Hs, *Homo sapiens* (Human); Mm, *Mus musculus* (Mouse); Ms, *Manduca sexta*; Os, *Oryza sativa*; Pm, *Phallusia mammilata* (ascidian); Sf, *Spodoptera frugiperda*; Sj, *Schistosoma japonicum* (Blood fluke); Ss, *Sus scrofa* (Pig); Th, *Trichoderma harzianum*; Tn, *Trichoplusia ni*; Tv, *Trichoderma virens*
REFERENCES

Fig. 1 (Tomiya et al.)

A

Fluorescence intensity

Elution time (min)

1 2 3

B

Fluorescence intensity

Elution time (min)

4 5 6

C

Hydrolysis (% of total)

Incubation time (h)

D

Hydrolysis (% of total)

Incubation time (h)
Fig. 2 (Tomiya et al.)

Cell viability (%)

Hexosaminidase activity (μU/mL; μU/1x10^6 cells)

Cell density (1x10^6) (□)

Day
Fig. 3 (Tomiya et al.)

- Hexosaminidase activity (mU/mL)
- Elution volume (mL)
- Protein (µg/mL)
Fig. 6 (Tomiya et al.)

<table>
<thead>
<tr>
<th></th>
<th>pIB/V5-His</th>
<th></th>
<th>pIB/V5-HisSfhex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>M</td>
<td>L</td>
<td>M</td>
<td>L</td>
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</table>

(kDa)

- 75
- 50
- 37
- 25
- 15

* and ** annotations indicate specific bands or markers on the gel.
Purification, characterization, and cloning of a spodoptera frugiperda SF9 
beta-N-acetylhexosaminidase that hydrolyzes terminal N-acetylglucosamine on 
N-glycan core
Noboru Tomiya, Someet Narang, Jung Park, Badarulhisam Abdul-Rahman, One Choi, 
Sundeep Singh, Jun Hiratake, Sakata Sakata, Michael J. Betenbaugh, Karen B. Palter and 
Yuan C. Lee

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