



Expression of C1 Esterase Inhibitor by the Baculovirus Expression Vector System: Preparation, Purification, and Characterization

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C1 esterase inhibitor (C1INH) is an important regulator of the classical complement pathway. Hereditary deficiency of C1INH causes angioedema of the skin, gut, and respiratory tissues that may be fatal. C1INH replacement therapy may be lifesaving for patients with this disorder. The objective of this study was to evaluate the use of the baculovirus expression vector system for mass producing biologically active human recombinant (rC1INH). A recombinant baculovirus was constructed coding the human native (nC1INH) sequence under control of the polyhedrin promoter. *Spodoptera frugiperda* Sf-9 insect cells were infected with this recombinant baculovirus in a medium-scale (10-L) bioreactor to produce rC1INH with a specific activity of 45 U/mg. Purification of rC1INH from the culture harvested at 60 h postinfection yielded 5.9 µg rC1INH/mL supernatant of a 75-kDa product with a specific activity of 31,000 U/mg purified rC1INH compared to 71,000 U/mg purified nC1INH from human serum using the same procedure. This rC1INH was about 25 kDa smaller than nC1INH, suggesting that Sf-9 cells express underglycosylated rC1INH. Glycan analysis showed that both N-glycan and O-glycan chains were present in rC1INH. The N-glycan chains, released using PNGaseF and fluorescently labeled, were analyzed using exoglycosidase treatment and

capillary electrophoresis. Their high-mannose structure was consistent with the known failure of the insect cell glycosylation pathway to afford the fully elaborated biantennary structures found on human native nC1INH. © 2001 Academic Press

C1 esterase inhibitor (C1INH),² a 104-kDa serine protease inhibitor, is the major regulator of activation of the classical complement activation pathway (1), inhibiting the proteolytic activity of C1 esterase by acting on the C1r and C1s subcomponents of C1 (2, 3). Insufficient functional C1INH allows uncontrolled activation of the classical pathway of complement. This is seen clinically in the human genetic condition called hereditary angioedema (HAE), an autosomal dominant disorder characterized by attacks of angioedema involving the face, extremities, and respiratory and gastrointestinal tracts

² Abbreviations used: AcNPV-C1INH, recombinant *Autographa californica* nucleopolyhedrosis virus expressing human C1 esterase inhibitor; AGA, 7-amino-1,3-naphthalene disulfonic acid; BEVS, baculovirus expression vector system; CE, capillary electrophoresis; C1INH, C1 esterase inhibitor; DO, dissolved oxygen; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; hpi, hours postinfection; HAE, hereditary angioedema; Man, mannose; m.o.i., multiplicity of infection; nC1INH, native C1 esterase inhibitor; rC1INH, recombinant C1 esterase inhibitor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Sf-9, *Spodoptera frugiperda*, insect cell line; SFM, serum-free medium; SLPM, standard liter per minute.

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(4–6). This defect is caused by either reduced levels of antigenic and functional C1INH protein in the plasma (type I HAE) or normal to elevated levels of an antigenic but dysfunctional mutant C1INH (type II HAE) (6,7). Replacement therapy, using human serum derived C1INH, is now under active investigation in the United States to treat hereditary angioedema (8); in other countries C1INH has been approved for use in treating HAE. Human derived material is not currently available in adequate quantities and at a sufficiently low cost to be administered regularly for prophylaxis of HAE attacks. In addition, human-derived material has the potential to cause viral infections. Finally, there are many other potential uses for C1INH such as to treat septic shock or transplant rejection. Thus, there is an important need to develop a safe, effective, and affordable recombinant C1INH (rC1INH) that is structurally similar to the human native C1INH (nC1INH).

Human nC1INH is a heavily glycosylated plasma protein with a total carbohydrate composition between 26 and 34% (1, 9, 10). Thirteen glycosylation sites, six N-linked and seven O-linked, have been identified (9). The N-glycans of the human plasma protein are a mixture of biantennary structures (11). A single trisaccharide O-glycan structure, NeuAc ($\alpha 2 \rightarrow 3$)Gal($\beta 1 \rightarrow 3$)GalNAc (1 \rightarrow O) has been identified (11). The peptide backbone of nC1INH accounts for only 51% of the apparent molecular weight (104 kDa) of the circulating protein (12). The remaining observed molecular mass is the result of posttranslational modification and the migration characteristics of the folded protein in SDS-PAGE and on gel permeation chromatography. Even if nonglycosylated rC1INH, prepared in bacterial expression systems, retains function as a C1 esterase inhibitor, its therapeutic potential is limited by the likelihood that it would both be antigenic and rapidly metabolized and cleared *in vivo*.

The baculovirus expression vector system (BEVS) has become a popular system for expressing eukaryotic proteins due to its demonstrated high expression levels and its ability to perform posttranslational modifications (13, 14). This report describes experiments designed to demonstrate that the BEVS can be used to produce large quantities of biologically active rC1INH. We describe the production, evaluation, and structural characterization of rC1INH produced by *Spodoptera frugiperda* Sf-9 insect cells infected with the recombinant baculovirus expressing full-length, glycosylated C1INH under the control of the polyhedrin promoter.

MATERIALS AND METHODS

Cells, Virus, and Medium

S. frugiperda (Sf-9) cells were obtained from Gibco BRL (Grand Island, NY) and were routinely subcultured in Sf-900 II serum-free medium (SFM) (Gibco

BRL). Cell counts were performed with a Coulter Counter Model ZM interfaced with a Coulter Channelyzer 256 (Coulter Electronics, Hialeah, FL) and viability was determined by the trypan blue exclusion method. Sf-900 II SFM was prepared from powder in 10-L batches following manufacturer directions. The pH was adjusted to 6.25 with 5 M NaOH and the osmolality was adjusted with a 15% (w/v) NaCl solution to 360 mOsm/kg.

The cDNA encoding the full-length human nC1INH was obtained as a gift from R. C. Strunk (Washington University, St. Louis, MO), sequenced, and inserted into the *Hind*III site of pBluescript II SK- (Stratagene, La Jolla, CA). Following amplification in *E. coli*, the cDNA insert was removed from the plasmid by digestion with *Hind*III (New England Biolabs, Inc., Beverly, MA) and gel purified on a 0.5% TAE-agarose gel (Fisher Biotech, Pittsburgh, PA). The 1.8-kb DNA fragment was excised, purified using the Qiaquick gel extraction kit (Qiagen, Inc., Valencia, CA), and inserted into the *Hind*III site of the baculovirus transfer vector pBlueBacIII (Invitrogen, Carlsbad, CA). Following amplification in *E. coli*, Sf-9 cells were transfected at 27°C with 2 μ g of this transfer vector and 0.5 μ g Baculogold DNA (PharMingen, San Diego, CA). The recombinant baculovirus (AcMNPV-C1INH) was then isolated by following the instructions given with the Baculogold kit. The resulting virus was titered by the TCID₅₀ method (15).

Bioreactor Production of rC1INH

A 2-L Biostat A bioreactor (B. Braun Biotech International, Allentown, PA) equipped with a marine impeller was used with a working volume of 1 L to produce the seeding stock of Sf-9 cells for the 10-L BioFlo 3000 bioreactor (New Brunswick Scientific Co., Edison, NJ). The 2-L bioreactor was inoculated at 5×10^5 cells/mL with uninfected Sf-9 cells (>95% viability) grown in 500-mL shaker flasks with a working volume of 50 mL. The shaker flasks were rotated at 100 rpm in an incubator shaker (New Brunswick Scientific) maintained at 27°C. The temperature and dissolved oxygen (DO) concentration in the bioreactor were controlled at 27°C and $\geq 50\%$ air saturation (by adjusting the flow rate of pure oxygen), respectively. In addition, the agitation was maintained at 100 to 150 rpm and the pH was maintained between 6.20 and 6.30 by the addition of 1 M KOH during the cell growth phase. The bioreactor headspace environment was continuously flushed with air at a flow rate of 1 standard liter per minute (SLPM) to avoid CO₂ accumulation (16). After a cell density of 5×10^6 cells/mL was reached, the cells were transferred to a 10-L BioFlo 3000 bioreactor (New Brunswick Scientific Co.) equipped with a marine impeller via a sterile tubing connection by a peristaltic pump. Thus, the starting density in the 10-L bioreactor was 5×10^5

cells/mL with a viability of >95%. The temperature, DO concentration, and pH were maintained at 27°C; 50% air saturation (by adjusting the flow rate of pure oxygen) and between 6.20 and 6.30, respectively. The bioreactor was sampled at 12-h intervals and cell density and viability were monitored. Cells were infected with the recombinant AcMNPV-C1INH baculovirus at an m.o.i. of ~10 at a cell density of 2.5×10^6 cells/mL. The bioreactor run was stopped approximately 96 hpi when the cell viability decreased to ~50%.

Assays for Total Protein and C1INH

Supernatant samples harvested from the bioreactor at timed intervals were centrifuged for 10 min at 200g and 4°C to remove any cellular debris, and 20 mL of each sample was concentrated approximately 20-fold using Amicon Centriprep 30 (Millipore, Bedford, MA). Total protein concentration of each concentrate was determined by Coomassie G-250 reagent (Pierce, Rockford, IL).

Antigenically detectable C1INH was determined by dot blot and Western blot assays. The dot blot assay was performed by dotting 1 μ L of the unconcentrated supernatant onto a nylon membrane (Nytran, Keene, NH) and the Western blot assay by loading 200 ng of protein per lane onto 10% polyacrylamide gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nylon membrane. The membranes were then probed for C1INH with a goat IgG anti-human C1-esterase inhibitor antibody (DiaSorin Inc., Stillwater, MN). Bound antibodies were detected by using alkaline phosphatase conjugated rabbit IgG anti-goat IgG antibody (Cappel, West Chester, PA) and Immun-Star substrate (Bio-Rad, Hercules, CA). C1INH was assayed by radial immunodiffusion assay (17) using goat IgG anti-human C1INH (DiaSorin Inc.) diluted 1:80 in veronal buffered saline that contained 1.5% agar and 0.01 mM EDTA. Functional C1INH activity was determined using traditional sheep red blood cell assays (18). nC1INH was purified from normal human serum (18) and used as a standard for antigenic and functional assays. SDS-PAGE (12% acrylamide) was performed using a Mini-Protean II system (Bio-Rad) and silver staining.

Purification of C1INH

Following removal of cells via centrifugation, the 10-L bioreactor supernatant was concentrated ~10-fold to 1 L by ultrafiltration. A 300-mL sample was further concentrated to 100 mL using 10000 MWCO Amicon and Centriprep concentrators (Amicon, Inc., Beverly, MA). rC1INH was purified by a modification of the method described by Pilatte *et al.* (19) for the purification of C1INH from serum. Briefly, 100 mL of concentrated supernatant was run over a jacalin-agarose

(Vector Laboratories, Burlingame, CA) column (6×1.5 cm) at a flow rate of 20 mL/h. The column was washed with 150 mL of PBS containing 10 mM EDTA and 25 μ M NPGB buffer at pH 7.0. The addition of 0.5 M NaCl to this buffer was required to eliminate nonspecific protein binding. rC1INH was eluted from the column using 0.125 M melibiose and was detected in fractions by using immunoblotting. Fractions containing rC1INH were pooled and concentrated to 20 mL by ultrafiltration and made up to 0.4 M in $(\text{NH}_4)_2\text{SO}_4$ by addition of 4 M solution of $(\text{NH}_4)_2\text{SO}_4$. Concentrated samples were then run over a 10-mL phenyl-Sepharose (Pharmacia, Piscataway, NJ) column equilibrated with PBS containing 10 mM EDTA, 0.4 M $(\text{NH}_4)_2\text{SO}_4$, and 25 μ M NPGB buffer (pH 7.0) and eluted with 150 mL of PBS containing 10 mM EDTA and 25 μ M NPGB buffer (pH 7.0). Fractions containing rC1INH were pooled and concentrated to a final volume of 2 mL containing 1.5 mg of protein.

Removal of Glycans from C1INH

Before releasing glycans for analysis, C1INH was further purified using a Sephacryl S-200 HR (Sigma, St. Louis, MO) column (45×1.5 cm) to remove low-molecular-weight contaminants that might interfere with analysis. C1INH was dissolved in column buffer (2 mM Hepes, 0.2% SDS, pH 7.5) and eluted from the Sephacryl S-200 HR column in void volume fractions. These fractions were pooled and the C1INH was precipitated by adding 9 vol of acetone and incubated overnight at -20°C and the precipitated protein was recovered by centrifugation. The acetone precipitated C1INH was resuspended in 0.2 mL of buffer 1 (20 mM Hepes, 1% SDS, pH 8.2) and β -mercaptoethanol (20 mM) was added. The sample was boiled for 5 min to dissolve the protein and then combined with 0.8 mL of buffer 2 (20 mM Hepes, 1.25% NP-40, pH 8.2) containing 50 mM EDTA. PNGase F (1.0 U/mL, Boehringer Mannheim, Indianapolis, IN) was added to remove the N-glycan chains from C1INH. The reaction was carried out overnight at 30°C and stopped by boiling for 3 min. The released N-glycans were recovered from the core protein by fractionation on an S-200 HR column (45×1.5 cm). Protein eluting from the column was monitored at 280 nm and the fractions containing released glycans were determined by reducing sugar assay (20).

O-Glycans were next released from the recovered C1INH protein using mild β -elimination conditions (21). In brief, 1 mg freeze-dried protein was dissolved in 1 mL of 0.5 M lithium hydroxide (nitrogen saturated) and the solution was kept in an ice bath for 48 h under nitrogen atmosphere. The reaction was stopped by addition of 1 mL of 0.5 M acetic acid. The released O-glycans were recovered from the protein by fractionation on an

S-200 HR column (45 × 1.5 cm). The fractions containing released *O*-glycan were determined by measuring absorbance at 210 nm.

After both the *N*-glycan and *O*-glycan fractions were individually pooled, 2% (v/v) saturated KCl was added and incubated at 4°C overnight. The precipitated potassium-SDS salt was removed by centrifugation and the glycans in the supernatant were passed over a 3-mL Biobead SM2 (Bio-Rad) column to remove the residual nonionic detergent. The column was washed with 5 column vol of water. The pooled fractions were freeze-dried and then individually desalted on a Sephadex G-10 column (Pharmacia, Sweden). Salt-free fractions containing *N*-glycans were pooled as were those containing the *O*-glycans and both samples were freeze-dried.

Fluorescent Labeling of Glycans and Preparation of Standards

Glycans were conjugated to 7-amino-1, 3-naphthalene disulfonic acid (AGA, Aldrich, Milwaukee, WI) by reductive amination (22, 23). The monopotassium salt of AGA (recrystallized from water) was dissolved in 15% acetic acid (5 mg/100 μ L), the purified glycan was added and the mixture was incubated for 1 h at room temperature. Sodium cyanoborohydride (100 μ L of 1.0 M) was added to the mixture and incubated for 12 h at 45°C. Excess AGA was removed using a Sephadex G-25 (Pharmacia, Sweden) column (45 × 1.5 cm) with detection at 247 nm. The fluorescently labeled glycan fractions were pooled and freeze-dried.

An oligomannose-type N-linked oligosaccharide, oligomannose 8 (Oxford Glyco Systems, Wakefield, MA) was fluorescently labeled with AGA as described above. Partial digestion of oligomannose 8-AGA with α -mannosidase (Oxford Glyco Systems, Wakefield, MA) (20 U/mL) in 10 mM sodium acetate buffer (pH 5.0) at 37°C was used to prepare a mixture of AGA-labeled mannosylated standards. To prepare the standard ladder, samples were treated with α -mannosidase for 10 min, 20 min, 1 h, 2 h, 3 h, 5 h, and 24 h, after which the digestions were stopped by boiling the sample for 5 min and the samples were combined. The denatured α -mannosidase was removed by centrifugation at 10,000g for 10 min and buffer salts were removed with a G-10 ultramicrospin column (AmiKa, Columbia, MD). AGA-labeled oligosaccharides containing one to eight mannose units were detected in the mixture of the products (Fig. 4D). AGA-labeled chitobiose and AGA-labeled biantennary glycan (Oxford) were also similarly prepared.

Capillary Electrophoresis (CE) and Exoglycosidase Treatment of C1INH N-Glycans

The AGA-labeled *N*-glycans obtained from rC1INH were digested with α -mannosidase (50 U/mL) in 10 mM

sodium acetate buffer (pH 5.0) at 37°C for 2 days. The digestion was terminated by boiling the sample for 5 min. The denatured α -mannosidase was removed by centrifugation at 10,000g for 10 min and buffer salts were removed with a G-10 ultramicrospin column. The AGA-labeled glycan mixtures were analyzed with a CE system (Dionex, Sunnyvale, CA) at 25 kV by fluorescent detection (λ 250 nm, λ_{em} 420 nm). Separation and analysis were carried out in a reversed polarity mode using a fused silica capillary tube (55 cm in length and 50 μ m). The separation buffer contained 20 mM sodium phosphate, pH 3.5 (23). The sample was pressure injected (5 s, 5 psi) resulting in an injection volume of 0.5 μ L. The identity of each peak was determined by coinjection of an AGA-labeled standard.

RESULTS

Bioreactor Preparation of C1INH

The 10-L bioreactor was inoculated with approximately 5×10^5 cells/mL with a viability of >95% (Figs. 1 and 2). High viability was maintained until viral infection began. The Sf-9 cells overcame the lag phase within the first 24 h and grew with a population doubling time of 24 h until infected with the virus. The run was continued for 94 hpi by which time the cell viability had dropped to 48%. At 94 hpi the glucose concentration in the supernatant was approximately 43 mM and the lactate concentration was 3.04 mM. Total extracellular protein concentration on a volumetric basis increased from 10 to 140 μ g/mL from the time of inoculation until harvest. This corresponded to a per cell increase from 1.63×10^{-5} to 2.57×10^{-5} mg/mL-cell.

Western blot analysis of aliquots obtained from a 10-L bioreactor run showed that rC1INH was detectable

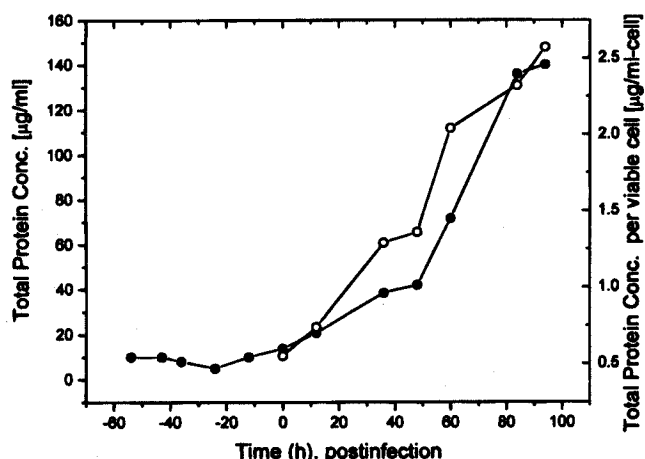


FIG. 1. Bioreactor cultivation of recombinant baculovirus AcMNPV-C1INH-infected Sf-9 insect cells. Total extracellular protein concentration per unit volume (●) and per viable cell (○) is plotted as a function of fermentation time postinfection.

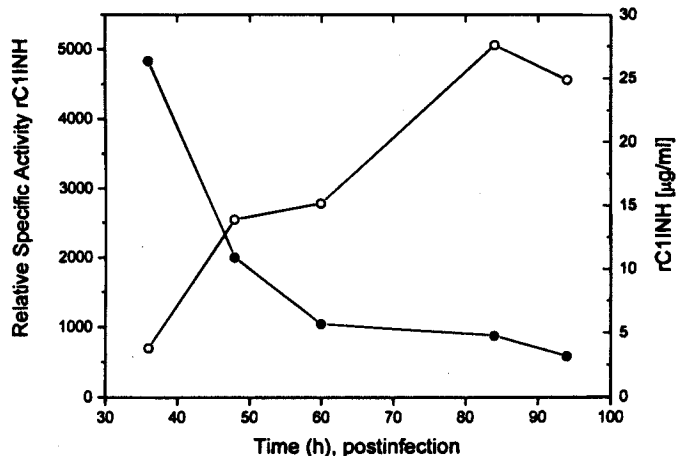


FIG. 2. Bioreactor cultivation of recombinant baculovirus AcMNPV-C1INH-infected Sf-9 insect cells. Specific activity of rC1INH (●) and concentration of rC1INH (○) is plotted as a function of time postinfection.

in the cell supernatant by 36 hpi. A prominent band appeared on the Western blot analysis (Fig. 3C) with an apparent molecular weight of 75 kDa, about 25 kDa smaller than purified human nC1INH. The highest functional rC1INH activity was observed at 36 hpi (Fig. 2). From 36 to 60 hpi, the specific activity dropped to 25% of its maximum value where it remained relatively constant. After 96 hpi the specific activity dropped to 10% of its maximum value (data not shown).

Purification of C1INH

The crude rC1INH obtained from the bioreactor had a very low biological activity (45 U/mg) and when analyzed by SDS-PAGE (Fig. 3A) showed numerous bands associated with contaminating protein and a very faint, diffuse band at ~75 kDa. The purification procedure involving ammonium sulfate precipitation, phenyl-

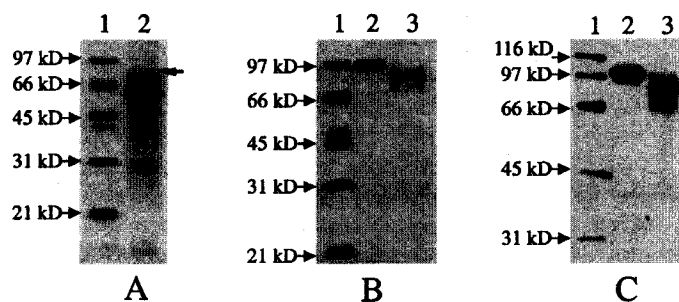


FIG. 3. SDS-PAGE and Western blot analysis of human nC1INH and rC1INH. (A) SDS-PAGE: lane 1, molecular weight markers; lane 2, crude rC1INH (a weak, diffuse band indicated with an arrow) before purification. (B) SDS-PAGE: lane 1, molecular weight markers; lane 2, purified nC1INH; lane 3, purified rC1INH. (C) Western blot with lane 1 containing prestained molecular weight markers; lane 2, purified nC1INH; lane 3, purified rC1INH.

Sepharose chromatography and jacalin-Sepharose affinity chromatography is essentially identical to that previously used for the purification of nC1INH from plasma (19). The bioassay used to determine C1INH activity measures its ability to suppress complement-mediated lysis of red blood cells. Because of the sensitivity of this assay to contaminants and reagents used in the intermediate steps of the purification, only the purity and activity of the final product was assessed. The final purified rC1INH showed a prominent diffuse band having an apparent molecular mass of ~75 kDa (Fig. 3B). Western analysis confirmed that the same band was recognized by antibodies generated against human nC1INH (Fig. 3C). The specific activity of the purified rC1INH was 31,000 U/mg comparable to an activity of 71,000 U/mg for the nC1INH isolated from human plasma using the same purification procedure.

Analysis of C1INH N-Glycans

Oligosaccharide maps of the released N-glycans were prepared (Figs. 4A-4D). It should be noted that exact alignment of peaks in a CE experiment is often difficult

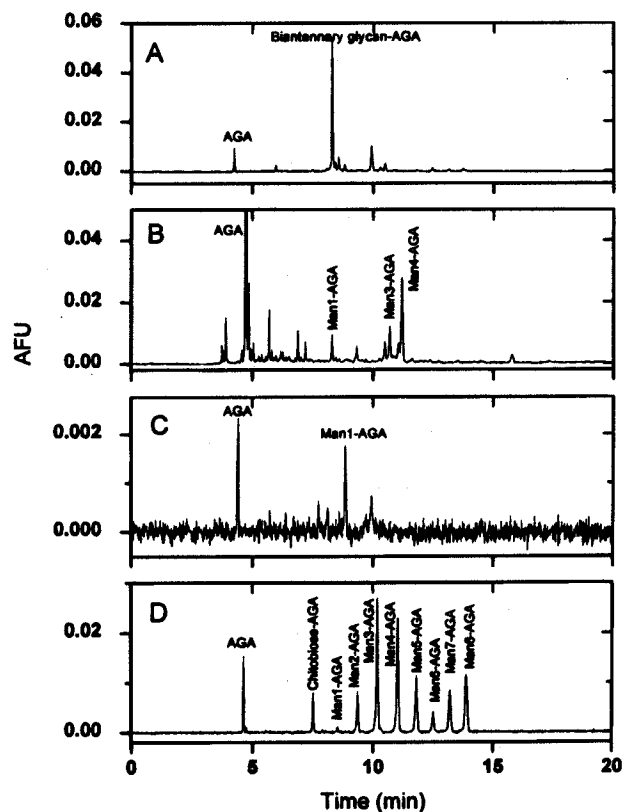


FIG. 4. CE analysis of AGA labeled N-glycans. (A) AGA-labeled N-glycans from nC1INH. (B) AGA-labeled N-glycans from rC1INH. (C) AGA-labeled N-glycans from rC1INH after treatment with α -mannosidase. (D) A mixture of AGA-labeled mannosylated N-glycans and AGA-labeled chitobiose standards. Peaks identified by comigration with known standards are labeled on each electropherogram.

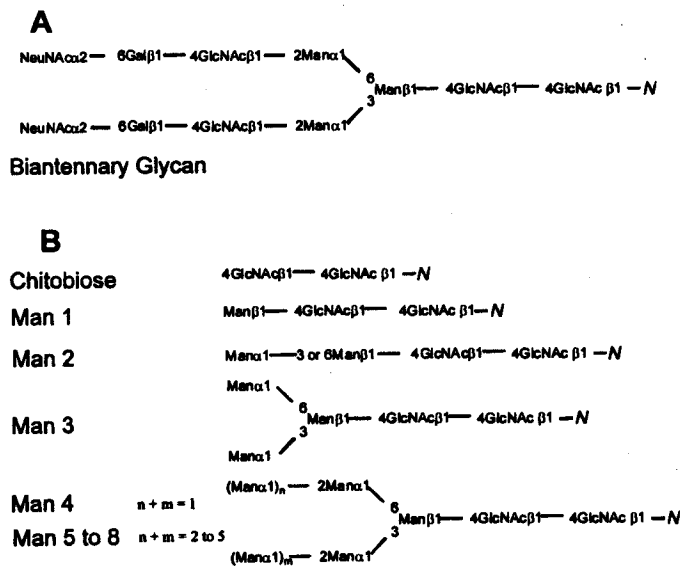


FIG. 5. Structure of *N*-glycans. (A) Structural variation reported for *N*-glycan from nC1INH (11). (B) Structures of glycan standards and Sf-9 insect cell glycans.

particularly in experiments involving small quantities of sample in the presence of buffers, salts, and enzymes. Therefore, comigration studies were performed in which AGA-labeled standards of known structure were coinjected with each sample to positively confirm the identity of each component. CE analysis of *N*-glycans released from nC1INH (Fig. 4A) afforded a single major peak comigrating with an AGA-labeled nonfucosylated biantennary standard (Fig. 5A), consistent with the structure reported by Strecker *et al.* (11). The electropherogram of rC1INH (Fig. 4B) was considerably more complex. A number of peaks were tentatively identified by comigration with AGA-labeled mannosylated glycan standards (Fig. 4B). Exhaustive treatment of *N*-glycans released from rC1INH with α -mannosidase, followed by CE analysis, afforded a single peak, corresponding to an AGA-labeled 4'- β -mannosyl chitobiose (Fig. 4C), as confirmed by coinjection migration with standard. These data demonstrate major *N*-glycans released from rC1INH are of the oligomannose type.

Analysis of C1INH O-Glycans

CE analysis (data not shown) demonstrated that nC1INH and rC1INH samples contained similar amounts of *O*-glycans. A complex mixture of *O*-glycans was removed from rC1INH with many different structures. Because of the absence of authentic *O*-glycan standards no further analysis was performed.

DISCUSSION

C1INH is an important regulator of the complement pathway that is deficient in HAE patients (4–6). Replacement therapy, an approach for treating HAE, is

under active investigation using plasma-derived C1INH (8). The current study looked to insect cell culture as a way to prepare safe and affordable rC1INH for clinical evaluation. In addition to preparing a biologically active rC1INH, it is also important that the protein be glycosylated to prevent its rapid *in vivo* catabolism or clearance and to decrease the possibility of an adverse immune response.

A recombinant baculovirus AcMNPV-C1INH was prepared and used to infect insect cells in a 10-L bioreactor. At harvest approximately 1.4 g of soluble protein was obtained containing rC1INH having a specific activity of 45 U/mg. The final purified rC1INH contained 1.5 mg of protein and had a specific activity of 31,000 U/mg, comparing favorably with the value observed for nC1INH of 71,000 U/mg purified from plasma using the same protocol. The rC1INH had an apparent molecular mass of 75 kDa, approximately 25 kDa smaller than human plasma nC1INH. This difference in molecular mass was attributed to differences in the level of glycosylation of both proteins. Human nC1INH glycosylation has been well studied. Thirteen glycosylation sites (six N- and seven O-linked) have been identified in the human protein by Perkins *et al.* (9). Ollier-Hartmann *et al.* (24) claim a N-glycosylation:O-glycosylation ratio of 1:4, which is in agreement with Harrison (1). An NMR study by Strecker *et al.* (11) demonstrated multiple biantennary *N*-glycan structures but only a single *O*-glycan structure. Several independent analyses of dysfunctional nC1INH indicate that the dysfunction in some cases may be due to changes in the glycosylation pattern (24–26). A minor band at ~70 kDa, running just below rC1INH on both SDS-PAGE and Western blot analysis (Figs. 3B and 3C, lane 3), might result from catabolism of rC1INH. The C1INH serpin is known to contain a reactive loop that may be susceptible to proteolysis.

Insect cells are not able to produce eukaryotic glycoproteins with complex N-linked glycans. While sites glycosylated in mammalian cells are usually glycosylated in insect cells, the insect cell glycan structures differ from mammalian derived glycans. N-linked glycans are mainly high-mannose-type glycans (27–31). Several studies have shown that insect cells are capable of producing biologically active recombinant glycoproteins (32–33) in spite of the fact that their glycosylation pattern differs from the native glycoprotein.

Examination of the C1INH samples was undertaken using CE analysis with exoglycosidase treatment. The *N*-glycan structure of the nC1INH was confirmed to primarily consist of a fully elaborated biantennary structure as previously reported (11). In contrast, rC1INH produced in Sf-9 insect cells contained a high-mannose structure consistent with the known pathway of insect cell glycosylation (34–37). Both nC1INH and rC1INH contained approximately the same quantity of O-linked glycans.

In summary, rC1INH could be prepared in high yields and specific activity in insect cell culture but had a lower apparent molecular mass due to incomplete elaboration of its glycan structure. The *N*-glycan structure of the Sf-9 insect cell preparation was high mannose type and contained no observable *N*-acetylglucosamine, galactose, or neuraminic acid at its nonreducing terminus. Future studies will be directed at utilizing insect cells with engineered glycosylation pathways to obtain rC1INH with human-like glycan structures for clinical evaluation. In addition, structures of rC1INH *O*-glycans will be elaborated. Regarding engineering of the insect cell glycosylation pathway, the Sf-9 cells have been stably transformed to express mammalian β 1,4-galactosyltransferase and/or α 2,6-sialyltransferase (34–37).

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