Glycosylation of a Recombinant Protein in the Tn5B1-4 Insect Cell Line: Influence of Ammonia, Time of Harvest, Temperature, and Dissolved Oxygen

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Abstract: Glycosylation is both cell line and protein dependent. Culture conditions can also influence the profile of glycoforms produced. To examine this possibility in the insect cell/baculovirus system, structures of N-linked oligosaccharides attached to SEAP (human secreted alkaline phosphatase), expressed under various culture conditions in BTI Tn5B1-4 cells, were characterized using FACE (fluorescence-assisted carbohydrate electrophoresis). Parameters varied were time of harvest, ammonia added during infection, dissolved oxygen, and temperature. It was found that glycosylation in the insect cell/baculovirus expression system is a robust, stable system that is less perturbed by variations in culture conditions than the level of protein expression. Addition of ammonia and low oxygen conditions affected SEAP expression, but not the oligosaccharide profile of SEAP. Time of SEAP harvest increased the amount of α-mannosidase resistant structures from 4.1% at 34 hours postinfection (h pi), to 5.0% at 100 h pi, and to 7.5% at 120 h pi. These structures were primarily sensitive to *N*-acetylhexosaminidase digest, although a small amount was insensitive to both mannosidase and *N*-acetylhexosaminidase digests. Lowering the temperature from 28°C to 24°C or even 20°C, resulted in a twofold increase in oligosaccharides containing terminal α(1,3)-mannose residues. This condition did not affect the amount of mannosidase-resistant structures. However, this could result in more complete glycosylation of recombinant proteins in the BTI Tn5B1-4 cell line, because more structures with the potential for further processing would be produced. *Biotechnol Bioeng* **63**: 255–262, 1999.
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INTRODUCTION

Although insect cell/baculovirus expression has proven to be valuable for production of small to moderate quantities of proteins, it has not been widely adopted for large-scale use. In spite of the capability of producing high concentrations of proteins and even secreted glycoproteins (see Dee et al., 1997), the lack of complete glycosylation precludes the use of insect cell culture for production of most therapeutic proteins due to the rapid clearance of asialoglycoproteins in the liver (Ashwell and Harford, 1982; Fukuda et al., 1989). Improvements in the level of glycosylation in insect cell baculovirus expression systems could lead to more widespread use of this system.

There are indications that culture conditions, such as ammonia, pH, dissolved oxygen, carbon source depletion, and age of the cells influence glycosylation in mammalian cells (Andersen and Goochee, 1995; Chotigeat et al., 1994; Goochee and Monica, 1990; Hahn and Goochee, 1992; Oda et al., 1988; Thorens and Vassalli, 1986). In addition to these environmental factors, investigators using the insect cell/baculovirus system, which features a lytic virus cycle, must consider the effect of harvest time due to the release of proteases and exoglycosidases (Licari et al., 1993). It also appears that glycoproteins produced earlier in the virus life cycle may be processed more completely (Jarvis et al., 1990, 1996; Sridhar et al., 1993) than those produced with very late polyhedrin promoter (Donaldson, 1998). This may be due to high throughput of proteins of the secretory pathway at late times postinfection; loss of integrity of the system because cellular proteins, including secretory pathway processing enzymes, are not replaced during viral infection; or depletion of other cellular resources. The decision of when to harvest the protein must take all of these factors into consideration.

Ammonia and other amines are known to disrupt intracellular vesicle trafficking. This has been shown to disrupt sialylation in cell culture (Andersen and Goochee, 1995; Oda et al., 1988), due to disruption of the pH in the Golgi apparatus. Increasing dissolved oxygen concentration increased the sialic acid content of human follicle-stimulating hormone in CHO cells (Chotigeat et al., 1994). Lower temperatures have been used in *E. coli* to increase the secretion

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of recombinant β-lactamase into the periplasmic space (Fu et al., 1993). These variables have been shown to affect other systems, but how these factors influence glycosylation in the insect cell/baculovirus system has not been systematically studied.

The time necessary to produce sufficient material for oligosaccharide analysis, purify over 20 separate protein preparations, and perform quantitative analysis of each oligosaccharide on each glycosylation site can be overwhelming with some proteins and oligosaccharide analysis methods. We used a human secreted alkaline phosphatase (SEAP), which only has one utilized glycosylation site; there is only one local glycoprotein environment to study in this case. We used fluorophore-assisted carbohydrate electrophoresis (FACE) (Jackson, 1991, 1994a,1994b; 1996; Jackson and Williams, 1991; Starr et al., 1996) for oligosaccharide analysis, which greatly reduced the time of analysis, allowed direct side-by-side comparison of oligosaccharide profiles, and still provided quantitative analysis of each type of oligosaccharide present on the protein.

With this system we explored the influence of added ammonia, time of harvest, temperature, and dissolved oxygen on the glycosylation of SEAP. Glycosylation of SEAP appeared to be very robust because very consistent results were observed over a wide range of conditions. Lowering the temperature and delaying harvest time had measurable effects on the oligosaccharide profile of SEAP.

MATERIALS AND METHODS

Cell Culture, Medium, Virus, and Routine Maintenance

Sf-21 cells (IPLB-Sf21AE) were grown in 50-mL spinner flasks at 130 rpm in Ex-Cell 400 medium (JRH Biosciences). Low-passage (70 total passages from isolation) BTI-Tn5B1-4 cells in TNM-FH media were obtained from Dr. R.R. Granados (Boyce Thompson Institute for Plant Research, Ithaca, NY). These cells were adapted to Ex-Cell 405 (JRH) by gradual reduction of the percentage of TNM-FH (50%, 25%, 10%, 5% TNM-FH) with two or three passages between each step decrease in TNM-FH, allowing time for a subpopulation to be naturally selected, which could grow well under the reduced serum conditions (Donaldson, 1998). Tn5B1-4 cell cultures were grown at 160 rpm in 50-mL spinner flasks (Bellco, Vineland, NJ) with a 40-mL working volume, using 100 μg/mL dextran sulfate (Sigma, dextran MW 5000) to prevent clumping (Dee et al., 1997). The screw-cap closures were replaced with silicone foam closures (Bellco) to ensure maximum gas exchange.

A Coulter counter (Model ZM, Coulter Electronics, Hialeah, FL) was used for total cell counts and viability was determined by Trypan blue exclusion.

A recombinant baculovirus expressing a secreted alkaline phosphatase (SEAP) was obtained from Dr. Alan Wood, BTI (Davis et al., 1992). The virus stock was plaque purified and amplified in Sf-21 cells. For virus production, Sf-21 cells were grown to midexponential phase, 2~3 × 10^6 cells/mL. Cells were centrifuged at 125*g* for 5 min, and resuspended in fresh medium at 2 × 10^6 cells/mL. Virus was added at a multiplicity of infection (MOI) of 2. After 48–60 h, virus was harvested by removing cells and debris by centrifugation at 125*g* for 5 min, followed by centrifugation at 1000*g* for 30 min. Viral stocks were stored at 4°C protected from light. Viral titers were determined by a modified endpoint dilution assay (Dee and Shuler, 1997b).

Infection Protocol

Tn5B1-4 cells grown to 3–4 × 10^6 cells/mL were pooled together, centrifuged at 250*g* for 5 min, resuspended in fresh medium, and infected at an MOI of 20 pfu/cell in a 250-mL spinner flask for 2 h. The cells were then seeded into individual 50-mL spinner flasks at the various conditions tested. Dextran sulfate was then added back to the culture. SEAP was assayed as described previously (Dee et al., 1997).

Ammonia Experiment

A 0.8 *M* stock of ammonium sulfate was prepared in water and filter sterilized. After 2 h to allow virus to adsorb and endocytose (99% of the virus will attach to Tn5B1-4 cells at 5 × 10^6 cells/mL in 60 min (Dee and Shuler, 1997a), five 50-mL spinner flasks were prepared with 0, 10, 20, 30, and 40 m*M* ammonia. Samples were taken at 36 and 72 h postinfection (pi) for ammonia analysis. The concentration of ammonia was measured using an ammonia electrode (Orion Model 95-12). Briefly, a clarified 0.5-mL sample was added to 19.5 mL of water and 0.4 mL of ISA buffer (Orion). A steady readout was generated by gentle agitation of the probe in the sample.

Oxygen Measurements

Tn5B1-4 cells were infected according to the aforementioned protocol and resuspended at 6 × 10^6 cells/mL. A low dissolved oxygen spinner flask was prepared by using the black phenolic caps screwed tightly closed. A high dissolved oxygen spinner flask was prepared by using the silicon foam closures and incubating the flask in an 80% oxygen environment. Dissolved oxygen was monitored with miniature dissolved oxygen probes (Model MI-730, Microelectrodes, Inc., Londonderry, NH) inserted through the top of the spinner flask. The probes were calibrated by sparging nitrogen and oxygen into medium at 28°C and allowing the signal to reach a steady state as monitored using a chart recorder (Model 1202, Cole Parmer, Chicago, IL).

Temperature Experiments

Tn5B1-4 cells were grown and infected using the protocol just described. Postinfection, 50-mL spinner flasks with infected cells were incubated at 20°, 24°, 28°, and 30°C. The

flask at 20°C was harvested at 96 h pi, because the infection proceeded slightly slower, whereas the other flasks were harvested at 72 h pi.

SEAP Purification and FACE Analysis

Recombinant SEAP protein was harvested and purified as described by Kulakosky et al. (in press). Briefly, secreted protein was harvested at the appropriate time postinfection and clarified by three centrifugal steps: 125*g* for 5 min; 1000*g* for 30 min; and 23,000*g* for 4 h. The clarified virus-free supernatant was then extensively dialyzed against 20 m*M* Tris, 1 m*M* $MgCl_2$, pH 8.0. SEAP was then purified by passing the supernatant over a phosphate affinity column (4-aminobenzylphosphonic acid diazotized by histidine to epoxy agarose (Kulakosky et al., in press), and then eluting using a 1 to 100 μM phosphate gradient. Peak fractions of SEAP activity were pooled, concentrated, and exchanged into distilled water using a Centricon-30 ultrafilter unit (Amicon, Danvers, MA). Greater than 80% of the total SEAP activity was recovered with >95% purity. Purified SEAP was stored at −70°C.

Enzyme Digest Conditions

Oligosaccharides were released from denatured SEAP by PNGaseF (peptide *N*-glycosidase F, Glyko, Novato, CA) digestion at 25°C, overnight, followed by 2 h at 37°C. Oligosaccharides were analyzed using the fluorescence-assisted carbohydrate electrophoresis (FACE) system (Glyko). Labeling with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) was done at 37°C overnight, according to Glyko's procedures. Labeled oligosaccharides were separated by gel electrophoresis using Glyko's N-linked oligosaccharide gels and Glyko's electrophoresis apparatus. Analysis was done with Glyko's analytical software and a digital camera.

RESULTS

To establish the baseline glycoform profile, the oligosaccharides of SEAP produced under standard conditions were characterized. Because lectins are easy to use and give rapid results, we used them for an initial, qualitative analysis. A positive response was seen with WGA lectin (Donaldson, 1998), which is specific for nonreducing terminal GlcNAc. To determine if this was a specific lectin recognition event, the lectin incubation was carried out in the presence of 0.5 *M N*-acetylglucosamine, using a method analogous to that used to characterize the oligosaccharides on gp64 (Jarvis and Finn, 1995). A strong response of WGA binding was again seen with three independently ordered samples (Fig. 1, lanes 1, 3, 5, and 7), while there was a clear inhibition of WGA lectin binding in all four lanes incubated with WGA in the presence of 0.5 *M N*-acetylglucosamine (Fig. 1, lanes 2, 4, 6, 8). This implies that there is specific binding of WGA to SEAP and, therefore, that these samples all contain terminal *N*-acetylglucosamine. Because the samples in lanes 2–7 of Figure 1 are clarified, virus-free, cell culture supernatant samples, there is a remote possibility of a comigrating protein binding with WGA instead of SEAP. However, the recombinant protein is the only protein present in such large quantities when producing recombinant proteins using Tn5B1-4 cells in serum-free medium; the secreted cellular protein background is low with this cell line (data not shown). To be certain of the results a quantitative analysis of the oligosaccharides from purified SEAP was carried out.

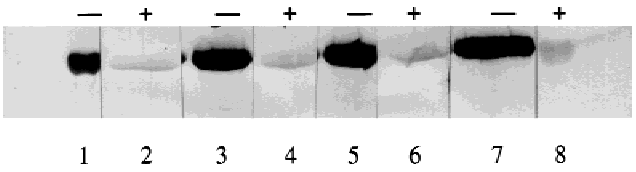

Figure 1. SEAP samples analyzed by WGA lectin blot. Lanes 1 and 8, equal amounts of 0 m*M* NH_3 control SEAP purified by (4-amino)benzylphosphonic acid affinity chromatography. Lanes 2 and 3, equal amounts of Tn5B1-4 produced SEAP supernatant sample 1. Lanes 4 and 5, equal amounts of Tn5B1-4 produced SEAP supernatant sample 2. Lanes 6 and 7, equal amounts of Tn5B1-4 produced SEAP supernatant sample 3. All supernatant samples were clarified by high speed centrifugation and virus-free. Lanes 1, 3, 5, and 7 were incubated with WGA following normal procedures. Lanes 2, 4, 6, and 8 were incubated with WGA in the presence of 0.5 *M N*-acetylglucosamine, which is a specific competitive inhibitor of WGA that recognizes terminal *N*-acetylglucosamine residues. (Note that the dimer or trimers of *N*-acetylglucosamine are even better inhibitors.)

A sample of SEAP produced in Tn5B1-4 cells, harvested at 100 h postinfection (h pi) was prepared for analysis by FACE. Initial analysis of the oligosaccharide profile revealed no differences from what has been reported for SEAP produced in Tn5B1-4 cells (Kulakosky et al., 1998). However, when the oligosaccharide profile was digested with α-mannosidase and also with β-*N*-acetylhexosaminidase in combination with the α-mannosidase, it was clear that three bands not digested by the α-mannosidase alone were eliminated in the combination digest (Fig. 2). This implies, first, that these structures were α-mannosidase-resistant (i.e., had terminal sugars other than mannose, which prevented digestion by α-mannosidase), and second, that the terminal sugar blocking α-mannosidase digestion was *N*-acetylglucosamine. This confirmed the result seen in the lectin blot: there are terminal GlcNAc moieties in some portion of the SEAP oligosaccharide profile.

The two bands that were β-*N*-acetylhexosaminidase-sensitive, with degrees of polymerization (DPs) of 5.4 and 4.7, are of the same size as $GlcNAc_2Man_3GlcNAc_2$ and $GlcNAc_1Man_3GlcNAc_2$, respectively. The third band, with a DP equal 4.2, is probably a partially digested structure containing terminal GlcNAc [such as $GlcNAc_1Man_2(Fuc)GlcNAc_2$], because this is too small for a normal oligosaccharide containing terminal GlcNAc. We have seen this same band in other extensive α-mannosidase digests in which this band remains resistant to digestion (data not shown).

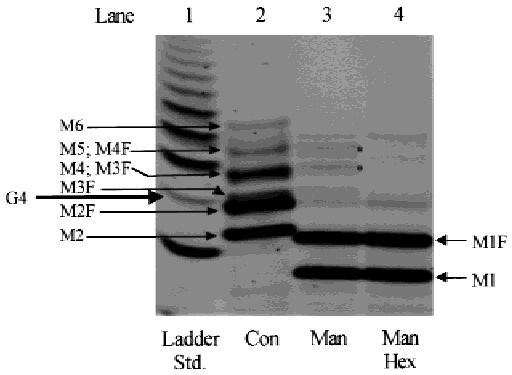

Figure 2. α-Mannosidase and combination α-mannosidase/β-*N*-acetylhexosaminidase digests of SEAP oligosaccharides harvested at 100 h pi. Con = incubated mock digest; Man = mannosidase; Hex = *N*-acetylhexosaminidase.

Quantitation of the bands revealed that 5% of the total lane luminance was resistant to mannosidase digest. The smallest faint band, of size 3.9 DPs, was not included in this 5%, because it was is not certain that this band contained terminal GlcNAc as it was not digested in the double digest (Fig. 2, lane 3). Of this 5%, 4.4% was in three β-*N*-acetylhexosaminidase-sensitive bands, with the rest being equally distributed between the fucosylated and nonfucosylated core structures, $Man_1(Fuc)GlcNAc_2$ and $Man_1GlcNAc_2$, respectively. It appears that this small portion of terminal GlcNAc is responsible for the very prominent staining with WGA lectin. A small amount (~1%) of the mannosidase-resistant material remained, even after the combination digest. This could either be due to incomplete digestion by the enzymes or the presence of other terminal sugars preventing digestion by these enzymes.

Time of Harvest

Once this baseline oligosaccharide profile was established by combining the aforementioned results with those previously reported (Kulakosky et al., in press), SEAP was harvested from Tn5B1-4 cell culture at 34, 48, 72, 96, and 120 h pi and analyzed. The oligosaccharide profiles from the purified SEAP is shown in Figure 3, with the structural representations shown for each band as previously determined (Kulakosky et al., in press). The number of bands is identical in each case, with some slight quantitative differences, particularly in the band of Manα(1,6)-Manα(1,6)-Manα(1,4)-$GlcNAc_2$(1,4)-GlcNAc which is acid labile.

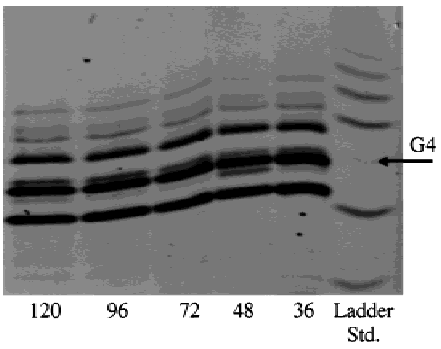

Figure 3. SEAP oligosaccharide profiles harvested at various timepoints from Tn5B1-4 cell culture.

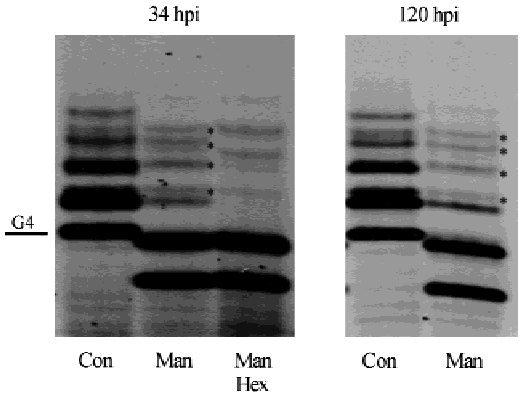

Figure 4. α-Mannosidase and combination α-mannosidase/β-*N*-acetylhexosaminidase digests of SEAP oligosaccharides harvested at 34 and 120 h pi. Con = incubated mock digest; Man = mannosidase; Hex = *N*-acetylhexosaminidase. A glucose ladder is not shown, but the location of G4 is indicated for reference.

Along with the 100-h pi sample, samples harvested at 34 and 120 h pi were digested with mannosidase and *N*-acetylhexosaminidase to determine if there were any quantitative differences in the mannosidase-resistant structures. The results of these digests are shown in Figure 4. Table I provides a quantitative distribution of these structures. A slight trend in increasing amounts of mannosidase-resistant structures was seen, with 4.4% of the lane luminance in resistant structures at 34 h pi, 4.8% at 100 h pi, and 7.2% at 120 hpi. Because each timepoint is a cumulative harvest, the amount of mannosidase-resistant structures produced between timepoints was calculated based on the amount of SEAP produced per cell between timepoints. There was little change up to 100 h pi, but this analysis showed that mannosidase-resistant oligosaccharides accounted for ap-

Table I. Bandwise quantitative analysis of α-mannosidase resistantoligosaccharides of SEAP harvested at 34, 100, and 120 h pi.[a]

34 h pi			100 h pi				120 h pi		
DP	% Lum.	% Lum.	DP	% Lum.	% Lum.	% Lum.	DP	% Lum.	% Lum.
6.0	1.0	0.9	6.1	0.7	0.8	0.8	5.8	1.3	1.5
5.6	0.9	0.8	5.4	1.3	1.3	1.2	5.4	1.8	1.9
4.9	2.0	1.9	4.7	2.4	1.9	1.5	4.8	2.7	2.8
4.2	0.8	0.5	4.2	1.0	1.0	0.5	4.1	1.1	1.3
Sum	4.7%	4.1%		5.4%	5.0%	4.0%		6.9%	7.5%
Avg.	4.4%			4.8%				7.2%	

[a]The percent luminance was measured with different exposures of the same gel for an estimate of the amount of error in the quantitative band analysis.

proximately 50% of the total oligosaccharides produced between 100 and 120 h pi. About 5% of the recombinant SEAP was produced between 100 and 120 h pi, so this remains a small contribution to the total pool of oligosaccharides. Medium exchange, as done by Davidson and Castellino (1991), was not performed, because it would introduce other variables into the system as well, and it is not a standard method of harvesting recombinant protein.

Dissolved Oxygen

The dissolved oxygen content of two flasks under normal air and 80% oxygen is shown in Figure 5. The closed screw caps significantly reduced the amount of oxygen in the flask for the initial infection period, remaining below 36% for the first 24 h with a gradually increasing concentration throughout the infection. The flask under elevated oxygen immediately went to almost 80% saturation and remained relatively stable throughout the infection period. Elevated O_2 resulted in a 47% decrease in SEAP production (data not shown). However, there was no effect on the SEAP oligosaccharide profile, as seen in Figure 6. Apparently, a much more stringent oxygen restriction would be necessary to effect glycosylation in these insect cells. A drop in protein production was seen before any effect in glycosylation was observed, showing that other cellular mechanisms are more sensitive to oxygen tension than glycosylation.

Ammonia

It has been shown that the Tn5B1-4 cell cultures in particular produce a relatively large amount of ammonia during growth and infection, up to 40 m*M* (Donaldson, 1998; Taticek, 1996; Yang et al., 1996). Therefore, we added ammonia, up to 40 m*M*, to the cultures after viral adsorption to see how much ammonia was necessary to disrupt normal glycosylation. When 40 m*M* ammonia was added, the final accumulation was 62 m*M* at harvest (72 h pi). The additional ammonia affected SEAP production, with a 66% reduction at 40 m*M*. When the oligosaccharides were examined (Fig. 7) there was no apparent change upon addition of up to to 30 m*M* ammonia. At 40 m*M* ammonia, a band appeared, which comigrated with the core standard ManGlcNAc(Fuc)GlcNAc. Also, the tri-mannosyl core band (comigrating with G4, top of the triplet bands), was more prominent. It is not clear whether the appearance of these bands was attributable to the ammonia added during infection, or if these bands were the result of some other factor. Even beyond the typical range of ammonia concentrations in culture, ammonia has very little effect on protein glycosylation in this cell system.

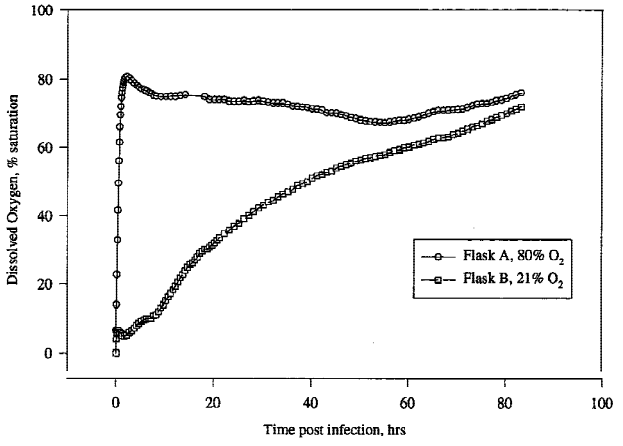

Figure 5. Dissolved oxygen concentration in two 50-mL spinner flasks, 160 rpm agitation, during SEAP baculovirus infection of Tn5B1-4 at 6×10^6 cells/mL. The low oxygen flask had both screw caps tightly closed. The high oxygen flask had silicon foam closures and was incubated in an 80% O_2 environment.

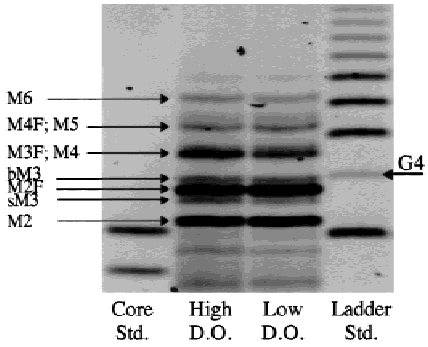

Figure 6. SEAP oligosaccharide profiles from Tn5B1-4 cell culture under the two different dissolved oxygen conditions shown in Figure 5.

Temperature

Temperature during infection was lowered to allow more time for passage through the ER and Golgi apparatus during the secretion of high amounts of recombinant protein. Flasks were incubated at 30°, 28°, 24°, and 20°C. Whereas changing the temperature of the infection produced no different oligosaccharides, there was a definite pattern in the quantitative amounts of the bands seen (see Fig. 8). When the bands were sorted on the basis of presence or absence of α(1,3)-linked mannose it is clear that, as temperature in-

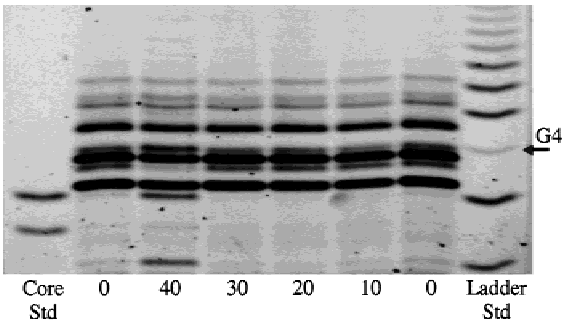

Figure 7. SEAP oligosaccharide profiles from Tn5B1-4 cell culture with 0, 10, 20, 30, and 40 m*M* ammonium sulfate added to the cultures after viral attachment and endocytosis.

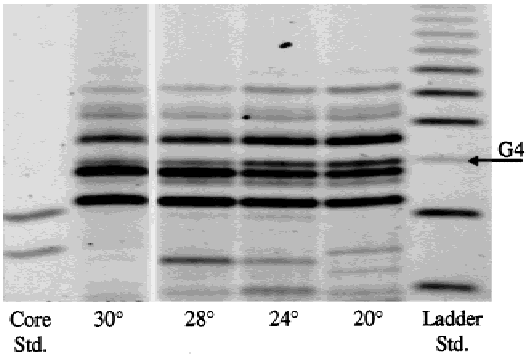

Figure 8. SEAP oligosaccharide profiles from Tn5B1-4 cell culture infected at 20°, 24°, 28°, and 30°C.

creased, there was a shift toward bands that did not contain α(1,3)-linked mannose (see Table II). The straight chain structure Manα(1,6)-Manα1,6)-ManGlcNAc$_2$ did not follow this pattern. Perhaps this band is more susceptible to normal α-mannosidase II processing at higher temperatures. As a percent of lane luminance, there was almost a twofold increase of bands containing α(1,3)-linked mannose at 20°C compared with 30°C, with almost all of the increase found between 24° and 28°C. This is significant because the structures without α(1,3)-linked mannose were dead-end structures and were unsuitable substrates for further processing by glycosyltransferases, especially for *N*-acetylglucosaminyl transferase I.

To determine if reduced α(1,3)-mannosidase activity at lower temperatures affected the amount of mannosidase-resistant structures, these samples were analyzed by mannosidase and *N*-acetylhexosaminidase digests. However, no trend in the data was seen, with the amount of mannosidase-resistant structures being 4.1% at 20°C, 4.6% at 24°C, 2.5% at 28°C, and 4.3% at 30°C. Actually, the data are quite consistent, increasing the significance of the time of harvest trend. The harvest time may have been the limiting factor in the amount of processing, rather than the temperature.

DISCUSSION

The effects of culture conditions on the glycosylation of proteins have been documented in mammalian cell culture systems, but have not been systematically explored in the insect cell/baculovirus system. It has been assumed that other factors play a more important role than culture conditions and that improving standard operating conditions, even if possible, would not improve glycosylation in the insect cell/baculovirus system.

This report confirms this assumption in some ways. Dissolved oxygen, ammonia, and time of harvest were more critical factors for quantity of protein produced than they were for the type of oligosaccharides added. Others have reported changes in the oligosaccharide profile as the viral infection progressed (Davidson and Castellino, 1991), and we found a small trend in this direction as well. The amounts of α-mannosidase-resistant structures increased from 4.4% at 34 h pi, to 4.8% at 100 h pi, and to 7.2% at 120 h pi. Approximately 50% of the oligosaccharides produced between 100 and 120 h pi must be mannosidase resistant for this increase to be seen in the total amount of mannosidase-resistant oligosaccharide structures. Because such a small amount of the total SEAP is produced between 100 and 120 h pi, the fraction of mannosidase-resistant structures produced between 100 and 120 h pi is sensitive to the amount of SEAP produced. We assumed there was no degradation of SEAP in the medium, and that SEAP production on a per-cell basis was consistent between different spinner flasks. Even if 10% of the total SEAP was produced between 100 and 120 h pi, the differential amount of mannosidase-resistant SEAP oligosaccharides would be approximately 25%. It has been our experience that production between 100 and 120 h pi is generally very low. If more production could be induced after 100 h pi this could have a large impact.

Evidence that this trend is real comes from two sources. First, multiple analyses of the same gels revealed that quantitation is sensitive and reproducible enough to detect this small trend in increasing amount of mannosidase-resistant

Table II. Quantitative changes in the oligosaccharide profile of SEAP by temperature shifts.[a]

	20°C	24°C	28°C	30°C	Change with increasing temp.
Man_6	5.1	3.7	2.2	2.1	Decrease
Man_5	1.4	1.0	0.8	1.2	Not signif.
Man_4Fuc	3.4	3.6	2.9	3.8	Not signif.
Man_4 or Man_3Fuc	25.1	20.9	14.8	14.5	Decrease
Man_3 core	10.3	8.9	5.1	5.1	Decrease
Man_2Fuc	20.2	19.4	30.4	39.4	Increase
Man_3α(1,6)-linked	4.5	5.2	1.5	1.3	Decrease
Man_2	24.6	26.8	33.2	31.5	Increase
Structures with α(1,3)man.	45.3	38.1	25.8	26.7	Decrease
Structures without α(1,3)man.	49.3	51.4	65.1	72.3	Increase

[a]Structures are shown in the order of appearance on the gel, top to bottom. Quantitation is given as a percent of total oligosaccharide luminance.

oligosaccharides with increasing time postinfection see Table I). Second, in analyses of seven other samples from cell culture and larval hemolymph the average amount of mannosidase-resistant structures was 2.3 ± 1.0% of the total lane luminance, with a range of 1% to 4 % (Kulakosky, unpublished data). Also, the mannosidase-resistant structures in samples analyzed at the various temperatures tested in this report accounted for an average of 3.9 ± 0.9% of the total lane luminance. On the basis of this evidence, this trend represents a physical phenomenon in the cells.

Most of the mannosidase-resistant structures were digested by *N*-acetylhexosaminidase, but a small amount, about 1% to 1.5%, remained resistant to digestion by both enzymes. We did not further determine the nature of these structures.

Overall, the insect cell system was more stable than might have been predicted based on the number of factors and conditions being explored. A very consistent oligosaccharide profile can be produced on a specific protein by this system, even under suboptimal expression conditions.

The expression of SEAP was examined previously in Tn5B1-4 and Sf-21 cells (Kulakosky et al., 1998). The investigators concluded that the main differences in the oligosaccharide profile in these two cell lines could be explained by the presence of an α(1,3)-mannosidase in the Tn5B1-4 cell line. This α(1,3)-mannosidase contributed to $Man_2GlcNAc_2$-truncated structures not seen in the SEAP expressed by the Sf-21 cell line, but seen in Tn5B1-4 cell culture here. These results were similar, even though TNM-FH medium supplemented with serum was used previously (Kulakosky et al., 1998); as noted earlier, we used Ex-Cell 405 serum-free medium in this report.

The almost twofold increase in α(1,3)-mannose-containing oligosaccharides produced by lowering the temperature from 28°C to 20°C during infection increased the amount of oligosaccharides, with potential for further processing. It must be acknowledged that all pausimannosidic structures smaller than a $Man_5GlcNAc_2$ structure are dead-end structures not suitable for further processing. So, there remains no practical advantage in producing Man_3 structures rather than Man_2 structures on SEAP. However, glycosylation can be different for other proteins expressed in this cell line, such as those seen with the N-linked oligosaccharides of a murine IgG-2a expressed in Tn5B1-4 cells, 35% of which were complex structures including some with terminal galactose (Hsu et al., 1997). A temperature shift to prevent this α(1,3)-mannosidase trimming may be quite useful in such a case. Furthermore, if genetic engineering were used to add or supplement low expression of glycosyltransferases (Jarvis and Finn, 1996; Wagner et al., 1996b) there would be a distinct advantage to producing structures suitable for processing by these added enzymes.

It is likely that any fucosylated structure was acted upon by *N*-acetylglucosaminyl transferase I (Altmann et al., 1993; Velardo et al., 1993) before being trimmed by α-mannosidase II and other α-mannosidases. Furthermore, the presence of $Man_2(Fuc)GlcNAc_2$ implies that the enzyme activity that removed the α(1,3)-mannose must have acted after α-mannosidase II, and also after a β-*N*-acetylhexosaminidase enzyme (Altmann et al., 1995; Wagner et al., 1996a), because the first GlcNAc is added to the α(1,3)-mannose. Thus, the processing proceeds in the order of GlcNAc transferase I, α-mannosidase II, fucosyltransferase, β-*N*-acetylhexosaminidase, and then α(1,3)-mannosidase.

It is of interest to note that the amount of $Man_5GlcNAc_2$ oligosaccharide is low in all cases tested, as was also seen in Sf-21 cells (Kulakosky et al., 1998). This small steady-state concentration indicates a reactive intermediate structure, which is quickly processed by *N*-acetylglucosaminyl transferase I, or by α-mannosidase II.

The presence of a small portion of terminal GlcNAc-type oligosaccharide is shown here both by lectin blot and by quantitative analysis. Although these structures were not rigorously sequenced, the data reported here are consistent with the existence of structures containing one- and two-terminal GlcNAcs. The presence of the oligosaccharide structure $GlcNAc_2Man_3GlcNAc_2$ requires GlcNAc transferases I and II, both of which have been detected in Sf-21 cells (Altmann et al., 1993), and are indicated to be present in Tn5B1-4 cells by the presence of terminal galactose as well as terminal GlcNAc in the oligosaccharides of a recombinant IgG-2a (Hsu et al., 1997).

Although most of the reports of glycosylation in insect cells have shown only terminal mannose structures, there is evidence that more potential exists in insect cells for further oligosaccharide processing. To take full advantage of this potential will require a combined effort from studies such as this one, to ensure the best possible environment for gene expression and genetic engineering approaches to enhance the endogenous genetic capability of the cells.

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